

**Fig. 7.** Maximum likelihood (ML) phylogeny of the replicase amino acid sequences of benyvirus and their related CaBRLSs and RpBRLSs. Phylogenetic trees were constructed using PhyML 3.0 based on the multiple amino acid sequence alignment of the methyltransferase (MTF) (A), RNA helicase (HEL) (B), and RNA-dependent RNA polymerase (RdRp) (C) domains shown in Supplementary Fig. S3E, F and G, respectively. A model LG with +I+G+F was selected as best-fit model for each alignment. The nodes with filled circles are supported by aLRT values of greater than 0.9. The scale bars represent the amino acid distances. Formal virus names and their GenBank accession numbers of the sequences (presented as acronyms) are listed in Supplementary Table S2. Asterisks show the viruses with unassigned species.

GACH01053674, GACH01115352;  $E$ -value =  $2e^{-5}$ – $4e^{-14}$ , identities = 40–47% in the TSA library of the Siam weed (*Chromolaena odorata*, family Asteraceae), one match (GeneBank accession JY146193;  $E$ -value =  $4e^{-10}$ , identity = 30%) in the TSA library of the European silver fir tree (*Abies alba*, family Pinaceae), and one match (GeneBank accession GACR01000094;  $E$ -value =  $1e^{-30}$ , identity = 40%) in the TSA library of a tree killing bark beetle (*Ips typographus*, family Scolytidae). In addition, one EST sequence (GeneBank accession FG537634) from the shoot apical meristem of the pea plant (*Pisum sativum*) also showed a moderate aa identity ( $E$ -value =  $7e^{-17}$ , identities = 32%) but with possible internal deletion. However, no significant hits for CAV or benyvirus protein-like sequences in the available databases of the algae were revealed. Instead, a sequence (GeneBank accession DV668917) in the EST library of a brown alga (*Sargassum binderi*, Phaeophyta) was detected to share a moderate level of identity with closteroviruses (family Closteroviridae), i.e. citrus tristeza virus ( $E$ -value =  $4e^{-19}$ , identity = 31%).

#### 4. Discussion

In this work, we have determined the complete nucleotide sequences of BdMoV that was isolated from burdock plants in Japan. On the basis of sequence similarities and genome organization, we propose that BdMoV is a new species in the genus *Benyvirus* and could represent the fourth member of this genus. Phylogenetic analyses show that BdMoV is more closely related to BNYVV and BSBMV than RSNV (Table 1 and Fig. 3A).

BNYVV and BSBMV usually require smaller RNA species RNA3 and RNA4 for disease development and viral survival in nature, whereas BdMoV and probably RSNV do not contain such smaller RNAs (Lozano and Morales, 2009), although this requires confirmation with a number of natural virus isolates. BSBMV supported BNYVV-RNA3 replication (Ratti et al., 2009), however attempts to superinfect BdMoV-infected plants at 20–30 days after inoculation

with RNA3 or RNA4 failed (T. Tamada, unpublished results). Considering that BNYVV RNA1 and RNA2 are sufficient for viral replication, assembly, movement and transmission (Richards and Tamada, 1992; Tamada, 1999), it is clear that the benyviruses intrinsically have a bipartite genome.

Three benyviruses BNYVV, BSBMV and RSNV are transmitted by *Polymyxa* species belonging to the family Plasmodiophoraceae. Our preliminary experiments showed that BdMoV was not transmitted by *Polymyxa betae*, which is a vector of BNYVV and BSBMV. We also found that the burdock plant is not a suitable host for *P. betae* (T. Tamada, unpublished results). Thus, it seems unlikely that *P. betae* is the natural vector of BdMoV. The vector of BdMoV still needs to be determined; however, owing to a similarity in the genome structure and especially the presence of the two transmembrane regions in the RT domain (Adams et al., 2001; Tamada and Kondo, 2013), it is possible that BdMoV is also transmitted by the plasmodiophorid species. In this respect, we could not rule out the possibility that BdMoV studied in this study carries a deletion in the RT domain that may have occurred during preparation of its inoculum from *C. quinoa* inoculated mechanically. Further studies using other natural isolates or infectious clones are required to verify the role of this domain in vector transmission.

In regard to cytopathic effects, BdMoV induces the formation of viroplasm-like inclusion bodies in the cytoplasm of infected cells. These inclusion bodies are spherical-shaped, electron-dense, and granular structures which contain electron-lucent voids with various sizes (Inouye, 1973; Yamashita et al., 2008). Rod-shaped particles of BdMoV are usually arranged radially from the surface of these inclusions (like sea urchin spine) but are not observed within the inclusion body. Apart from the inclusion body, small bundles of particles which are arranged in parallel are occasionally observed in the cytoplasm of infected cells (Inouye, 1973). So far it has been unknown whether these kinds of viroplasm-like structures are induced by infection of BNYVV or other rod-shaped viruses (Yamashita et al., 2008). In the case of BNYVV, viral particles are usually scattered or clustered in aggregates in the cytoplasm of

infected cells, but sometimes dense masses of particles arranged in parallel or angle-layer arrays are observed (Putz and Vuittenez, 1980; Tamada, 2002).

Compared to other benyviruses, a most notable feature of BdMoV is the presence of the AlkB-like domain in the replicase protein (Fig. 2). The AlkB-like domain is not present in rod-shaped viruses belonging to the family *Virgaviridae*. Viral AlkB proteins and their homologues have been identified in a number of plant RNA viruses, which include some viruses in the *Alfalexiviridae* and most viruses in the *Betaflexiviridae* (Bratlie and Drablos, 2005; Martelli et al., 2007). It is noted that AlkB is present in some, but not all, viruses that belong to the same genus. Outside of these two families, only a few numbers of known AlkB-containing viruses are recognized; these include grapevine leafroll-associated virus-3 and little cherry virus-1 (family *Closteroviridae*), blackberry virus Y (family *Potyviridae*), and black raspberry necrosis virus (BRNV, genus *Sadwavirus*) (Halgren et al., 2007; Susaimuthu et al., 2008). The viral AlkB proteins are reported to have a possible role in maintaining the integrity of the viral RNA genome through removal of deleterious RNA damage (van den Born et al., 2008).

The common features of AlkB-containing viruses are: (1) they have filamentous virions with a single exception of BRNV with isometric particles, (2) the AlkB domain is present in the replicase polyprotein of the viruses, (3) most, if not all, AlkB-containing viruses infect woody or perennial plants, and (4) the viral AlkB phylogeny does not reflect the viral species phylogeny, suggesting that AlkB may have been randomly distributed during virus evolution (van den Born et al., 2008). Indeed, the BdMoV AlkB-like domain is clearly present in the replicase polyprotein, and its sequence forms a clade with the viral AlKBs (Fig. 3B). Thus, BdMoV differs from the above AlkB-containing viruses in having rod-shaped particles and infecting a biennial burdock plant (not woody or perennial plant). It has been suggested that viral AlKBs might be acquired into the viral genome via a recombination event between two co-infecting viruses in the same host plant (Martelli et al., 2007).

The finding of the presence of BRLSs in plant and insect genomes provides additional evidence for benyvirus evolution. NRVs that share sequence similarities to the genome of viruses in the families *Hepeviridae* and *Alphatetraviridae* have not been identified so far in the genomes of their possible hosts and, therefore, this is the first evidence of the endogenization of their associated viral sequences into a host genome. Although benyviruses have a limited host range of plants and are transmitted by specific plasmodiphorid vectors, footprints of their ancestral viral infections in both plants and insects are recorded in the form of endogenous molecular fossils (BRLSs). This implies the presence of unforeseen benyviruses in a wide range of host organisms. Interestingly, it has been reported that NRVs having a sequence similarity to plant viruses with plus-sense RNA genomes are integrated into the genomes of diverse insects, including mosquitoes, fruit flies, bees, ants, silkworms, pea aphids, monarch butterflies, and wasps (Cui and Holmes, 2012). These NRVs are closely or distantly related to the sequences of cileviruses or tobamoviruses, respectively (Cui and Holmes, 2012). Although it is unknown how such NRVs were endogenized, it is speculated that progenitor viral sequences might have been integrated after reverse-transcription in aid of retrotransposons, because some NRVs are embedded in or adjacent to sequence of transposable elements as is the case for CaBRLS2 (Fig. 5B) (Ballinger et al., 2012; Geuking et al., 2009). Indeed, evidence for recombination of retrotransposons and exogenous RNA viruses during infection/reverse transcription is reported and, moreover, such a recombinant has a potential to be integrated into the genome of host cells (Geuking et al., 2009; Goic et al., 2013; Horie et al., 2010).

NRVs of benyvirus (BRLSs) were discovered in chickpea and blood-sucking bug chromosomes, and benyvirus-like transcript sequences were also identified in Siam weed, pea, European silver

fir and bark beetle from NCBI TSA or EST databases, which might be derived from the genomic RNA of extant novel viruses or from endogenous transcripts corresponding to integrated host chromosomal virus-like sequences. Taken together with the close relationship of benyviruses to CAV isolated from algae (Fig. 7), these findings suggest that ancestral and extant benyviruses may have infected a broad range of hosts (algae, plants and insects). Thus, our findings provide an interesting insight into the origin and evolution of benyviruses and their related viruses.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.virusres.2013.07.015>.

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# Identification of a Bacteria-Like Ferrochelatase in *Strongyloides venezuelensis*, an Animal Parasitic Nematode

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## Abstract

Heme is an essential molecule for vast majority of organisms serving as a prosthetic group for various hemoproteins. Although most organisms synthesize heme from 5-aminolevulinic acid through a conserved heme biosynthetic pathway composed of seven consecutive enzymatic reactions, nematodes are known to be natural heme auxotrophs. The completely sequenced *Caenorhabditis elegans* genome, for example, lacks all seven genes for heme biosynthesis. However, genome/transcriptome sequencing of *Strongyloides venezuelensis*, an important model nematode species for studying human strongyloidiasis, indicated the presence of a gene for ferrochelatase (FeCH), which catalyzes the terminal step of heme biosynthesis, whereas the other six heme biosynthesis genes are apparently missing. Phylogenetic analyses indicated that nematode FeCH genes, including that of *S. venezuelensis* (SvFeCH) have a fundamentally different evolutionary origin from the FeCH genes of non-nematode metazoa. Although all non-nematode metazoan FeCH genes appear to be inherited vertically from an ancestral opisthokont, nematode FeCH may have been acquired from an alpha-proteobacterium, horizontally. The identified SvFeCH sequence was found to function as FeCH as expected based on both *in vitro* chelatase assays using recombinant SvFeCH and *in vivo* complementation experiments using an FeCH-deficient strain of *Escherichia coli*. Messenger RNA expression levels during the *S. venezuelensis* lifecycle were examined by real-time RT-PCR. SvFeCH mRNA was expressed at all the stages examined with a marked reduction at the infective third-stage larvae. Our study demonstrates the presence of a bacteria-like FeCH gene in the *S. venezuelensis* genome. It appeared that *S. venezuelensis* and some other animal parasitic nematodes reacquired the once-lost FeCH gene. Although the underlying evolutionary pressures that necessitated this reacquisition remain to be investigated, it is interesting that the presence of FeCH genes in the absence of other heme biosynthesis genes has been reported only for animal pathogens, and this finding may be related to nutritional availability in animal hosts.

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## Introduction

Heme is essential for the vast majority of life serving as a prosthetic group for many hemoproteins such as catalase, cytochrome, hemoglobin, myoglobin, and peroxidase [1]. Although most aerobic organisms possess a complete biosynthetic pathway for this compound [2], certain organisms are deficient in heme biosynthesis, lacking some or all genes for the heme biosynthetic pathway. Some anaerobic protists, such as *Giardia intestinalis*, *Trichomonas vaginalis*, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Blastocystis hominis*, and *Encephalitozoon cuniculi* do not possess any heme biosynthetic genes [3]. Members of the family Trypanosomatidae lost some or the entire set of heme biosynthesis genes. They acquire heme or heme precursors from their diet [3,4]. In Trypanosomatidae, members of the genus *Trypanosoma* lack all the heme biosynthesis genes [3,5,6,7], whereas other members such as

*Leishmania* spp. possess the genes for the last three steps which were horizontally acquired from a gamma-proteobacterium [3]. Insect trypanosomatid species (*Blastocrithidia culicis* and *Crithidia oncopelti*) cannot synthesize heme by themselves but harbor bacterial endosymbionts that generate and donate heme or heme precursors to the host (trypanosomatid) cells [4,8]. More peculiar is the case of *Phylomonas serpens*, a plant kinetoplastid [9]. This organism lacks most of the known hemoproteins including respiratory cytochromes and does not require heme for viability despite its dependence on oxidative metabolism [9]. The draft genome of *P. serpens* does not appear to contain heme biosynthesis genes other than ferrochelatase (FeCH, EC 4.99.1.1) [9].

Another important and interesting group of organisms that lack the ability to synthesize heme is the nematodes. Nematodes, or roundworms, are typically small, diverse, and highly abundant metazoan organisms [10]. Although free-living species are found

CTGCTATTAAAGTAATCTA  
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ATGTCGCTCTAACAATAATGTTAAAATTCATCTTATTAGTTTATCTAAAATCACTTGT
1  M S S N N N N V K I Q S Y L V Y L K S L V
TCCAATCATGTAAACTACCCGTAATTAATAGTATCAAGAGAACATCAAAGACTGGT
21  S N H V K R L P V N I N S I K R T S K T G
ATTATTATTATAACAGGTACCTAAGTCTTATGGATACCTGGGACTTAAGAAGATAT
41  I I I I N T G T P K S Y G Y W D L R R Y
CTTGAAGAATTTTAAACCGATCAAAGAGTTATAGAAATCAGTAAATTTATATGGTATCCT
61  L E E F L T D Q R V I E I S K F I W Y P
ATACTTTATCTTTTATCTTCCAATTCGCTCTTTAAAAAGAGAAATGTTATAAAAGT
81  I L Y L F I L P I R P F K K R N C Y K S
ATCTGGAATATGGAGAACGATCAATCACCATTATTAACCTTATCTAGAAATCAATGTGAT
101  I W N M E K D E S P L L T L S R N Q C D
AAAAATTTGAAAATTTATCTAGTAAAATTAATCTCCCTTCATTCGATTCGGGCAATTT
121  K I I E N L S S K I K S P F I V D W A F
AGATATGGACCACACAATTTAGGAGAGAAATTAATGTTCTTGTTAATGAAGTTGTGAC
141  R Y G P H N I E E R I N V L V N E G C D
AAGTTGGTAATCTTACCCTTTTCCACATTATAGTCAAGTACTGTTGGTGGAGATGT
161  K L V I L P L F P H Y S Q A T V G G A C
GATGAG (gLaagattgatttaaaatattatataaaacaatatttttag) GTA
181  D E <-----
                    intron
                    >----- V
TACAGAACATGCTGAAATTAAGATATCAACCTGCCATTACGTATAGTCCCCCATACTAT
184  Y R T M L K L R Y Q P A L R I V P P Y Y
AAAATTTGAAAATATATAGAAGTATTGCGTAATTCAGTATGAAAAACGACAAATGAT
204  K I E K Y I E V I G N S V L K K L T N D
AACATTCACCTTGAAGTACTTATTTTTCATATCATGGAATACCATTAAAATATAGTCAA
224  N I P L E V L I F S V H G I P L K Y S Q
AAAGCGGATCCATATGCATATCAATGCATGAAACCACTGAATATATACAATATATC
244  K G D P Y G Y Q C H E T T E Y I T N Y I
AAAAACATTATGAAAAGAACCGTCAAAGTATAACCGACTCCCATATACCGTGACATCT
264  K N I I E K E P S K Y N P L P Y T V T S
TATTCAAGTAGATTTGGTCCATTAGAATCGCTAAAACCATACAGATGATGTTGTACG
284  Y S S R F G P L E W L K P Y T D D V V T
AATCTTGGAAAAGGAGGATGTAATCATTGGGAATATATACCTTCCTCCATACATGAT
304  N L G K K G C K S L G I I S P S F H T D
TGTCTTGAACATGGGAAGAAGTATAGGATGAATAGGGGAAGCTTTTATTAATCACTGAT
324  C L E T W E E L R D E L G E L F I K L S
AATGGTGGAAATTTCCGTTTTATAGATTCATTAATGATACTAAGATGCAATTTGATCTT
344  N G G N F G F I D S L N D T K D S I D L
CTATGCAATTAATGATAGTAACTTTTAGATAATTATTTTAAAGTAAATTAAT
364  L C Q L I D S N N F *
AATATAAATACAAAATAA

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**Figure 1. Genomic DNA and cDNA sequences of the *Strongyloides venezuelensis* FeCH gene.** Both sequences were identical excluding the intronic region, which existed only in the genomic DNA, and a nucleotide at the 54th codon (single-underlined), which was cytosine in the cDNA sequence but was thymidine in the genomic DNA sequences (silent mutation). The deduced amino acid sequence is shown below the nucleotide sequence. In-frame stop codons are indicated by asterisks. 5'- and 3'-splice junction sites that obey the GT-AG rule of eukaryotic introns are indicated by double lines. doi:10.1371/journal.pone.0058458.g001

in nearly all habitats (marine, freshwater, and soil), nematodes are also parasites of vertebrate and invertebrate animals as well as plants. Molecular phylogenetics have defined five major nematode clades (I through V), within which parasitism has arisen multiple times [11]. The genome of *Caenorhabditis elegans*, which was the first metazoan genome to be completely sequenced [12], appears to lack all seven genes necessary to synthesize heme from 5-aminolevulinic acid [13].

Some hemoproteins of animal parasitic nematodes are particularly well studied because of the interests in their roles in low-oxygen environment (host intestine). One such protein is peritenteric hemoglobin of *Ascaris lumbricoides* (parasitic nematode of humans), which has an extraordinary high oxygen affinity, approximately 10,000-fold higher than that of the host's globin [14]. The proposed functions of this oxygen-avid hemoglobin include oxygen detoxification by a reaction driven by nitric oxide [15] and maintenance of body wall O<sub>2</sub> tension by creating an inward-decreasing O<sub>2</sub> gradient that is considered important for oxygen unloading from body wall myoglobin, another heme-

containing protein [16]. Another example of well-studied nematode hemoproteins is cytochrome *b* in the mitochondrial respiratory complex II of *Ascaris suum* (swine parasitic nematode). *A. suum* larvae utilize classic mammalian-type respiration, expressing a small subunit of larval cytochrome *b* (CybS<sup>L</sup>) [17]. In contrast, adult worms live in the host small intestine, where oxygen tension is low and utilize an anaerobic NADH-fumarate reductase system expressing a different small subunit of cytochrome *b* (CybS<sup>A</sup>) instead of CybS<sup>L</sup> [17]. Given the important roles played by the hemoproteins in animal parasitic nematodes, it is interesting to know how heme molecules are synthesized or acquired from the animal hosts.

*Strongyloides* is a genus of obligate gastrointestinal parasites of vertebrates that belong to nematode clade IV [18]. Among more than 50 documented species, two are known to cause human infections, namely *Strongyloides stercoralis* and *Strongyloides juelleborni* [18]. It is estimated that 30–100 million individuals are infected with *Strongyloides* worldwide primarily in tropic and subtropic regions [19]. Symptoms are usually absent or mild in immunocompetent hosts. However, in impaired host immunity, severe manifestations can develop, and fatalities may ensue [20].

To study strongyloidiasis, *Strongyloides venezuelensis*, which is native to rats but can also infect mice, has been widely used as a model [21]. In a transcriptome sequencing project of this *Strongyloides* species, we identified a partial cDNA sequence that most likely encodes a gene for FeCH [22]. FeCH catalyzes the terminal step of heme biosynthesis [23]. The existence of FeCH sequences was noticed in the genomes of *Brugia malayi* (another animal parasitic nematode belonging to nematode clade III) and its bacterial endosymbiont, (*Wolbachia*). However, further analysis was conducted only on the FeCH gene in the endosymbiont genome [24,25].

In the present study, we cloned the entire cDNA sequence of the FeCH gene from *S. venezuelensis* (*SvFeCH*). Our BLAST search on publicly available databases revealed that only a fraction of nematode species possesses the FeCH gene. Interestingly, all these species were parasites of mammals. Surprisingly, in our phylogenetic analysis, nematode FeCH formed a distinctive clade, and it was placed distantly from the clade that contains non-nematode metazoan FeCH, suggesting that the origin of nematode FeCH genes are different from those of non-nematode metazoan FeCH. The chelatase activity of the *SvFeCH* was confirmed by an *in vitro* assay using recombinant protein and a gene complementation assay using an FeCH-deficient *Escherichia coli*.

Nematode genes for heme biosynthesis have not been cloned or characterized to date, essentially because of the nonexistence of these genes in species commonly used in laboratories such as *C. elegans*. Thus, the present study represents the first report of a cloned active FeCH from organisms in the phylum *Nematoda*.

Although the biological significance of carrying only the FeCH gene among other heme biosynthesis genes is unclear, the presence of this gene only in animal parasites suggests a possible role for this gene in nutritional adaptation to the animal host environment.

## Materials and Methods

### Ethics Statement

*S. venezuelensis* has been maintained over serial passages in male Wistar rats purchased from Kyudo Co. Ltd. (Kumamoto, Japan). The animals were housed and handled in the Division of Parasitology, Department of Infectious Diseases, University of Miyazaki [26]. All animal studies were conducted under the applicable laws and guidelines for the care and use of laboratory animals in the University of Miyazaki and approved by the Animal





**Figure 2. Multiple sequence alignment of FeCH sequences.** The FeCH sequences were taken from the NCBI protein database together with the *S. venezuelensis* sequence (this study); *Homo sapiens* (CAB65962), *Drosophila melanogaster* (AAC26225), *Saccharomyces cerevisiae* (EDV10759), *Brugia malayi* 1 (ADI33748), *Brugia malayi* 2 (ADI33749), *Acanthocheilonevma viteae* (ADI33750), *Dirofilaria immitis* (ADI33752), *Onchocerca volvulus* (ADI33751), *Escherichia coli* (AP\_001124), and *Bacillus subtilis* (NP\_388894). The sequences were computationally aligned by the ClustalX program [55]. The catalytic core and the C-terminal extension are boxed by red and green dotted lines, respectively. A histidine residue reported to be critical for metal substrate binding (H263, human sequence numbering) is indicated by an inverted triangle. A cluster of three acidic residues are marked with asterisks. Two residues at the active site that were reported to be identical in all known FeCH sequences (Y276 and W310) [56] are indicated by number (#) marks.

doi:10.1371/journal.pone.0058458.g002

Experiment Committee of the University, as specified in the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan, 2006.

### 5'- and 3'-rapid Amplification of cDNA Ends (RACE)

To determine the sequences of the 3'- and 5'-ends of FeCH cDNA, RACE experiments were performed [27,28]. The priming sites used for these experiments were based on a contig sequence obtained from our *S. venezuelensis* transcriptome sequencing project [22]. For 3'-RACE, a PrimeScript RT-PCR kit (Takara, Japan) was used with oligo(dT) adaptor primers to synthesize cDNA from total RNA prepared from parasitic adult worms. Using this 3'-RACE-ready cDNA as a template, hemi-nested PCR was performed first with primer pairs ENM059/ENM008, followed by ENM060/ENM008. The primer sequences used in this study are summarized in Table S1. The resultant PCR products were cloned into pCR2.1 TOPO (Invitrogen, Carlsbad, CA, USA) for DNA sequencing.

For 5'-RACE, a gene specific-primer (reverse) ENM070 was used to synthesize cDNA from total RNA prepared from adult worms. The addition of a homopolymeric A-tail to the 3'-end of the synthesized first-strand cDNA was performed using dATP and terminal transferase. The dA-tailed cDNA was used as a template for hemi-nested PCR first with primers ENM5\_6\_7, and ENM008/ENM071, then with primers ENM008/ENM072. The resultant PCR products were cloned into pCR2.1 TOPO for DNA sequencing.

Based on the sequence information obtained from the 5'- and 3'-RACE experiments, a PCR primer pair (ENM073/ENM074) was designed to amplify the entire ORF of the *SvFeCH* gene. The PCR products obtained using an adult-stage cDNA sample as a template were cloned into pCR2.1 TOPO vectors to determine the sequence. The resultant full-length ORF sequence was deposited into DNA Data Bank of Japan under the accession number AB710465, which can be accessed through GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>), and used to deduce the amino acid sequence of the *SvFeCH*.

### BLAST Homology Search

To search for heme biosynthesis genes, BLAST homology searches [29] were performed against predicted protein sequence data from published nematode genome projects (*Caenorhabditis briggsae* (nematode clade V) [30], *C. elegans* (V) [12], *Pristionchus pacificus* (V) [31], *Meloidogyne incognita* (IV) [31], *Meloidogyne hapla* (IV) [32], *Bursaphelenchus xylophilus* (IV) [33], *B. malayi* (III) [25], *Ascaris suum* (III) [34], and *Trichinella spiralis* (I) [35]) and nematode expressed sequence tags (ESTs) from NEMBASE4 [36], and *S. venezuelensis* genome (obtained by the Roche-454 pyrosequencing platform [37] with an estimated coverage of more than 20, unpublished), and transcriptome [22] datasets, using human sequences as queries with cutoff value of  $1 \times 10^{-4}$ . For the FeCH gene, the *S. venezuelensis* protein sequence, deduced from the cDNA

sequence, was also used as a query to search for potential orthologs against the aforementioned set of nematode genome and EST datasets, as well as the NCBI nonredundant protein database. Similarly, nematode heme biosynthesis gene sequences identified during these database searches were used as queries, instead of the human sequences, to search for potential orthologs in our *S. venezuelensis* genome and transcriptome datasets.

### Phylogenetic Analyses

We retrieved the gene sequences encoding FeCH of 71 bacterial and 65 eukaryotic species from the GENBANK nonredundant protein database (note that some eukaryotes possess more than two FeCH homologs). These amino acid sequences and those of the *S. venezuelensis* homolog were firstly aligned using MAFFT [38], and the resultant alignment was edited manually. After the exclusion of ambiguously aligned positions, the final FeCH alignment containing 71 eukaryotic and 71 bacterial homologs with 177 amino acid positions was subjected to phylogenetic analyses, as described below. Taxonomic affiliation and accession numbers for the sequences considered in our FeCH alignment are listed in Table S2.

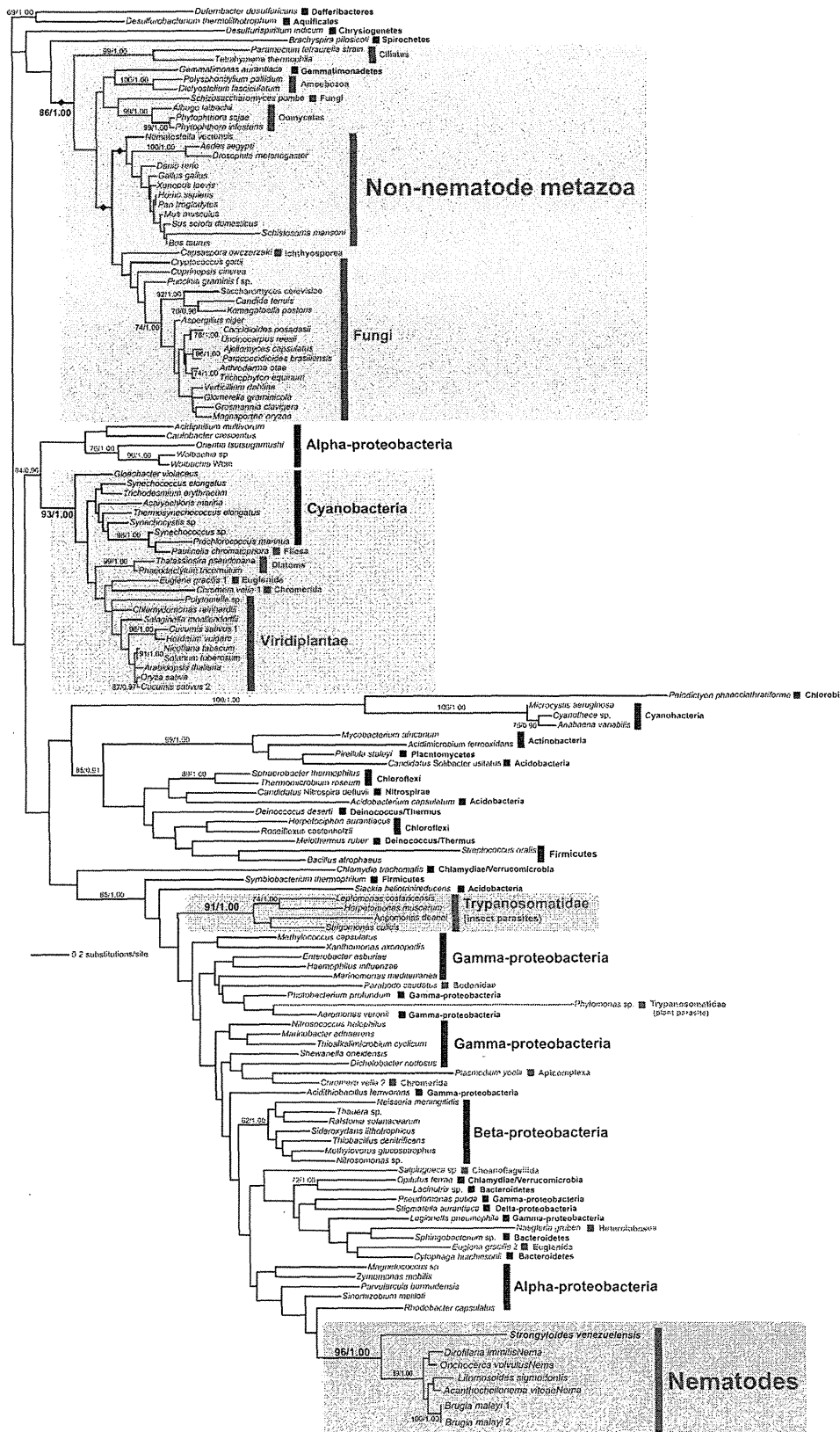
Maximum likelihood (ML) phylogenetic analyses were performed using RAXML 7.2.8 [39]. The substitution model used was the LG model incorporating the among-site rate variation approximated with a discrete gamma distribution with four categories (LG+Γ). This particular substitution model was selected as the most appropriate model for the FeCH alignment using Aminosan [40]. The ML tree was selected from heuristic tree search initiated from 20 distinctive parsimonious trees. In ML bootstrap analysis (with 100 replicates), a single tree search was performed per replicate.

Bayesian analysis based on the LG+Γ model was also conducted using MrBayes 3.2.1 [41]. Four parallel Metropolis-coupled Markov chain Monte Carlo runs, each consisting of one cold and seven heated chains with a chain temperature of 0.1, were run for 5,000,000 generations. Log-likelihood scores and trees with branch lengths were sampled every 1000 generations. The first 1,250,000 generations were excluded as burn-in, and the remaining trees were summarized to obtain Bayesian posterior probabilities.

### Bacterial Expression of Recombinant *SvFeCH* and Measurement of Porphyrin-metal Chelatase Activity

A cDNA sequence corresponding to the entire catalytic core region of *SvFeCH* (amino acid positions 29–373) was obtained by PCR using the primer pair TKT001/TKT002. The PCR product was cloned into pET-21a (+), an *E. coli* expression vector (Merck, Darmstadt, Germany), and the plasmid obtained was transferred to *E. coli* BL21. The bacteria were grown in LB medium for 16 h, and then the culture medium was diluted by 10-fold in fresh LB medium. The enzyme was expressed with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C for 2 h.

The cells were harvested by centrifugation and suspended in 20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM DTT, 0.1%





**Figure 3. Phylogenetic analysis of FeCH sequences.** The ML phylogeny inferred from FeCH amino acid sequences from 71 bacteria and 65 eukaryotes. Numerical values at the nodes represent MLBPs and BPPs. Only MLBPs greater than 60% are shown. The majority of the FeCH homologs sampled from eukaryotes (colored in red) were separated into four clades shaded in blue, green, pink, and orange. The homologs from *Strongyloides venezuelensis* identified in this study formed the 'orange' clade with those of other nematodes. We compared the ML tree and the alternative hypotheses for the origin of the nematode FeCH genes by pruning and regrafting the entire nematode clade (shaded in orange) to the branches marked by diamonds in the 'blue' clade.

Tween 20, and 0.3 M NaCl. Cells were disrupted by sonication and centrifuged at 5000×g at 4°C for 10 min. The supernatants were used for the enzyme assay.

The FeCH activity was determined by measuring the insertion of zinc ions into mesoporphyrin, as described previously [42]. After incubation at 30°C for 30 min, the protoporphyrin or zinc-protoporphyrin formed was measured fluorophotometrically.

#### Genetic Complementation Assay of hemH (Bacterial FeCH) Deficient *E. coli*

*E. coli* strain VS200 ( $\Delta$ hemH), a deletion mutant for hemH gene [43] was provided by the National Bioresource Project of MEXT, Japan.

The entire ORF of *Sv*FeCH, obtained by RT-PCR with the primer pair ENM089/ENM098, was cloned into the *XhoI/BglIII* restriction site of pFLAG-CTC plasmid, an *E. coli* expression vector containing a tac promoter (Sigma-Aldrich, St. Louis, MO, USA). The resultant plasmid pFLAG-CTC-*Sv*FeCH was tested as a gene complementation vector. The original pFLAG-CTC plasmid served as a control.

$\Delta$ hemH was transformed with pFLAG-CTC-*Sv*FeCH or with pFLAG-CTC. The transformed and untransformed *E. coli*  $\Delta$ hemH strains were cultured overnight in LB medium supplemented with hemin (10  $\mu$ g/ml).

For the culture of the transformed  $\Delta$ hemH, ampicillin was also added at a concentration of 50  $\mu$ g/ml. The bacteria from the overnight culture were pelleted by centrifugation and washed thrice with LB medium. After washing, the bacteria pellets were resuspended to give an OD<sub>600</sub> of 0.1 in hemin-containing (10  $\mu$ g/ml) or hemin-free LB medium with (for the transformed  $\Delta$ hemH)

or without (for the untransformed  $\Delta$ hemH) ampicillin, and incubated at 37°C with rocking. O.D. 600 of each culture was measured every hour up to 20 h.

#### Real-time RT-PCR Analysis

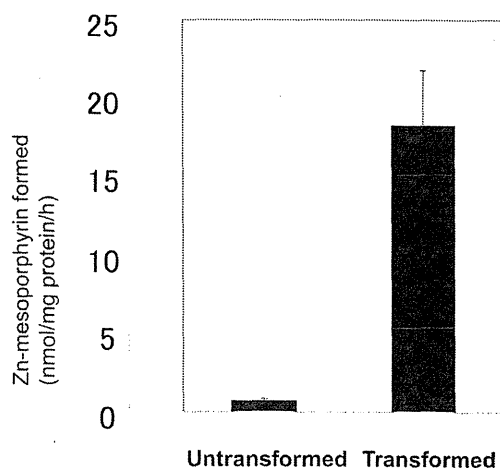
Total RNA samples were prepared from eggs, a mixture of first- and second-stage larvae (L1/L2), third-stage infective larvae (L3i), lung third-stage larvae (LL3), mucosal larvae (ML) and parasitic adult stages. Eggs were obtained by the floatation method with saturated salt solution from rat feces. L1/L2 and L3i were prepared from fecal culture. LL3 and ML were collected from infected male ICR mice 72 and 85 h after infection, respectively. Parasitic adults were collected from the small intestine of rats 10 days after infection. Eggs and worms were washed extensively with PBS, pelleted by centrifugation and stored at -80°C until used.

Frozen eggs or worms were crushed with a crushing device (SK-200) purchased from Tokken, Japan. Trizol (Invitrogen) was used for total RNA preparation following the manufacturer's instructions. After DNase I treatment, cDNA was synthesized using PrimeScript RT-PCR kit. Real-time RT-PCR was performed by the GoTaq qPCR system (Promega, Madison, WI, USA) using specific primer pairs (ENM056/ENM057 for *Sv*FeCH and 377F/501R for 18S ribosomal RNA genes). The real-time RT-PCR analyses were performed using biological triplicate samples.

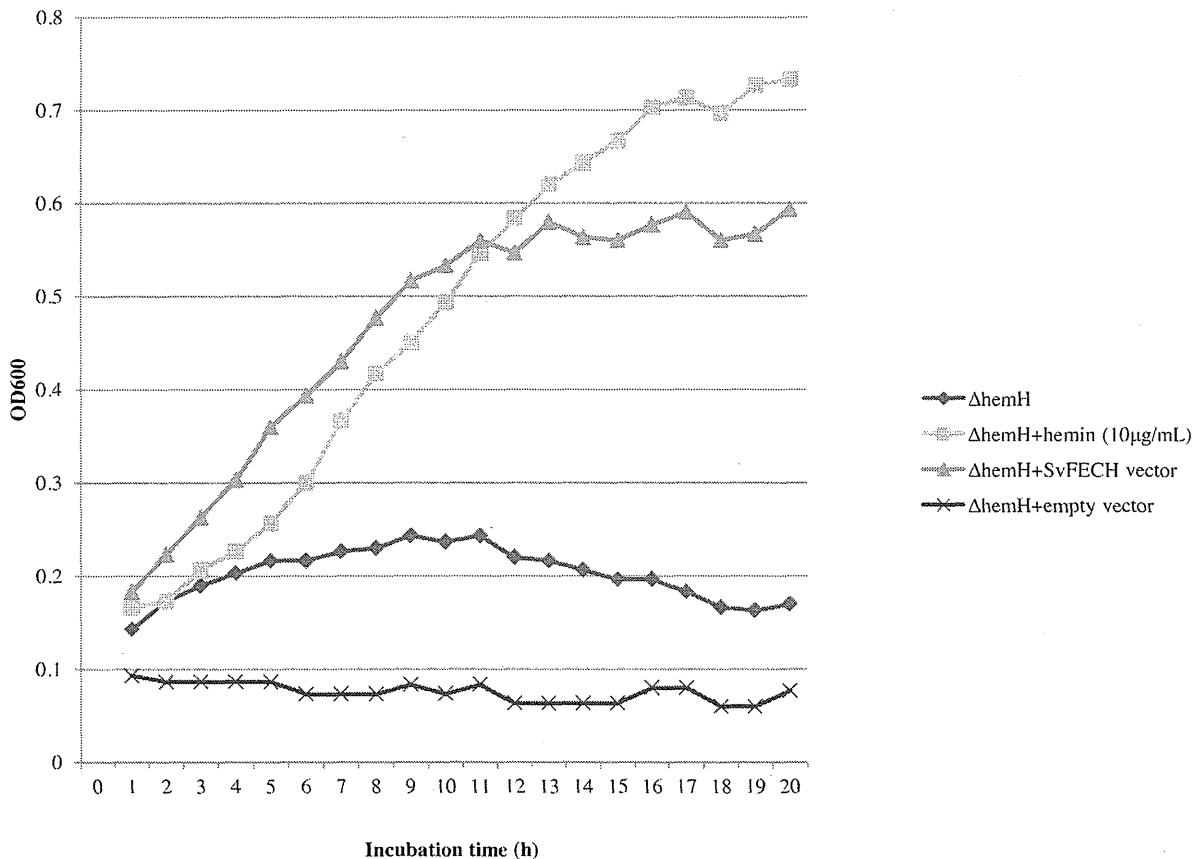
#### Results

Initially, we identified an EST contig that appeared to represent a transcript from *Sv*FeCH gene [22]. The entire cDNA sequence was determined by 3'- and 5'- RACE experiments. This sequence could be mapped to the genomic DNA sequence of this organism obtained from our genome sequencing project, the details of which will be published elsewhere. The genomic and cDNA sequences of the *Sv*FeCH gene are presented in Figure 1. The length of the coding region was 1122 bp including the stop codon. There was one short (49 bp) intron. The deduced amino acid sequence had a length of 373 residues and an expected molecular mass of 43.3 kDa.

In our search for the presence of other heme biosynthesis genes, BLAST homology searches were performed against nematode genome and EST databases, using human heme biosynthesis gene sequences as queries (Tables S3 and S4). Overall, many nematodes appeared to lack all the heme biosynthesis genes, as reported for *C. elegans* [13]. However, some exceptions were also noticed, including the presence of the aminolevulinic acid dehydrogenase (ALAD) gene in several species and the uroporphyrinogen decarboxylase (UROD) gene in *Meloidogyne paranaensis*, the coproporphyrinogen oxidase (CPOX) gene in *Ancylostoma caninum*, and the FeCH gene in *B. malayi* and *Strongyloides ratti*. No heme biosynthesis gene other than FeCH was found in our *S. venezuelensis* genome and transcriptome data using the human sequences as queries. We did not obtain any significant hit from the BLAST analyses for *S. venezuelensis* genome and transcriptome datasets using the ALAD, UROD, and CPOX gene sequences identified in the nematode genome/EST datasets (see above) as queries. When the *Sv*FeCH protein sequence was used as a query for the BLAST analysis, two additional species were found to carry FeCH gene

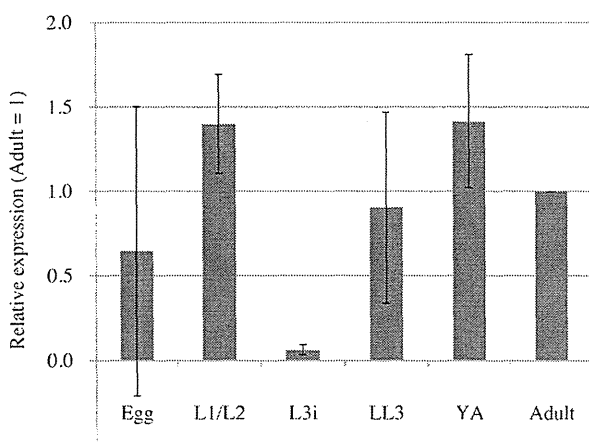


**Figure 4. Chelatase assay using bacterially expressed recombinant *Sv*FeCH.** The cell extracts were incubated with 20 mM Tris-HCl, pH 8.0, 0.1% Tween 20, 15  $\mu$ M mesoporphyrin IX, and 40  $\mu$ M zinc acetate in a final volume of 200  $\mu$ l at 30°C for 60 min. The formation of zinc mesoporphyrin was measured. Data are expressed as the mean  $\pm$  SD of triplicate experiments.



**Figure 5. Genetic complementation assay of  $\Delta$ hemH *E. coli*.** An untransformed  $\Delta$ hemH strain of *E. coli* was grown in the absence (diamond) or presence 10  $\mu$ g/ml hemin (square). In the same experiment, a transformed  $\Delta$ hemH strain of *E. coli* either with SvFECH gene expression vector (triangle) or with empty vector (x-mark) was cultured in the absence of hemin. OD<sub>600</sub> was measured every hour up to 20 h to monitor bacterial growth.

doi:10.1371/journal.pone.0058458.g005



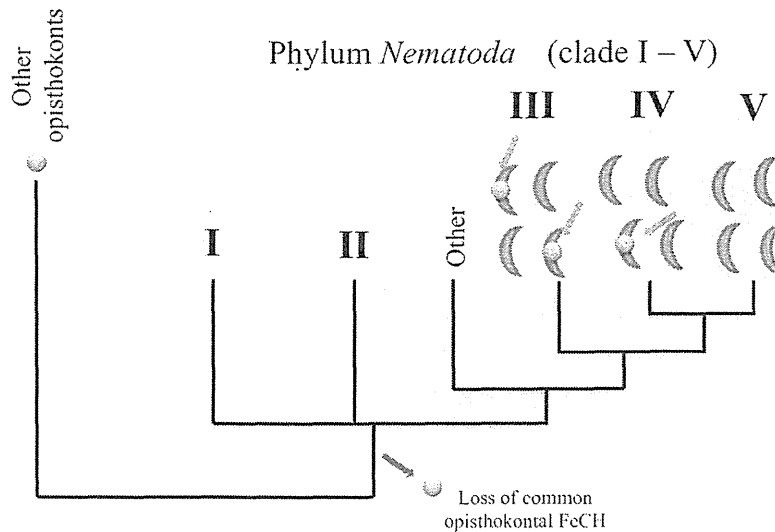
**Figure 6. Expression analysis of SvFECH gene by real-time RT-PCR.** mRNA abundance is shown *relative* to the expression level at the adult stage, after normalizing to 18S rRNA expression levels. The bars represent the means and standard deviations ( $\pm$ ) of biological triplicates. Real-time RT-PCR was performed in triplicate wells for each biological replicate.

doi:10.1371/journal.pone.0058458.g006

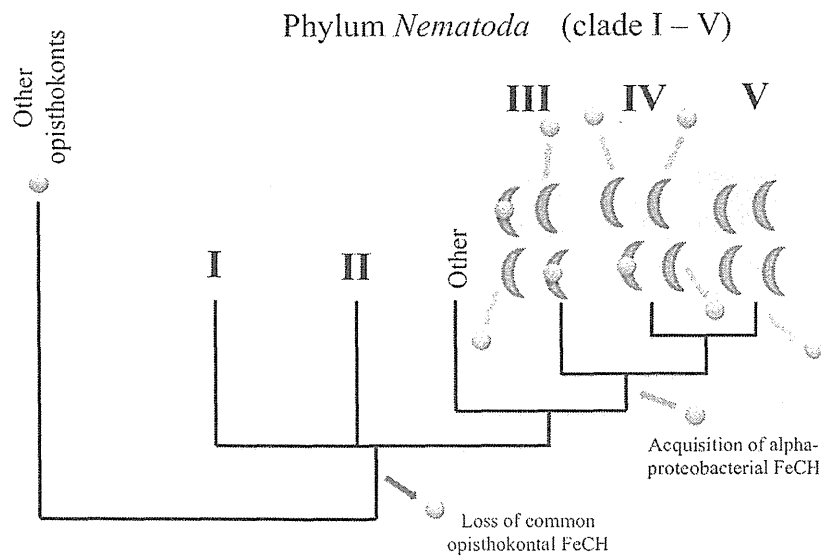
(Table S5), namely *Litomosoides sigmodontis* and *Onchocerca volvulus*. The *S. venezuelensis* sequence was also used for BLAST searches against NCBI non-redundant protein database, which led to the identification of two more nematode species that carry FeCH, namely *Dirofilaria immitis* and *Acanthocheilonema viteae*. These results are interesting because all the species found to carry the FeCH gene were animal parasites (filarial nematodes in clade III and *Strongyloides* in clade IV).

A multiple sequence alignment of FeCH protein sequences from selected organisms is presented in Figure 2. Amino acid residues in the catalytic core (boxed by a red dotted line) displayed moderate similarity. Key residues for FeCH activity, such as H263 (human sequence numbering), which was proposed to be involved in metal substrate binding [23,44], were well conserved. Characteristically, nematode (*S. venezuelensis* and *B. malayi*) FeCH lacked a protein region called the "C-terminal extension," a short (approximately 30–50 amino acid residues) stretch of sequences at the C-terminus of the protein that is commonly present in the FeCH of non-nematode opisthokonts [23,45] (boxed by a green dotted line in Figure 2). To measure the similarities of these selected sequences, BLAST scores and amino acid identities were retrieved by the BLASTP program (Table S6). All the nematode FeCH sequences had higher BLAST scores and percent similarity values to the *E. coli* sequence (BLAST score: 202–221; similarity: 33.6%–36.8%)

a)



b)



● FeCH gene (common opisthokontal)

● FeCH gene (alpha-proteobacterial)

**Figure 7. Proposed hypotheses for the loss of the original (common opisthokontal) FeCH gene and the re-acquisition of alpha-proteobacterial FeCH in the evolution of the phylum *Nematoda*.** The initial loss of the common opisthokontal FeCH gene may have occurred at the common ancestor level (red arrows). (a) Scenario 1: The first scenario hypothesizes that alpha-proteobacterial FeCH was acquired independently by some species in clades III and IV (green arrows). (b) Scenario 2: Reacquisition of FeCH from an alpha-proteobacterium may have occurred at the common ancestor level of clades III, IV and V (blue arrow) followed by a secondary loss in some species in clade III and IV and in the branch leading to clade V (pink arrows). The phylogenetic relationships of the nematode clades are based on Sommer and Streit [10]. doi:10.1371/journal.pone.0058458.g007

than to human (92–106 and 26.8%–27.5%, respectively), *Drosophila* (93–102 and 24.0%–25.9%, respectively) and *Saccharomyces* sequences (74–96 and 23.0%–27.2%, respectively). When BLAST homology searches were conducted using the *SvFeCH* protein sequence as a query against the NCBI non-redundant protein as described above, virtually all the top hits were bacterial sequences excluding the sequences of filarial nematodes (data not shown). These findings prompted us to conduct a phylogenetic analysis to better clarify the evolutionary origin of nematode FeCH genes.

### Phylogenetic Analysis

The amino acid alignment of FeCH sampled from 71 eukaryotic and 71 bacterial species was phylogenetically analyzed by ML and Bayesian methods (Figure 3). Overall, the FeCH trees inferred by the ML and Bayesian methods were concordant with each other as well as the results of previously published FeCH phylogenies [7,46,47,48]. Four major clades including the FeCH homologues sampled from eukaryotes were reconstructed with ML bootstrap support values (MLBPs) of 86%–96% and a Bayesian posterior probability (BPP) of 1.00 (shaded in blue, green, pink, and orange; Figure 3): (1) a ‘blue’ clade comprising a single bacterial homolog (*Gemmatimonas aurantiaca*) and those of eukaryotes–non-nematode metazoans, fungi, *Capsaspora ovczarzaki*, oomycetes, amoebozoans, and ciliates; (2) a ‘green’ clade of the homolog of cyanobacteria including an obligate endosymbiont in the testate amoeba, *Paulinella chromatophora* [49], and putative plastid homolog in photosynthetic eukaryotes; (3) a ‘pink’ clade comprising the homolog of insect trypanosomatids [3]; (4) an ‘orange’ clade comprising the homolog of parasitic nematodes including *S. venezuelensis*. Other homologs sampled from eukaryotes were scattered amongst the bacterial homologues, and they exhibited no specific evolutionary affinity to other homologs.

The FeCH phylogeny suggested that the homologs from non-nematode metazoans nested in the ‘blue’ clade and those of nematodes forming the ‘orange’ clade were distantly related to each other. Although they received little support from the ML bootstrap and Bayesian analyses, the homologs from non-nematode metazoans, *Capsaspora*, and fungi were grouped together, corresponding to members of Opisthokonta, a well-established monophyletic assemblage [50]. Curiously, the nematode FeCH homologs formed a robust clade with an MLBP of 96% and BPP of 1.00, being distinct from other metazoan homologs. This tree topology can be rationalized by the vertical inheritance of FeCH genes from the ancestral opisthokont species to non-nematode metazoans and horizontal transfer of a FeCH gene between the ancestral nematodes and a non-metazoan organism. This conjecture was further supported by a topology test comparing the ML tree shown in Figure 3 with three alternative trees, in which the nematode homologs were enforced to branch at the base of (1) the non-nematode metazoan clade, (2) the clade of the opisthokont homologues (excluding that of *Schizosaccharomyces pombe*), and (3) the ‘blue’ clade composed of the eukaryotic and *Gemmatimonas* homologs (highlighted by diamonds in Figure 3). Importantly, all the alternative trees were successfully rejected with very small *p* values ( $2.0 \times 10^{-78}$ – $2.0 \times 10^{-36}$ ).

### Chelatase Assay using Recombinant *SvFeCH*

To determine whether the FeCH gene of *S. venezuelensis* identified in the present study encodes an active enzyme, we conducted a chelatase assay using a bacterially expressed recombinant *SvFeCH*. We constructed an expression plasmid, pET-*SvFeCH*, which was used to transform *E. coli* strain BL21. Protein expression was induced by incubation with 0.3 mM IPTG at 30°C for 2 h. The enzyme activity was measured using the cell

extracts of untransformed and transformed bacteria. The FeCH activity in transformed bacteria, which was derived from over-expressed *SvFeCH* and endogenous *E. coli* FeCH, was much higher than that in the untransformed control, which originated solely from endogenous FeCH, indicating that the enzyme was active (Figure. 4).

### Genetic Complementation Assay of hemH Deficient *E. coli*

The VS200 strain of *E. coli* K12, a hemH null-mutant, was used for the gene complementation assay, and the results are shown in Figure 5. VS200 could not grow in LB medium, unless hemin (10 µg/ml) was supplemented. The expression of *SvFeCH* by pFLAG-CTC-*SvFeCH* made the bacteria capable of growing in the LB medium in the absence of hemin. Transforming the bacteria with the control vector (pFLAG-CTC) did not have such an effect. Therefore, it was concluded that *SvFeCH* is an active enzyme that can function as FeCH.

### Expression of FeCH during the Life Cycle of *S. venezuelensis*

The relative expression levels of *SvFeCH* mRNA were assessed by real-time RT-PCR analysis using RNA samples prepared from the six major developmental stages of *S. venezuelensis* (Figure 6). It was observed that although *SvFeCH* mRNA expression was present throughout the stages, it was relatively low in L3i.

### Discussion

We demonstrated that a gene for FeCH exists in the *S. venezuelensis* genome. Although the presence of the FeCH gene in the draft genome of *B. malayi* was reported previously [24,25], no further characterization was reported. The present study represents the first cloning and characterization of nematode FeCH, particularly in an evolutionary context.

Phylogenetic analyses revealed that nematode FeCH forms a distinct clade from that of non-nematode metazoans, indicating that the evolutionary origin of nematode FeCH is fundamentally different from that of the FeCH genes of other metazoan organisms. In the ML phylogeny, the nematode clade was placed within the homologs from a subset of alpha-proteobacteria, although the statistical support for this hypothesis is inconclusive. If the affinity between the nematode and alpha-proteobacterial FeCH homologs is genuine, then an as-yet-unknown alpha-proteobacterium was the source of the FeCH homologs working in the extant nematodes. This hypothesis is intriguing because replacement of the eukaryotic FeCH gene by a bacterial FeCH gene had been suggested only for unicellular eukaryotes, such as apicomplexan parasites (*Plasmodium falciparum*, *P. chabaudi*, *P. berghei*, *Eimeria tenella*, *Toxoplasma gondii*, and *Neospora caninum*) [47,48,51], the chromerid *Chromera velia* [47], rhodophytes (*Cyanidioschyzon merolae*, *Porphyra yezoensis*, and *Galdieria sulphuraria*) [48], and the euglenid *Euglena gracilis* [46].

BLAST analysis of the sequenced nematode genomes and transcriptomes revealed that the FeCH gene is present only in *Strongyloides* (clade IV) and filarial parasites (clade III). It is still not clear at which point of nematode evolution the proposed horizontal gene transfer event occurred. Regarding *B. malayi* and related filarial nematodes, horizontal gene transfer from *Wolbachia*, a bacterial symbiont, is known to have occurred [52]. However, the FeCH sequences present in nematode genomes do not appear to originate from *Wolbachia* based on the positions of the *Wolbachia* species in the phylogenetic tree (Figure 3).

We hypothesize two possible scenarios concerning the evolutionary histories of FeCH genes in nematodes, using a current view of the phylogenetic relationship of nematode clades [10]. Because no nematode species possesses the 'blue clade' FeCH commonly found in opisthokonts, it can be speculated that this type of FeCH was lost early in nematode evolution (Figure. 7). *Strongyloides* and the filarias may have acquired FeCH genes from alpha-proteobacteria independently. Alternatively, a common ancestral lineage leading to clades III, IV, and V may have received such an alpha-proteobacterial FeCH gene (scenarios 1 and 2, respectively: Fig. 7a and 7b). For scenario 1 to be true, the hypothetical alpha-proteobacterial species that provided FeCH genes to *Strongyloides* and filarias, need to be closely related to each other, because the nematode homologs were robustly grouped together in the FeCH phylogeny (Figure. 3). In scenario 2, the lateral transfer of a bacterial FeCH gene occurred through an ancestor leading to species that belong to clades III, IV, and V, and again, the FeCH gene disappeared in some species in clades III and IV such as *Ascaris* and *Meloidogyne* and in the branch leading to clade V (Figure. 7b).

Among the parasitic nematodes, the reason why only *Strongyloides* and filarias needed to reacquire (scenario 1) or retain (scenario 2) FeCH gene is unclear, particularly when the other six heme biosynthesis genes are still absent. This situation (the presence of FeCH gene in the absence of other heme biosynthesis genes) has been documented for a limited number of organisms, such as *Haemophilus influenzae* [53] and *P. serpens* [9]. As was suggested for *H. influenzae* [9,54], there may be a possibility that FeCH is used to obtain Fe<sup>2+</sup> through its reverse activity rather than obtain heme from protoporphyrin IX using its forward activity.

## Supporting Information

**Table S1 List of primers used in this study.**

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(PDF)

**Table S2 Taxonomic affiliation and accession numbers for the sequences considered in the phylogenetic analysis.**

(PDF)

**Table S3 BLAST homology search against predicted proteins from nematode genome projects.**

(PDF)

**Table S4 BLAST homology search against nematode EST database (NEMBASE 4).**

(PDF)

**Table S5 BLAST homology search against nematode EST database (NEMBASE 4) using *Strongyloides venezuelensis* ferrochelatase sequence as a query.**

(PDF)

**Table S6 Sequence similarities of FeCH proteins assessed by BLAST program.**

(PDF)

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## Author Contributions

Conceived and designed the experiments: EN HM. Performed the experiments: EN ST GC AY. Analyzed the data: SI YI. Wrote the paper: EN SI YI HM.

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## Efficacy and safety of paromomycin for treating amebiasis in Japan

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### ABSTRACT

The clinical management of amebiasis is a growing concern, particularly among human immunodeficiency virus (HIV)-infected individuals who are predisposed to severe illness. Treatment with a luminal amebicide is strongly recommended following acute-stage treatment with a nitroimidazole. In 2004, the Japanese Research Group on Chemotherapy of Tropical Diseases introduced paromomycin, which was not nationally licensed, and offered it to a number of patients. From 2004 to 2011, 143 case records of amebiasis (123 with amebic colitis, 16 with amebic liver abscess, and 4 with both) in which patients were treated with paromomycin, mainly 1500 mg/day for 9 or 10 days following metronidazole treatment, were submitted. Among 123 evaluable cases, 23 (18.7%) experienced possible adverse effects, the most common being diarrhea (17/123, 13.8%) and other gastrointestinal problems that were resolved after the completion or discontinuation of treatment. In addition, single cases of bloody stools associated with *Clostridium difficile* colitis, skin rash, and the elevation of liver enzymes were also reported, although the causal relationship was not clear. HIV infection did not appear to increase the incidence of adverse drug effects. Each of the 11 asymptomatic or mildly symptomatic amebic colitis cases became negative for stool cysts after paromomycin treatment. Paromomycin was shown to be safe and well tolerated, as well as effective in a special subset of amebic colitis cases.

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### 1. Introduction

Amebiasis is a protozoan infection caused by *Entamoeba histolytica* that consists of two types, intestinal amebiasis (i.e., amebic colitis) and extraintestinal amebiasis, the latter including amebic liver abscess and rare manifestations such as pulmonary, cardiac, or brain involvement [1]. Both types can cause severe complications such as intestinal hemorrhage, ileus, intestinal perforation, or peritonitis in cases of amebic colitis, and rupture to the peritoneal, pleural, or pericardial cavity in cases of amebic liver abscess [2,3]. In developed countries, most amebiasis cases used to occur in travelers returning from developing countries [4,5]; however, recently, domestically infected cases among males who have sex with men (MSM) are increasing [6–8]. This is a concern given that MSM also constitute a risk group for human immunodeficiency virus (HIV) infection and amebiasis may develop into severe disease in HIV-infected individuals [9,10].

Currently, acute-stage treatment with a nitroimidazole (metronidazole or tinidazole) is followed by a luminal amebicide (paromomycin,

diloxanide furoate, or iodoquinol), which is strongly recommended to eradicate the possibility of residual protozoan parasites [11,12]. This is because even after nitroimidazole treatment relieves acute symptoms and leads to negative stool tests for *E. histolytica*, a very small number of protozoa may survive in the intestine and can cause a relapse [1]. Luminal agents are poorly absorbed through the intestinal mucosa, thus attaining high intra-luminal drug concentrations. Apart from their use as anti-relapse therapy, luminal agents can be used as a monotherapy for treating asymptomatic (i.e., cyst passer) or mildly symptomatic amebic colitis [13].

The Japanese Research Group on Chemotherapy of Tropical Diseases, of which the authors are the principal members, imports nationally unlicensed medicines for tropical and parasitic diseases, and enables their use in patients in Japan when it is considered necessary and appropriate. This system is indispensable for the appropriate treatment of Japanese patients who contract exotic diseases against which there are only a limited number of nationally licensed medicines [14,15]. Previously, the research group had imported diloxanide furoate as a luminal agent and offered it to a number of patients with amebiasis. However, because diloxanide furoate became unavailable, the research group initiated the importation of paromomycin instead in 2004.

With the increased number of cases treated with this agent, we aimed to clarify the safety profile of paromomycin when used in Japan mainly as an anti-relapse amebicide, and also to evaluate its efficacy in asymptomatic or mildly symptomatic amebic colitis, although the

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cases might be few. These data have been obtained outside of Japan; however, the available Japanese data are very limited. Although this study was not a formal clinical trial, it could contribute to the delineation of how this agent could be utilized in Japan.

## 2. Patients and methods

### 2.1. The research group

The research group was established in 1980, and is currently funded by the Japanese Ministry of Health, Labour and Welfare. At present, the research group imports more than 15 nationally unlicensed medicines, among which 6 are antimalarial drugs. Imported drugs are stocked at the central storage facility, the Institute of Medical Science, University of Tokyo, Tokyo, and provided to 25 registered medical facilities upon request. Members of the registered medical facilities have obtained approval for participating in this program from the research ethics committee of each facility based on the approval obtained at the chief investigator's institution (M. Kimura, No. 09002). The drugs are used at those registered facilities after obtaining the patient's informed consent, which clearly states that the drugs are not licensed in Japan. In exceptional cases in which the patients cannot be referred to one of these medical facilities (e.g., due to severity of the disease), drugs are used outside the registered medical facilities on a humanitarian basis. Following treatments, physicians in charge fill in the patient records that were formulated by the research group and send them to us.

### 2.2. Treatments and analyses

Paromomycin in 250 mg capsules (Humatin®, manufactured by Parke-Davis, Germany) was purchased from the German pharmacy Paesel & Lorei. All of the moderately to severely symptomatic amebiasis cases were first treated with a nitroimidazole at the acute stage, followed by paromomycin for the prevention of relapse. The daily dose and duration of paromomycin administration were primarily determined by the physicians in charge, although in some cases advice was given from one of the authors.

Analysis was conducted using the patient records submitted by the physicians in charge. When necessary, direct contact with the physicians was made in order to gain more detailed information. Adverse effects (AEs) were evaluated by the physicians' descriptions, as well as by our own judgments on the laboratory data shown in the patient records. Cases that could not be followed up after treatment were excluded from the drug safety analysis.

### 2.3. Efficacy study

Asymptomatic or mildly symptomatic colitis cases were enrolled for the efficacy analysis if they seemed to be due to *E. histolytica*, but not due to *Entamoeba dispar*, which is morphologically undistinguishable from *E. histolytica* in the cyst form, but a non-pathogenic protozoan, and if they were treated with paromomycin, but no other amebicides, at least within 1 month. Specifically, cases were presumed to be amebic colitis if stool microscopy had previously shown typical features of *E. histolytica* trophozoites (e.g., ingestion of red blood cells), a colonoscopy had shown characteristic features with or without histology compatible with the disease, a stool had tested positive with a species-specific PCR, or serum antibodies had been raised [16]. Treatment success was defined mostly as the disappearance of cysts from the stool.

## 3. Results

### 3.1. Patients and treatments

From 2004 to 2011, 199 cases were treated with paromomycin, with 145 patient records being submitted and enrolled in this study.

**Table 1**  
Characteristics of patients (n = 145).

Age <sup>a</sup>	Median: 45 (IQR: 37–57)		
Sex	Male	124	(85.5%)
	Female	19	(13.1%)
	Unknown	2	(1.4%)
Nationality	Japanese	141	(97.2%)
	Other	3	(2.1%)
	Unknown	1	(0.7%)
Illness	Amebic colitis alone	123	(84.8%)
	Amebic liver abscess alone	16	(11.0%)
	Both amebic colitis and liver abscess	4	(2.8%)
	Giardiasis	2	(1.4%)
HIV status	Reported to be positive	31	(21.4%)
	Not reported to be positive (i.e., negative or unknown)	114	(78.4%)

<sup>a</sup> Age was not reported in 2 cases. HIV, human immunodeficiency virus; IQR, inter-quartile range.

As shown in Table 1, the median age of the patients was 45 years, with 2 of them being under 20 years old (2 and 6 years old). The majority of patients were male, and most were of Japanese nationality. The majority were treated for amebic colitis alone, and a few received treatment for amebic liver abscess alone or for both diseases. Exceptionally, 2 cases were treated for giardiasis. Although the HIV status of the patients was not requested in the patient records, approximately 1 in 5 cases were reported to be HIV positive.

The majority (90/145, 62.1%) of patients were treated with a daily dose of 1500 mg for 8–10 days, 20 cases (20/145, 13.8%) with 1500 mg for 5–7 days, 4 cases (4/145, 2.8%) with 1500 mg for 1–4 days, 5 cases (5/145, 3.4%) with 1500 mg for unknown period, and 3 (3/145, 2.1%) cases with 750 mg for 3 days. In 11 cases, each received individually different combination of the daily dosage and the period (including cases treated for unknown period), and in the remaining 12 cases (12/145, 8.2%), daily dosages were not described. Among them, the 2-year-old child was treated with 360 mg/day for 10 days, and the 6-year-old child received 600 mg/day for an unknown period.

### 3.2. Safety of paromomycin

A total of 123 cases could be assessed for drug safety, among which 23 cases (23/123, 18.7%) were reported to have AEs (Table 2). Most of the reported AEs were gastrointestinal symptoms, mainly diarrhea (17/123, 13.8%), which were relieved after the completion or discontinuation of paromomycin administration (Table 3). Paromomycin was discontinued due to AEs in 7 cases (5.6%), including the 3 cases described below and another 4 diarrheal cases in which the diarrhea resolved soon after the discontinuation of the drug. No AEs were reported in either the 2- or 6-year-old child.

The first discontinued case involved a 51-year-old HIV-positive male with amebic liver abscess who first received metronidazole, followed by 1500 mg/day of paromomycin. On day 5 of drug administration, he developed diarrhea and bloody stools. His stools tested positive for *Clostridium difficile* toxin A and a colonofiberscopy revealed features of pseudomembranous colitis. However, the patient had received other antibiotics including cefepime, ciprofloxacin, clindamycin, aztreonam, and cefmetazole in 1 month before the onset of the symptoms, and the physician in charge suggested that the illness might be due to those antibiotics. He was lost to follow-up. Second, a 59-year-old male with amebic

**Table 2**  
Reported presence or absence of adverse effects (n = 123).

Adverse effects	n	%
+	23	18.7
–	100	81.3

Evaluation could not be performed in 22 cases.

**Table 3**  
Reported adverse effects (n = 123).

	n	%
Diarrhea	17	13.8
Nausea	2	1.6
Anorexia	1	0.8
Abdominal bloating	1	0.8
Excessive flatulence	1	0.8
Heartburn	1	0.8
Bloody stools ( <i>Clostridium difficile</i> colitis)	1	0.8
Headache	1	0.8
Drowsiness	1	0.8
Skin rash	1	0.8
Elevation of liver enzymes	1	0.8

Evaluation could not be performed in 22 cases.

colitis first received metronidazole, followed by 1500 mg/day of paromomycin. Before paromomycin treatment, abnormal liver function levels of 62 IU/L  $\gamma$ -GTP and 2.7 mg/dL total bilirubin were noted; on day 5 of paromomycin treatment, AST, ALT, and LDH levels were found to be 88 IU/L, 115 IU/L, and 263 IU/L, respectively. Paromomycin was discontinued and the liver enzymes became normalized after 10 days. Lastly, a 47-year-old male with amebic colitis first received metronidazole, followed by 1500 mg/day of paromomycin. On day 2 of paromomycin treatment, he developed erythema and urticaria on the trunk and limbs, and was treated with a single injection of glycyrrhizin, followed by oral betamethasone and *d*-chlorpheniramine for 3 days, after which the rash resolved.

Among the evaluable 22 cases with AE, the daily dosages were 1500 mg in 21 cases and 750 mg in one case, while among the evaluable 88 cases without any AE, they were 1500 mg in 80 cases, 750–1250 mg in 4 cases, and 1750–2250 mg in 4 cases. The median daily dosages were not significantly different between individuals with and without AE ( $p = 0.56$ , Mann–Whitney's *U*-test).

Among the 22 evaluable cases that were reported to be HIV positive, 4 (4/22, 18.2%) patients had AEs. Among the 101 evaluable cases that were not reported to be HIV positive (i.e., the HIV status was negative or unknown), 19 (19/101, 18.8%) had AEs.

### 3.3. Efficacy of paromomycin

A total of 11 asymptomatic or mildly symptomatic cases of amebic colitis were analyzed for the efficacy of paromomycin when given alone (Table 4). Most cases were treated with 1500 mg/day for 9–10 days, and parasite clearance was achieved in all cases. The 6-year-old boy was also found to be negative for cysts in the stool after paromomycin treatment.

Exceptionally, 2 patients with giardiasis received paromomycin, with one of them being evaluable. The patient had previously received metronidazole twice unsuccessfully (500 mg/day for 10 days

and 2250 mg/day for 21 days), and was reported to be cured with 750 mg/day of paromomycin given for 5 days.

## 4. Discussion

Since 2009, the Japanese Ministry of Health, Labour and Welfare has been taking action to dissolve the so-called “drug lag,” and data obtained from studies other than formal clinical trials have become useful in making a previously unlicensed medicine licensed in Japan. This move prompted us to analyze the safety (and if possible the efficacy) of paromomycin, in an effort to contribute to licensing the medicine in Japan where the clinical management of amebiasis is increasingly recognized as important. From the 1960s to 1982 and 1998, this agent was licensed in Japan as 2 different brands for treating certain cases of bacterial enterocolitis, respectively. In fact, it was used most frequently for treating tapeworm infestations and most reports supported the safety of paromomycin when used for this purpose [17–19], although this may not guarantee the safety of the drug when used for the treatment of amebiasis for longer periods. Thus, in a Japanese multicenter case series, 118 cases of fish tapeworm received 50 mg/kg paromomycin either as a single dose or divided into 2 doses 30 minutes apart [19]. Four patients reported a total of 5 AEs, i.e., nausea in 2 patients, anorexia in one patient, and transient/slight tinnitus plus speech disturbance appearing in one patient. These patients were given almost concomitantly magnesium sulfate as a laxative to facilitate purging the tapeworm, and therefore drug-induced diarrhea could not be assessed properly in this study.

The current study was not conducted as a formal clinical trial and is, therefore, subject to some limits in interpretation. First, the evaluation of AEs and of the effectiveness of the drug may have not been conducted uniformly by the physicians in charge. Second, post-treatment follow-up periods may have varied between cases; for example, foreign visitors to Japan may have been observed only for a short period of time prior to returning to their original country. However, it is also plausible that the physicians established close relationships with their patients due to the unique nature of this trial, resulting in most of the unusual events, such as delayed AEs, being reported even after the patient record was completed and submitted. Thus, despite these limitations, the data seem to contribute to the efficacy and safety evaluation of paromomycin in Japan.

Studies on the efficacy and safety of paromomycin were reported mostly in the 1960s, with almost all reports showing mild and acceptable symptoms. These older studies may have included *E. dispar* infections, and thus, may not be adequate for analyzing drug efficacy; however, drug safety could be assessed. In an Ethiopian study, 96 patients with amebiasis aged 1–75 years received paromomycin, mostly either 30, 15, or 7.5 mg/kg/day for 5 days. Diarrhea occurred on days 2–3 of treatment, but most cases improved on day 5 [20]. In the US, 79 patients with amebiasis or *Dientamoeba fragilis* received paromomycin, 1,750 mg/day for 2, 3, 4, or 5 days, or 4 doses of 1,000 mg each for one day. Totally, diarrhea

**Table 4**  
Asymptomatic or mildly symptomatic amebic colitis cases treated with paromomycin.

Age	Sex	Evidence of <i>E. histolytica</i> infection	Treatment	Findings	
				Before treatment	After treatment
53	M	Trophozoites in stool	1500 mg/day, 10 days	Stool cyst (+)	Stool cyst (–)
49	M	Colonoscopy	1500 mg/day, 10 days	Stool cyst (+)	Stool cyst (–)
42	M	Colonoscopy	1500 mg/day, 10 days	Trophozoites in colonic mucosa	Stool trophozoite (–), cyst (–), improved histology
32	M	Colonoscopy	1500 mg/day, 10 days	Stool cyst (+)	Stool cyst (–)
37	F	Trophozoites in stool	1500 mg/day, 9 days	Stool cyst (+)	Stool cyst (–)
65	M	Colonoscopy	1500 mg/day, 10 days	Stool cyst (+)	Stool cyst (–)
47	M	Colonoscopy, serum antibodies	1500 mg/day, 10 days	Stool cyst (+)	Stool cyst (–)
61	F	Colonoscopy	1500 mg/day, 9 days	Stool cyst (+)	Stool cyst (–)
ND	M	Colonoscopy	1500 mg/day, 9 days	Stool cyst (+)	Stool cyst (–)
47	M	Trophozoites in stool	1500 mg/day, 9 days	Stool cyst (+)	Stool cyst (–)
6	M	Stool PCR	600 mg/day, for unknown period	Stool cyst (+)	Stool cyst (–)

ND, not described.

was noted in 13 cases (16.4%), nausea in 5 cases (6.3%), headache in 3 cases (3.8%), dizziness or abdominal cramps/bloating each in 2 cases (2.5%), and itching, urticaria, heartburn, or insomnia each in 1 case (1.3%), and except for a lower frequency in the one-day treatment arm, frequencies of the AEs did not correlate with duration of the treatments [21]. Another US study on 114 MSM patients with amebic colitis, receiving 25–35 mg/kg daily in 3 divided doses for 7 days, reported a high incidence of soft stools/diarrhea (67%), with most of the cases being mild, as well as nausea in 2 cases and constipation or dizziness each in 1 case [22]. The symptoms were reported to disappear after the completion of paromomycin treatment. A Kenyan study randomly allocated 417 amebic colitis patients aged 6–80 years to several treatment groups, including a paromomycin group in which adults and children received daily doses of 1,000 mg and 30 mg/kg, respectively, in 2 divided doses for 5 days [23]. The overall tolerability was reported to be excellent in the paromomycin group, with poor tolerability found only in 1% of cases.

Our data were largely consistent with previous reports in that most of the AEs consisted of mild gastrointestinal symptoms, and were mainly diarrhea that was resolved after completion or discontinuation of the agent. The case of *C. difficile* colitis might be due to the multiple antibiotics that had been given before paromomycin. The patient with liver function disturbance might have had an underlying liver disorder, and to our knowledge, there are no other reports on drug-induced hepatotoxicity with paromomycin. We found only 1 report on skin eruption, which was urticaria, and thus the dermatological AE does not seem to be unacceptable [21]. It is reassuring that HIV infection did not appear to increase the risk of drug-related AEs, although this issue was not formally addressed in this study.

The excellent efficacy of paromomycin monotherapy observed in our asymptomatic or mildly symptomatic cases deserves mentioning. This is consistent with the study results comparing paromomycin and diloxanide furoate given randomly to patients with PCR-confirmed asymptomatic *E. histolytica* infections in Vietnam (both 500 mg t.i.d. for 10 days) that showed the superiority of paromomycin over diloxanide furoate, with cure rates of 85% and 51%, respectively [24]. Most of our patients received paromomycin for preventing a relapse after successful acute-stage treatment; however, such efficacy of the agent could not be addressed in our study due to lack of the adequate control subjects. In addition, there have been no other reports with conclusive results on the relapse-preventing effect of the drug. A recent study of Japanese HIV-positive men with amebiasis showed that the recurrence rates of amebiasis were not different between those who took a luminal amebicide (paromomycin or diloxanide furoate) and those who did not [25]. However, this may be influenced by reinfections occurring more frequently among MSM. Apart from its use in the treatment of amebiasis, paromomycin has been suggested to be effective in patients with giardiasis or cryptosporidiosis that are refractory to the first-line treatment, as shown in the giardiasis case in this study [26,27].

Just recently, in December 2012, paromomycin was licensed for the treatment of amebiasis in Japan and is expected to be commercially available in mid-2013. As this licensing was decided without a formal clinical trial and based exclusively on the data shown here, we must remain vigilant as to whether a very rare, but potentially serious AE may occur with wider use of the agent. We will also attempt to determine its efficacy in protozoan diseases other than amebiasis in an effort to have it approved for the treatment of such diseases in the near future.

In conclusion, paromomycin was shown to be safe and well tolerated, as well as effective when used in the treatment of asymptomatic or mildly symptomatic amebic colitis in Japan. This agent was licensed just recently, and we will continue to further monitor its efficacy and safety.

#### Conflict of interest

T.M. is currently affiliated with ViiV Healthcare K.K., which was established by GlaxoSmithKline and Pfizer. Others have no potential conflicts of interest.

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## II 感染性疾患

### 寄生虫感染症

# 吸虫症

Fluke infections

Key words: 脳肺吸虫症, 神経型住血吸虫症, 瘧疾, 麻痺, プラジカンテル

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感染性疾患

## はじめに

吸虫は、条虫類と同じく扁形動物門に属する寄生性の動物で、生活史は基本的に2つの中間宿主を必要とし、第1中間宿主は陸産・淡水産の巻貝、第2中間宿主は吸虫の種によって様々である。終宿主は脊椎動物で、ヒトは吸虫にとっては終宿主になる。吸虫感染症で神経症状をきたすことはまれだが、肺吸虫症と住血吸虫症で比較的多く報告されている。

## 1. 概念・定義

吸虫類の人体内での寄生部位は、消化管か消化管と解剖学的につながっている肺や胆道系が主で、神経系は本来の寄生部位ではない。吸虫症における神経症状は、虫体が神経系に「迷入」するか、あるいは成虫が産卵した虫卵が血行性に中枢神経に到達することによって引き起こされる。

脳肺吸虫症は、肺吸虫の成虫が中枢神経系に迷入して起きる。かつて肺吸虫症が西日本を中心に多数みられたときには、小児を中心として大きな問題であった。一方、神経型住血吸虫症は、寄生部位である門脈系血管内に産卵された虫卵が中枢の小血管を塞栓し肉芽腫を形成するのが病態の中心をなす。

## 2. 疫学と病因

### 1) 肺吸虫症

肺吸虫属の中でこれまで人体寄生が確認されているのは7種で、そのうち神経系への迷入が報告されているのは、ウエステルマン肺吸虫(*Paragonimus westermani*)、宮崎肺吸虫(*P. miyazakii*)、*P. mexicanus*、*P. africanus*である。宮崎肺吸虫は今日ではスクリアピン肺吸虫(*P. skrjabini*)の亜種あるいは種内変異と見なされるようになったが、本稿では宮崎肺吸虫の用語を採用する。

肺吸虫症は多くの吸虫疾患と同様、食品由来の寄生虫感染症である。第2中間宿主の淡水産の甲殻類(我が国ではサワガニとモクズガニ、韓国ではザリガニが主)を加熱不十分な調理法で摂食すると、感染型の幼虫(メタセルカリア)が人体に取り込まれ感染が成立する。甲殻類の生食による感染以外に、イノシシ肉を刺身で食べてウエステルマン肺吸虫に感染する症例もみられる。イノシシが肺吸虫に感染したモクズガニやサワガニを食べると、メタセルカリアはイノシシの体内で成虫になれず、筋肉内でメタセルカリアないし幼若成虫のまま長期間とどまり感染源になる。

脳肺吸虫症の特徴として、理由は不明だが患者は子どもや若年層の男性に多いことが知られている。かつての我が国のウエステルマン肺吸

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