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In-silico and Phylogenetic Analysis of Acetate: Succinate COA-Transferase (ASCT) from *Angiostrongylus malaysiensis*

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ABSTRACT

The zoonotic capability of *Angiostrongylus malaysiensis* was recently observed after several years of doubt. This parasite was found in a high burden of Malaysian rats, which is alarming. There is currently no effective treatment for human neuroangiostrongyliasis. Acetate: succinate CoA-transferase (ASCT) enzyme catalyses acetate production in helminth parasites. ASCT was classified into three subfamilies within the family I CoA-transferases (IA, IB, and IC). Acetate is an essential metabolic end product of many parasites, making it an attractive drug target since it is absent in mammalian hosts. The current study describes the in-silico analysis conducted for the identification and phylogenetic characterisation of *A. malaysiensis* ASCT and genetic variations between subfamilies of ASCT. The *Am*ASCT was identified from the ongoing de novo transcriptome assembly and annotation

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of adult *A. malaysiensis*. The analysis of *Am*ASCT physiochemical properties, multiple sequence alignment and phylogenetic relations with the ASCTs of other helminths are conducted using standard bioinformatic tools. Pairwise comparisons between subfamilies of ASCT have also been conducted in silico. *Am*ASCT has the conserved regions of the family I CoA-transferases and is clustered with subfamily IB of ASCT. From the pairwise analysis, subfamilies IB and IC were most closely related between the three subfamilies. *Am*ASCT was predicted to be overall hydrophilic and stable in a neutral to slightly alkaline environment within the parasite. The phylogenetic analysis confirmed that *Am*ASCT belongs to subfamily IB of ASCTs. Further study on the biochemical activity of ASCT in *A. malaysiensis* is required to determine its enzymatic function.

Keywords: Acetate: succinate CoA-transferase (ASCT), acetate production, *Angiostrongylus malaysiensis*

INTRODUCTION

Human neuroangiostrongyliasis, caused by the rat lungworm, is a food-borne parasitic zoonosis distributed worldwide and endemic to Asia and the Pacific Basin. However, it has been reported in new regions beyond its traditional endemic range (Eamsobhana, 2014). The prime causative agent of human neuroangiostrongyliasis is *Angiostrongylus cantonensis* (Wang et al., 2008). Recently, *A. malaysiensis*, formerly known as the Malaysian strain of *A. cantonensis*, was revealed to be zoonotic after several years of doubt (Watthanakulpanich et al., 2021), increasing the risk of human neuroangiostrongyliasis infection. The newly found zoonotic capability of *A. malaysiensis* is alarming, given its high prevalence in rats in Malaysia (Low et al., 2023). There is currently no effective treatment for human neuroangiostrongyliasis. Current treatment focuses on alleviating pain, inflammation, and intracranial pressure in infected individuals (Sawanyawisuth et al., 2008; Slom et al., 2002). Although anthelmintics have been extensively used, their effectiveness is debatable (Murphy et al., 2013), as they leave the disease without an efficient cure. A new, effective approach is needed for treating this disease.

The complex life cycle of parasites enables them to inhabit more than one host organism throughout their life cycle. Most parasites have a complex life cycle, including free-living and distinct stages inhabiting one or more host organisms. As a result, parasites are forced to modify their metabolism to survive in habitats where nutrients and oxygen are scarce. These parasites have evolved metabolic strategies that produce acetate from acetyl-CoA as an essential metabolic product (Tielen et al., 2002). Two pathways of acetate formation from acetyl-CoA have been found in parasites and are catalysed by either a cytosolic acetyl-CoA synthetase (ACS) or an organellar acetate: succinate CoA-transferase (ASCT). Furthermore, the ACS pathway has been reported only in parasitic protists (Reeves et al., 1977; Sánchez et al., 2000; Stechmann et al., 2008), while the ASCT pathway was previously found in both helminths and protists (Steinbüchel & Müller, 1986; Saz et al., 1996; van Hellemond et al., 1998).

To date, all acetate/succinate CoA-transferases (ASCTs) found in eukaryotes are family I CoA-transferases that are distinguished by the catalysis of CoA group transfer, which involves the presence of a glutamate residue in the enzyme's active site (Heider, 2001). Recently, the corresponding ASCT genes were identified and characterised in two protists, *Trypanosoma brucei* (Rivière et al., 2004) and *Trichomonas vaginalis* (van Grinsven et al., 2008), and in the helminth *Fasciola hepatica* (van Grinsven et al., 2009). The ASCT genes from the three parasites showed little sequence homology, classifying them into three different subfamilies of family I CoA-transferases: *T. brucei* in subfamily IA, *F. hepatica* in subfamily IB, and *T. vaginalis* in subfamily IC (Tielens et al., 2010).

Acetate is a prime candidate for developing new antiparasitic medications because it is a crucial byproduct of the energy metabolism of many parasites absent in their mammalian hosts. Identifying and characterising acetate-producing enzymes in rat lungworms is crucial for developing new and effective drugs to treat human neuroangiostrongyliasis. Here, we conducted a phylogenetic and physiochemical characterisation of the identified putative gene sequence of *A. malaysiensis* ASCT (AmASCT) obtained from our ongoing transcriptomic data analysis and further analysed the genetic variations among the three subfamilies of ASCTs.

METHODS

In-silico Analysis

Identification of Putative A. malaysiensis *ASCT (AmASCT)*

Putative A*m*ASCT was identified through ongoing transcriptomic analysis of adult *A. malaysiensis*. In short, the raw RNA-seq reads obtained were subjected to quality control using Trimmomatic (Galaxy version 0.39) (Bolger et al., 2014) and de novo transcriptome assembly using Trinity (Grabherr et al., 2011). Coding regions were predicted using TransDecoder (Galaxy version 5.5.0) (Haas et al., 2013). Subsequently, the assembled transcripts and predicted coding regions were subjected to homology-based similarity search using BLASTx and BLASTp against the latest non-redundant protein sequence (nr) database in the DIAMOND alignment tool (Galaxy version 2.0.15) (Buchfink et al., 2014). Hmmscan (Galaxy version 3.4) (Finn et al., 2011) searches were conducted using the protein family database (Pfam-A) database as a query, and TMHMM software (Galaxy version 0.0.17) (Cock et al. 2013) was used for transmembrane domains detection. Trinotate (Galaxy version 3.2.2) (Grabherr et al., 2011) integrated the BLAST, hmmscan, and TMHMM results.

A BLASTp search (Camacho et al., 2009) using *F. hepatica*'s ASCT gene (accession no. ACF06126.1) (van Grinsven et al., 2009) as a query was conducted against the nr database of *A. cantonensis*. The GenBank accession number of the top BLASTp hit was retrieved

and searched against the Trinotate integrated annotation results previously obtained. The gene sequence of the putative *Am*ASCT was retrieved from the Trinotate result for further analysis.

*Validation of the Obtained A*m*ASCT*

Several bioinformatic tools and molecular techniques were employed to validate the identified *Am*ASCT. The initial bioinformatic validation involved utilising BLASTx search by using the obtained putative *Am*ASCT gene sequence as a query against the nr database of *F. hepatica* to ensure whether the putative *Am*ASCT is a homolog to the previously characterised ASCT of *F. hepatica* (accession no. ACF06126.1) (van Grinsven et al., 2009). In addition, ORFfinder in the National Center for Biotechnology Information (NCBI) websites (https://www.ncbi.nlm.nih.gov/orffinder/) was used to identify and analyse the open reading frame (ORF) of the *Am*ASCT gene, aiding in the prediction of coding regions and potential functional domains.

Following the bioinformatic validation, molecular techniques were employed to confirm the presence of the putative *Am*ASCT gene at the transcript and deoxyribonucleic acid (DNA) levels. Polymerase chain reaction (PCR), agarose gel electrophoresis, and Sanger sequencing were conducted. Briefly, adult *A. malaysiensis* worms collected from Low et al. (2023) were used for total ribonucleic acid (RNA) extraction using Trizol reagent (Thermo Fisher Scientific, USA), followed by cDNA synthesis using RevertAid™ First Strand cDNA Synthesis Kit (Toyobo, Japan) according to the manufacturer's instructions. Subsequently, the cDNA was used in polymerase chain reaction (PCR) amplification using a set of primers (forward: 5'-ATGCTGTGTCGGCTCTCATCC-3' and reverse: 5'-TTAGTCCACTTCAAGGCATC-3'). PCR was carried out in a thermal cycler with the cycling conditions set as follows: 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. The cycling condition was ended with a final extension at 72°C for 5 min. Next, 1.5% agarose mixed with 1x TAE buffer was prepared, and the PCR product was electrophoresed (400W/80V) for 60 minutes and visualised under a UV transilluminator. Each primer's PCR product and 10 µl (10 µM) were outsourced to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for purification and sanger sequencing.

*Physiochemical Profiling of A*m*ASCT*

The putative *Am*ASCT's gene sequence was retrieved and subjected to physiochemical characterisation using ExPASy's ProtParam tool (Wilkins et al., 1999). The default configuration of the ProtParam tool was utilised to obtain the putative *Am*ASCT's molecular mass, theoretical isoelectric point (pI), amino acid composition, aliphatic index, and grand average of hydropathy (GRAVY).

Phylogenetic Analysis

Retrieval of ASCT Protein Sequences from Other Helminths

Before multiple sequence alignment and phylogenetic analysis, ASCT gene sequences from other helminth parasites were retrieved from the NCBI GenBank. BLASTp searches using previously characterised ASCTs of *T. brucei* (accession no. EAN79240) (Rivière et al., 2004), *F. hepatica* (accession no. ACF06126.1) (van Grinsven, 2009), and *T. vaginalis* (accession no. XP_001330176) (van Grinsven, 2008) were conducted against the nr database of helminth parasites. Sequences with an E value smaller than 20 (E^{-20}) were retrieved. If multiple parasites of the same species were found as a hit, only the sequence with the highest hit was retrieved.

Multiple Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignment was conducted using the putative *Am*ASCT obtained in this study, the previously characterised ASCT protein sequences of *T. brucei*, *F. hepatica*, and *T. vaginalis*, and the retrieved ASCT protein sequences from other helminth parasites. Multiple sequence alignment was carried out using ClustalW (Thompson et al., 1994), and all sequences without the conserved ExG and GxGGxxD motifs of the family I CoA-transferases were removed. The aligned protein sequences of ASCTs were used for phylogenetic tree construction through the neighbour-joining (NJ) method in Molecular Evolutionary Genetics Analysis version 11 (MEGA11) software (Tamura et al., 2021). Pairwise comparisons to infer genetic variation within and between the three subfamilies of the family I CoA-transferases were also performed using MEGA11 software.

RESULTS AND DISCUSSION

Identification of the *A. malaysiensis* ASCT Gene Sequence

The ASCT sequence of *A. malaysiensis* was identified from our ongoing transcriptomic data analysis of adult *A. malaysiensis*. A BLASTP search conducted against the nr database of A. cantonensis using the previously characterised ASCT of *F. hepatica* (accession no. ACF06126.1) resulted in one blast hit (accession no. KAE9415559.1) that could be the putative ASCT of *A. cantonensis*. The previously characterised ASCT of *F. hepatica* was used as a query since it is a helminth parasite that could have genetic information similar to that of *A. malaysiensis*. In contrast, the other previously characterised parasites, *T. brucei* and *T. vaginalis*, are parasitic protists. Moreover, the nr database of *A. cantonensis* was searched against this gene due to the minimal protein and nucleotide sequences of *A. malaysiensis* being available in public databases. In addition, the reference genome of *A. malaysiensis* has yet to be sequenced. In contrast, the reference genome of *A. cantonensis*

is available in the NCBI GenBank (accession no. MQTX01000177.1, Xu et al., 2019). In addition, *A. cantonensis* is closely related to *A. malaysiensis,* and their morphological and genetic information is highly similar (Chan et al., 2020; Eamsobhana et al., 2015).

The gene annotation data from the transcriptomic data analysis of *A. malaysiensis* were searched for the gene homolog to the obtained putative *A. cantonensis* ASCT (accession no: KAE9415559.1). However, no similarities were found. For this reason, the putative *A. cantonensis* ASCT (accession no: KAE9415559.1) was further used as a query protein in a BLASTP search against the nr database to find other homologues. The top five hits of the BLASTP search (accession no. KAJ1347515.1, EYC13181.1, KIH58849.1, EYC13183.1, and EYC13182.1) were used further to search the transcriptomic gene annotation data of *A. malaysiensis*. A gene was found to be the homolog of the accession no. EYC13181.1, which could be the putative *A. malaysiensis* ASCT (*Am*ASCT). The complete nucleotide length and the open reading frame (ORF) amino acid sequence length of the putative AmASCT gene were 1413 base pairs (bp) and 470 amino acids (aa), respectively (Figure 1). A BLASTP search conducted using the putative *Am*ASCT protein sequence as a query revealed high similarity with a few other helminth parasites, with *A. cantonensis* (accession no. KAE9415559.1) exhibiting the highest similarity, followed by *Parelaphostrongylus tenuis* (KAJ1347515.1) and *Ancylostoma ceylanicum* (EYC13181). However, these homolog protein hits were described as hypothetical proteins because the gene has never been characterised in any of these helminths.

The putative AmASCTs were annotated as acetyl-CoA hydrolases or acetyl-CoA transferase N-terminal domains according to the protein family database (Pfam) annotation from the transcriptomic gene annotation data. Acetyl-CoA hydrolase (ACH) is one of the four known enzymes catalysing acetate formation from acetyl-CoA. The activity of ACH has been described in plants and animals (Hunt & Alexson, 2008; Zeiher & Randall, 1990). Although ACH activity has been reported in *Ascaris suum parasites* (de Mata et al., 1997), the complementary gene of this enzyme in this nematode parasite has yet to be identified. The CoA-transferase enzyme was previously confused with ACH in *Saccharomyces cerevisiae* (Lee et al., 1989, 1990); this enzyme was subsequently recharacterised as a CoA-transferase due to its minor hydrolase activity and was found to catalyse the transfer of the CoA moiety from succinyl-CoA to acetate, which is a particular characteristic of CoA-transferases (Fleck & Brock, 2009). One of the apparent characteristics of CoA-transferases that is absent in CoA-hydrolases is the glutamate active site for the formation of CoA-ester intermediates via a ping-pong bibi mechanism (Heider, 2001; White & Jencks, 1976). When the glutamate active site was replaced, CoA-transferase was found to be converted into a CoA-hydrolase (Mack & Buckel, 1997).

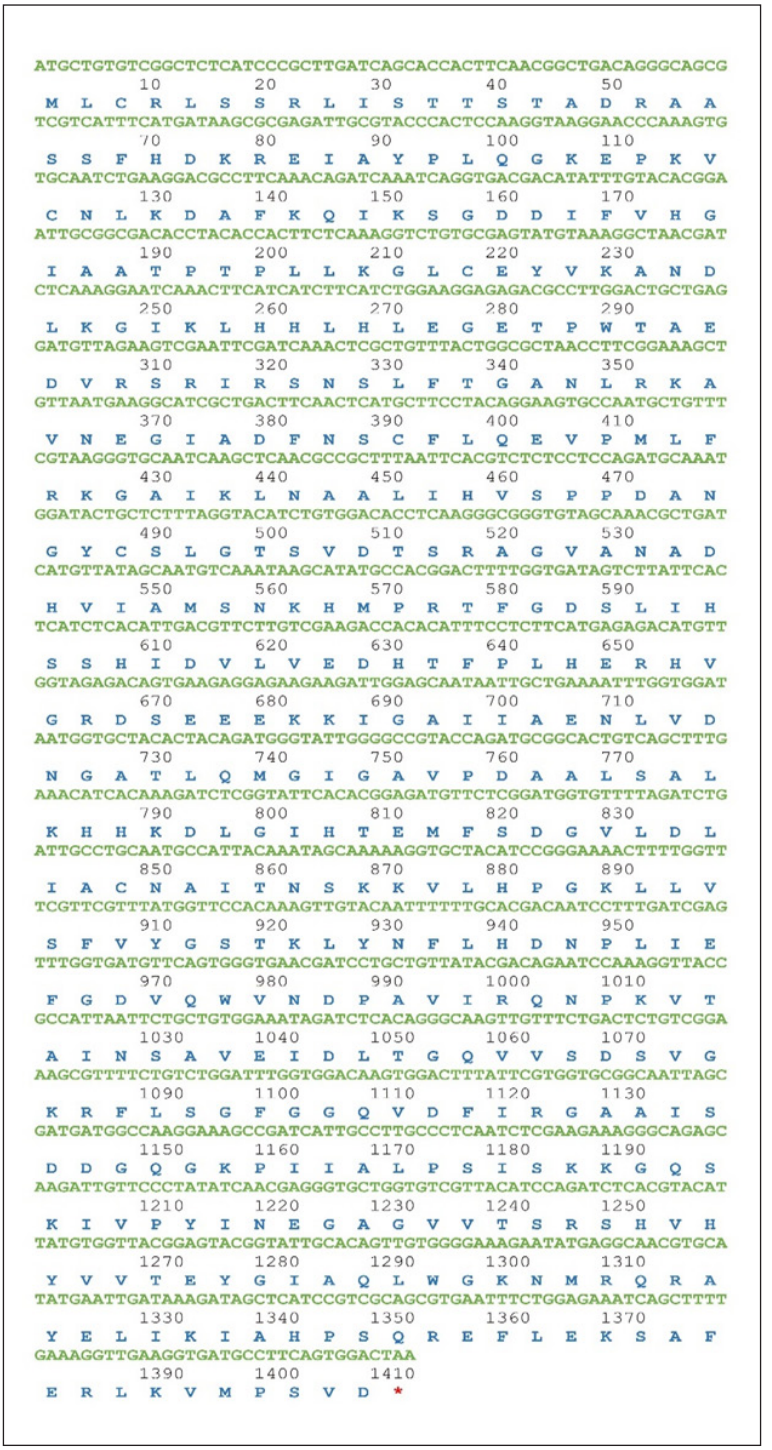

Figure 1. Full-length nucleotide sequence and ORF of the putative *Angiostrongylus malaysiensis* ASCT. The coding region comprises 1413 bp (green) of nucleotides and 470 aa (blue)

Validation of The Obtained Putative *A. malaysiensis* ASCT

Upon conducting the BLASTx search, the putative *Am*ASCT exhibited the ASCT gene of *F. hepatica* as the top hit, indicating a significant alignment and similarity between the two sequences. This BLASTx result demonstrates a high degree of homology between the putative *Am*ASCT and the previously characterised ASCT gene of *F. hepatica* (accession no. ACF06126.1). The positive amplification of the *Am*ASCT gene was confirmed through both PCR and agarose gel electrophoresis. Upon subjecting the PCR product to electrophoresis and visualising it under a UV transilluminator, a distinct and well-defined band was observed at the expected size, indicative of successful amplification of the *Am*ASCT gene and the presence of the gene in transcript and DNA levels. In addition, the obtained Sanger sequencing result of the amplified PCR product confirmed the identity of the putative *Am*ASCT, providing a similar representation of the coding sequence of the putative *Am*ASCT obtained from the de novo transcriptome annotation of adult *A. malaysiensis*. These bioinformatic and molecular approach results validate the reliability of the obtained putative *Am*ASCT gene sequence from the de novo transcriptome annotation data.

Physiochemical Profiling of AmASCT

The physiochemical properties of *Am*ASCT were analysed to understand and predict the enzyme function and behaviour under different conditions. The physiochemical profiling of *Am*ASCT conducted using ExPASy's ProtParam tool revealed several crucial molecular characteristics of *Am*ASCT (Table 1). The computational analysis predicted that *Am*ASCT has a molecular weight of 51.33 kDA, suggesting that the enzyme has a moderate size. The theoretical isoelectric point (pI) of 7.74 indicates that the *Am*ASCT is likely to be neutral or slightly alkaline, which suggests that *Am*ASCT is most stable under such conditions within the parasite. The high aliphatic index (93.79) implies that *Am*ASCT is enriched in hydrophobic amino acids and its adaptation and stability to functioning in hydrophobic environments. Also, the GRAVY score (-0.162) of *Am*ASCT indicates the hydrophilic nature of the enzyme, contributing to its solubility in aqueous environment. Despite containing enriched hydrophobic amino acids as indicated by the high aliphatic index, the overall hydrophilicity of *Am*ASCT may be because of the distribution of hydrophilic residues and charged or polar amino acids presence on its surface.

Table 1
Physiochemical profiling of AmASCT

No. of amino acids	Molecular weight (kDA)	Theoretical isoelectric point (pI)	Aliphatic index	GRAVY
470	51.33	7.74	93.79	- 0.162

Retrieval of ASCT from Helminth Parasites from GenBank

The retrieved amino acid sequences of helminth parasites were subjected to BLASTP searches against the non-redundant helminth protein database using *F. hepatica* (accession no. ACF06126.1), *T. vaginalis* (accession no. XP_001330176), and *T. brucei* (accession no. EAN79240), which represent the three subfamilies of the family I CoA-transferase, as tabulated in Table 1. BLASTP searches revealed 15, 22 and two amino acid sequences of subfamilies IA, IB and IC, respectively, of the family I CoA-transferase. The retrieved amino acid sequence of the helminth parasites in Table 2 shows that most helminth parasites

Table 2
Helminth parasites and their accession numbers of the family I CoA-transferase amino acid sequences with E values less than $1e^{-20}$ *retrieved from the NCBI GenBank*

Subfamily A	Subfamily B		Subfamily C
Ancylostoma ceylanicum (EYC45313)	*Fasciola hepatica* (ACF06126)	*Litomosoides sigmodontis* (VDK78337)	*Caenorhabditis remanei* (EFO99043)
Aphelenchoides bicaudatus (KAI6175034)	*Fasciola gigantica* (TPP58613)	*Toxocara canis* (KHN81999)	*Diploscapter pachys* (PAV69794)
Bursaphelenchus okinawaensis (CAD5205821)	*Heterobilharzia americana* (CAH8437700)	*Bursaphelenchus xylophilus* (CAD5213378)	
Nippostrongylus brasiliensis (VDL75362)	*Schistosoma mansoni* (XP_018648509)	*Acanthocheilonema viteae* (VBB34274)	
Caenorhabditis briggsae (XP_002629737)	*Trichobilharzia regent* (CAH8830533)	*Brugia malayi* (XP_042934641)	
Caenorhabditis elegans (NP_496144)	*Strongyloides ratti* (XP_024501800)	*Pristionchus pacificus* (KAF8362703)	
Meloidogyne enterolobii (CAD2169927)	*Caenorhabditis remanei* (EFP07577)	*Mesocestoides corti* (VDD82102)	
Parelaphostrongylus tenuis (KAJ1371690)	*Parelaphostrongylus tenuis* (KAJ1347515)	*Taenia asiatica* (VDK22328)	
Pristionchus pacificus (KAF8360361)	*Angiostrongylus cantonensis* (KAE9415559)	*Hymenolepis microstoma* (CUU98198)	
Ditylenchus destructor (KAI1702014)	*Enterobius vermicularis* (VDD96370)	*Echinococcus multilocularis* (CDS43154)	
Dracunculus medinensis (VDN53078)	*Steinernema carpocapsae* (TMS35399)	*Rodentolepis nana* (VDO13213)	
Strongyloides ratti (XP_024505624)			
Paragonimus westermani (KAF8572391)			
Clonorchis sinensis (KAG5445983)			
Opisthorchis felineus (TGZ64319)			

have the CoA-transferase enzyme of subfamily IB, followed by subfamily IA and only two in subfamily IC. This finding is in agreement with that of Tielens et al. (2010), who reported that the subfamily IA is found only in trypanosomatid parasites and some metazoan parasites, while the subfamily IC of ASCT is usually possessed only by prokaryotes and fungi. On the other hand, most of the ASCT enzymes of helminth parasites were previously reported to be in subfamily IB of family I CoA-transferases (Tielens et al., 2010).

Multiple Sequence Alignment of Helminth ASCT

Figure 2 presents the multiple amino acid sequence alignments of the putative ASCT gene of *A. malaysiensis* obtained in this study with amino acid sequences of the ASCT gene of other helminth parasites retrieved from the BLASTP searches and the ASCTs of previously characterised parasites. The helminth parasites were grouped into subfamilies based on the query ASCT sequence. Note that although *T. brucei* and *T. vaginalis* are parasitic protists, their ASCT proteins were included in this study as references since their ASCT genes were previously characterised.

According to the multiple amino acid sequence alignments, all the ASCT enzymes of helminth parasites included in this study contained the conserved ExG and GxGGxxD motifs of the ASCTs in the family I CoA-transferases (Tielens et al., 2010). The subfamily

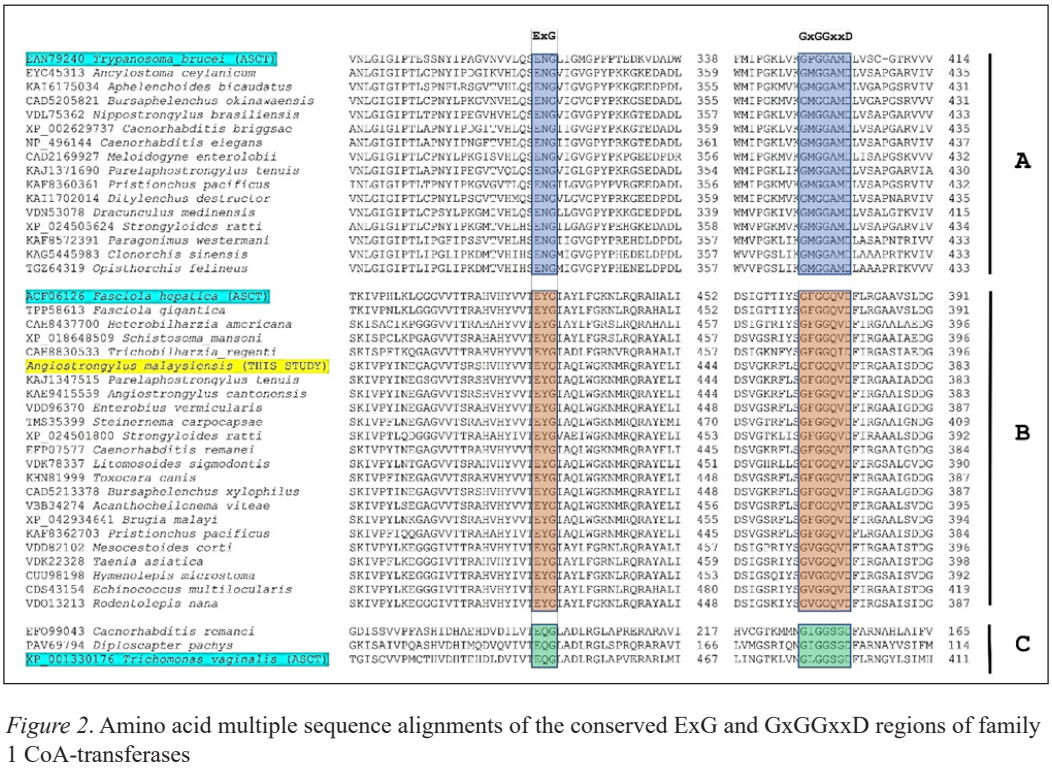

Figure 2. Amino acid multiple sequence alignments of the conserved ExG and GxGGxxD regions of family 1 CoA-transferases

IAs of ASCTs have a broader SENG motif since they are grouped with the mammalian succinyl-CoA:3-ketoacid CoA transferase (SCOT) enzyme (Rivière et al., 2004). This conserved SENG motif contains the glutamate residue active site of CoA-transferase (Rangarajan et al., 2005). On the other hand, ASCTs in subfamily IB and subfamily IC do not have the conserved SENG motif but instead possess a conserved ExG motif, which is believed to be a constituent of the more prominent SENG motif found within subfamily IA (Tielens et al., 2010). All the subfamilies of ASCT have the conserved GxGGxxD motif, an integral component of the oxyanion hole (Rangarajan et al., 2005; Tielens et al., 2010). The relative position of the GxGGxxD motif to the ExG motif was consistent between subfamily IB and IC. However, there was a notable difference in the expression of subfamily IA. In subfamily IA, the ExG motif was located proximal to the N-terminal region, whereas the GxGGxxD motif was situated toward the C-terminal region. The opposite trend occurs in subfamilies IB and IC.

Phylogenetic Analysis of Helminth ASCT

The phylogenetic tree constructed from the multiple amino acid alignment of ASCTs from helminth parasites is presented in Figure 3. The phylogenetic tree showed that the ASCTs of helminth parasites consisted of two clades: subfamilies IB and IC were in the same clade, while subfamily IA was in the other clade. Subfamilies IB and IC were divided into two distinct clusters within their clades. The phylogenetic tree in Figure 2 shows that subfamilies IB and IC are closer to each other than to subfamily IA. The putative *Am*ASCT obtained in this study clustered with the previously characterised ASCT of *F. hepatica* in subfamily IB.

The CoA-transferases responsible for the generation of acetate in parasites have been well-recognised as succinate-dependent enzymes and are thus referred to as acetate/succinate CoA-transferases. The CoA moiety of acetyl-CoA was transferred to succinate, resulting in the production of acetate and succinyl-CoA. To date, all eukaryotic ASCTs found are family I CoA-transferases. These enzymes are distinguished by their ability to transfer CoA groups, which involve a glutamate residue located in the active region of the enzyme (Heider, 2001). The identification and characterisation of the ASCT gene have been conducted in the helminth parasite *F. hepatica* (van Grinsven et al., 2009) and two other protist parasites, *T. brucei* (Rivière et al., 2004) and *T. vaginalis* (van Grinsven et al., 2008). The ASCT genes from these three parasites were found to share little homology, thus characterising them into three different subfamilies of the family I CoA-transferases (Tielens et al., 2010).

The ASCT enzymes of *T. brucei* were classified into subfamily IA of the family I CoA-transferases. ASCT in subfamily IA was found to exhibit significant homology to SCOT. This enzyme plays a crucial role in using ketone bodies inside the mitochondria of the human brain and muscle (Fukao et al., 2004). Among the three subfamilies, only

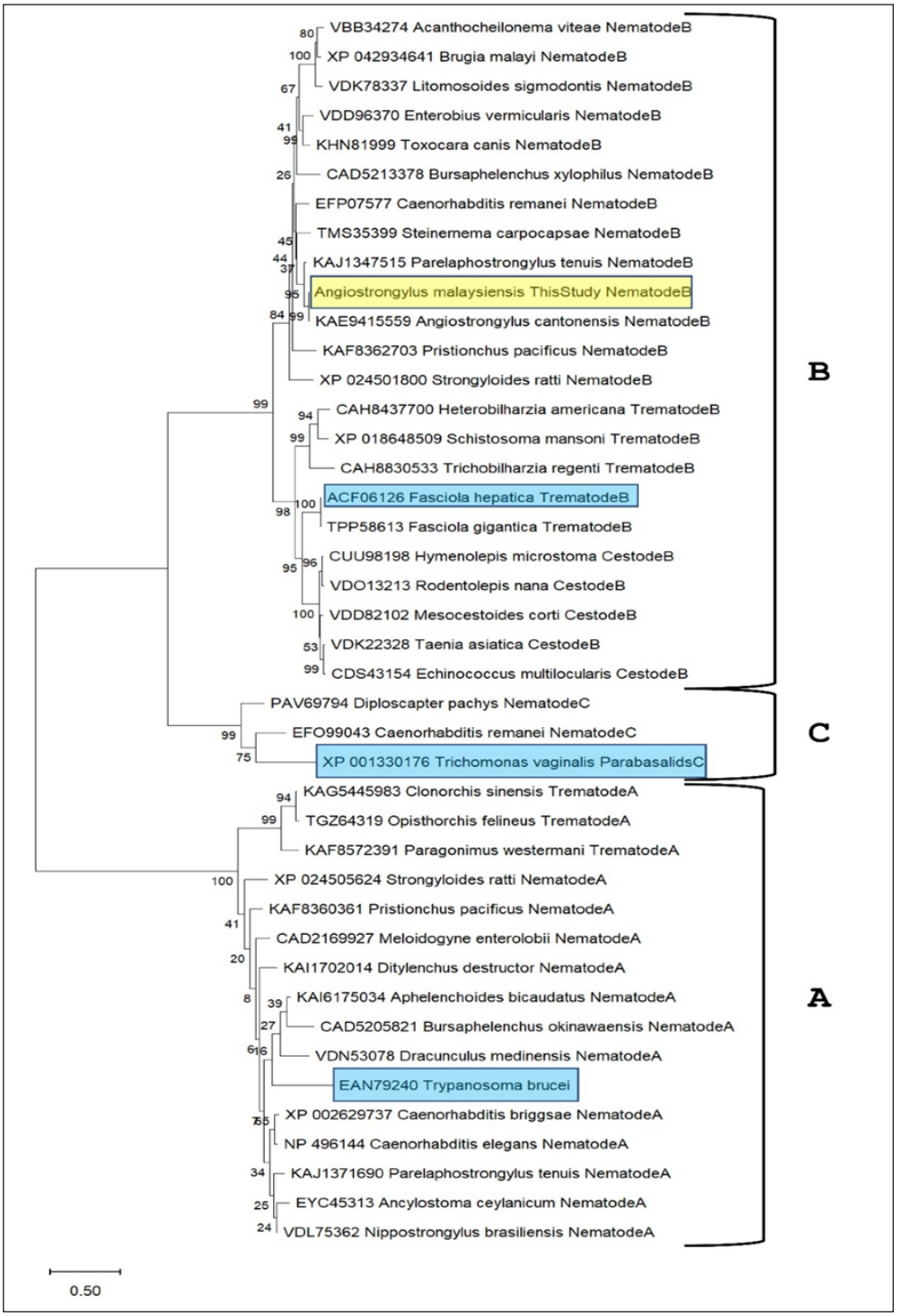

Figure 3. Phylogenetic tree of the ASCT gene of helminth parasites

subfamily IA exhibited homology to enzymes present in mammals. ASCT in subfamily IA occurs in the aerobic mitochondria of trypanosomatids and other metazoans (Rivière et al., 2004). The ASCT enzyme of *F. hepatica* was previously classified into subfamily IB and occurs in the anaerobically functioning mitochondria of metazoan organisms. The ASCTs of parasitic helminths mainly belong to subfamily IB of the family I CoA-transferases (Tielens et al., 2010). ASCT of *T. vaginalis* was classified into the subfamily IC. Unlike those in subfamilies IA and IB, ASCT in subfamily IC did not occur in mitochondria but in anaerobic hydrogenosomes (van Grinsven et al., 2008). Hydrogenosomes are membrane-bound organelles that are involved in the production of hydrogen. These organelles are closely linked to mitochondria, although they develop independently in different protists (Cavalier-Smith & Chao, 1996).

A previous study revealed the possibility that hydrogenosomes and mitochondria are evolutionarily related and that hydrogenosomes have undergone evolutionary changes due to adaptation to anaerobic environments (Martin & Muller, 1998; Roger et al., 1996). The exact evolutionary relationship between mitochondria and hydrogenosomes is currently disputed in the academic community. ASCT coupled with the succinyl-CoA synthase (SCS) cycle is the only catabolic pathway identified in mitochondria and hydrogenosomes. Nevertheless, based on the phylogenetic tree (Figure 3), the mitochondrial ASCT of *T. brucei* in subfamily IA lacks similarity with the hydrogenosomal ASCT of *T. vaginalis* in subfamily IC, as does the mitochondrial ASCT of *F. hepatica* in subfamily IB. Hence, if both mitochondria and hydrogenosomes are evolutionarily related, it may be inferred that the occurrence of ASCT enzyme activity within organelles derived from endosymbiotic events has separately undergone convergent evolution on at least two separate occasions. Additionally, based on the multiple sequence alignment previously discussed in this study (Figure 2), there was a significant difference in the relative location of the oxyanion hole GxGGxxD motif and the active site ExG motif between subfamily IA and subfamilies IB and IC. The positioning of the two conserved motifs is the same in subfamilies IB and IC but different in subfamily IA (Tielens et al., 2010). This relative location of both conserved regions may contribute to positioning the three subfamilies in the phylogenetic tree.

Pairwise analyses were also conducted to infer genetic variations between and within the three subfamilies of the family I CoA-transferases. The genetic variations within subfamilies ranged from 0.04% to 0.85% for subfamily IA (Table 3), 0.005% to 0.78% for subfamily IB (Table 4), and 0.56% to 0.63% for subfamily IC (Table 5). In subfamily IA, the highest genetic variation between helminths was observed between *Caenorhabditis briggsae* and *Opistorchis felineus* (0.760%), while the lowest was observed between *Clonorchis sinensis* and *Opisthorchis felineus* (0.039%). In subfamily IB, the highest genetic variation between helminths was observed between *Trichobilharzia regenti* and *P. pacificus* (0.781%), while the lowest variation was observed between *Fasciola*

Table 3
Pairwise genetic variation in percentage (%) for helminth parasites in subfamily IA*

	T.b	**A.c**	**A.b**	**B.o**	***N.b***	***C.b***	***C.e***	***M.e***	***P.t***	***P.p***	***D.d***	***D.m***	***S.r***	***P.w***	***C.s***	***O.f***
*T.*b																
A.c	0.663															
A.b	0.648	0.341														
B.o	0.665	0.351	0.228													
N.b	0.664	0.112	0.341	0.355												
C.b	0.713	0.149	0.355	0.355	0.176											
C.e	0.720	0.141	0.342	0.348	0.174	0.061										
M.e	0.663	0.324	0.307	0.315	0.329	0.359	0.354									
P.t	0.671	0.172	0.345	0.361	0.163	0.183	0.187	0.345								
P.p	0.680	0.211	0.342	0.338	0.209	0.206	0.197	0.315	0.262							
D.d	0.696	0.314	0.318	0.341	0.325	0.332	0.324	0.268	0.321	0.277						
D.m	0.670	0.336	0.363	0.375	0.346	0.352	0.341	0.361	0.368	0.318	0.335					
S.r	0.706	0.351	0.411	0.410	0.378	0.353	0.335	0.396	0.368	0.366	0.408	0.395				
P.w	0.802	0.671	0.591	0.645	0.651	0.717	0.707	0.664	0.679	0.681	0.662	0.643	0.658			
C.s	0.827	0.682	0.623	0.669	0.683	0.747	0.709	0.663	0.670	0.691	0.678	0.638	0.684	0.289		
O.f	0.846	0.680	0.628	0.667	0.673	0.760	0.725	0.656	0.676	0.702	0.685	0.649	0.706	0.272	0.039	

Note. T.*b = Trypanosoma brucei*, *A.c = Ancylostoma ceylanicum*, *A.b = Aphelenchoides bicaudatus*, *B.o = Bursaphelenchus okinawaensis*, *N.b = Nippostrongylus brasiliensis*, *C.b = Caenorhabditis briggsae*, *C.e = Caenorhabditis elegans*, *M.e = Meloidogyne enterolobii*, *P.t = Parelaphostrongylus tenuis*, *P.p = Pistionchus pacificus*, *D.d = Ditylenchus destructor*, *D.m = Dracunculus medinensis*, *S.r = Strongyloides ratti*, *P.w = Paragonimus westermani*, *C.s = Clonorchis sinensis*, *O.f = Opisthorchis felineus*

Table 4
Pairwise genetic variation in percentage (%) for helminth parasites in subfamily IB*

	F.h	**F.g**	**H.a**	**S.m**	**T.r**	**A.m**	**P.t**	**A.c**	**E.v**	**S.c**	**S.r**	**C.r**	**L.s**	**T.c**	**B.x**	**A.v**	**B.m**	**P.p**	**M.c**	**T.a**	**H.m**	**E.m**	**R.n**
F.h																							
F.g	0.005																						
H.a	0.388	0.392																					
S.m	0.378	0.375	0.170																				
T.r	0.463	0.468	0.277	0.362																			
A.m	0.597	0.597	0.680	0.637	0.689																		
P.t	0.583	0.583	0.680	0.667	0.701	0.057																	
A.c	0.597	0.596	0.681	0.636	0.690	0.005	0.062																
E.v	0.588	0.593	0.667	0.658	0.692	0.242	0.240	0.246															
S.c	0.611	0.617	0.683	0.653	0.705	0.185	0.169	0.189	0.252														
S.r	0.631	0.629	0.698	0.689	0.737	0.346	0.327	0.349	0.355	0.345													
C.r	0.632	0.629	0.669	0.645	0.671	0.174	0.175	0.177	0.281	0.201	0.322												
L.s	0.645	0.644	0.680	0.657	0.714	0.303	0.317	0.302	0.266	0.333	0.381	0.306											
T.c	0.602	0.607	0.670	0.652	0.687	0.198	0.179	0.202	0.129	0.224	0.331	0.220	0.247										
B.x	0.648	0.644	0.690	0.704	0.710	0.281	0.264	0.280	0.290	0.291	0.341	0.291	0.330	0.272									
A.v	0.642	0.641	0.680	0.640	0.720	0.307	0.326	0.304	0.277	0.337	0.386	0.326	0.113	0.271	0.352								
B.m	0.645	0.644	0.682	0.648	0.728	0.300	0.308	0.297	0.247	0.325	0.374	0.290	0.098	0.241	0.328	0.082							
P.p	0.696	0.702	0.725	0.714	0.781	0.316	0.318	0.321	0.295	0.291	0.353	0.275	0.374	0.291	0.401	0.372	0.348						
M.c	0.296	0.295	0.441	0.465	0.505	0.614	0.594	0.610	0.622	0.602	0.632	0.629	0.657	0.633	0.661	0.640	0.646	0.691					
T.a	0.292	0.292	0.432	0.447	0.519	0.634	0.612	0.630	0.642	0.613	0.647	0.656	0.669	0.639	0.658	0.652	0.658	0.701	0.062				
H.m	0.291	0.290	0.444	0.461	0.518	0.613	0.594	0.610	0.614	0.608	0.629	0.629	0.644	0.621	0.664	0.635	0.641	0.693	0.078	0.081			
E.m	0.299	0.298	0.442	0.457	0.521	0.642	0.626	0.637	0.646	0.629	0.653	0.658	0.675	0.643	0.663	0.658	0.666	0.702	0.062	0.025	0.081		
R.n	0.295	0.294	0.463	0.465	0.522	0.620	0.602	0.615	0.623	0.612	0.631	0.639	0.652	0.636	0.671	0.640	0.647	0.695	0.070	0.073	0.028	0.070	

Note. F.h = Fasciola hepatica, F.g = Fasciola gigantica; *H.a = Heterobilharzia americana*; *S.m = Schistosoma mansoni*; *T.r = Trichobilharzia regent*; *A.m = Angiostrongylus malaysiensis*; *P.t = Parelaphostrongylus tenuis*; *A.c = Angiostrongylus cantonensis*; *E.v = Enterobius vermicularis*; *S.c = Steinernema carpocapsae*; *S.r = Strongyloides ratti*; *C.r = Caenorhabditis remanei*; *L.s = Litomosoides sigmodontis*; *T.c = Toxocara canis*; *B.x = Bursaphelenchus xylophilus*; *A.v = Acanthocheilonema viteae*; *B.m = Brugia malayi*; *P.p = Pristionchus pacificus*; *M.c =Mesocestoides corti*; *T.a = Taenia asiatica*; *H.m = Hymenolepis microstoma*; *E.m = Echinococcus multilocularis*; *R.n = Rodentolepis nana*

hepatica and *Fasciola gigantica* (0.005%). Similarly, low variation was observed between *Angiostrongylus malaysiensis* and *Angiostrongylus cantonensis* (0.005%). Although both *C. briggsae* and *O. felineus* are in subfamily IA, *C. briggsae* is a nematode helminth, while *O. felineus* is a trematode. The same is true for *T. regenti* and *P. pacificus* in subfamily IB; *T. regenti* is a trematode, while *P. pacificus* is a nematode. Moreover, *Clonorchis sinensis* and *Opisthorchis felineus* in subfamily IA are both trematodes. In subfamily IB, *F. hepatica* and *F. gigantica* are parasites of the same genus, similar to *A. malaysiensis* and *A. cantonensis*, which explains the slight variation observed between these parasites. There was high genetic variation between parasites in the subfamily IC (Table 5). However, only two ASCT sequences of helminth parasites were found with the addition of the previously characterised ASCT of *T. vaginalis*.

Table 5
Pairwise genetic variation in percentage (%) for helminth parasites in subfamily IC*

	C.r	**D.p**	**T.v**
C.r			
D.p	0.555		
T.v	0.627	0.625	

Note. C.r = Caenorhabditis remanei; *D.p = Diploscapter pachys*; *T.v = Trichomonas vaginalis*

Table 6
Pairwise genetic variation between subfamilies IA, IB, and IC

	IA	**IB**	**IC**
IA			
IB	3.828		
IC	3.651	1.933	

A pairwise genetic comparison between the subfamilies was also conducted, where subfamilies IB and IC were found to be most closely related to each other, with a mean genetic variation between the two groups of 1.933%. Moreover, subfamily IA was found to have more significant genetic variation than both subfamily IB (3.828%) and subfamily IC (3.651) (Table 6). The three subfamilies of the family I CoA-transferases exhibit only remote genetic relationships. Notably, subfamilies 1B and 1C demonstrated a closer genetic affinity to each other than to subfamily 1A.

CONCLUSION

The putative *Am*ASCT identified in this study possessed the ExG motif, which contains the active site's conserved glutamate residue, and the conserved GxGGxxD motif, which is part of the oxyanion hole. Both motifs are characteristic of the family I CoA-transferases. The phylogenetic tree showed that the putative *Am*ASCT gene was clustered with the previously characterised subfamily IB gene of F. hepatica ASCT, suggesting that the putative *Am*ASCT gene is a subfamily IB gene of the family I CoA-transferase. Acetate, a significant byproduct of energy metabolism in many parasites but not in their mammalian hosts, presents an appealing opportunity to advance novel antiparasitic medications. Further biochemical characterisation of this enzyme in rat lungworms and other parasites

is crucial for further understanding its role in parasite energy metabolism for survival in mammalian hosts.

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