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-aminolevulic 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 evolutionarily 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 *Phytomonas 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...
and maintenance of body wall O₂ tension by creating an oxygen detoxification by a reaction driven by nitric oxide [14]. The proposed functions of this oxygen-avid hemoglobin are particularly well studied because of the interests in their roles in low-oxygen environments [16]. Another example of well-characterized nematode hemoproteins is cytochrome b in the mitochondrial respiratory complex II of *Ascaris lumbricoides* (parasitic nematode). *A. suum* larvae utilize classic mammalian-type respiration, expressing a small subunit of cytochrome b (CybS) instead of Cybs [17]. Given the important roles played by the hemoproteins in animal parasitic nematodes, it is interesting to know how these 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 stercoralis* füehebursi [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 endosymbiotic genome [24,25].

In the present study, we cloned the entire cDNA sequence of the FeCH gene from *S. venezuelensis* (*Sv*FeCH). Our BLAST search on publically 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 metazoa FeCH, suggesting that the origin of nematode FeCH genes are different from those of non-nematode metazoa FeCH. The chelatase activity of the *Sv*FeCH was confirmed by an FeCH-deficient assay using a cloned active FeCH from organisms in the phylum Nematoda [26].

**Figure 1. Genomic DNA and cDNA sequences of the *Strongyloides venezuelensis* FeCH gene.** Both sequences were identical excluding the intronic region, which only existed 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, excluding the intronic region, which existed only in the genomic DNA. *S. venezuelensis* FeCH was confirmed by an FeCH-deficient assay using a cloned active FeCH from organisms in the phylum Nematoda.

**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 Ethics Committee.

**Conflict of Interest**

The authors have declared that no competing interests exist.

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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 ENM5_6_7, and terminal transferase. The dA-tailed cDNA was used as a template the synthesized first-strand cDNA was performed using dATP and

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.

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

A cDNA sequence corresponding to the entire catalytic core region of S. venezuelensis FeCH (amino acid positions 29–373) was obtained by PCR using the primer pair TKT001/TKT002. The PCR product was cloned into pET-21a (Merck, 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%
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 (ΔhemH), a deletion mutant for hemH gene [43] was provided by the National Bioresource Project of MEXT, Japan.

The entire ORF of S. FeCH, obtained by RT-PCR with the primer pair ENM089/ENM098, was cloned into the XhoI/BglII 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-SFeCH was tested as a gene complementation vector. The original pFLAG-CTC plasmid served as a control.

ΔhemH was transformed with pFLAG-CTC-SFeCH or with pFLAG-CTC. The transformed and untransformed E. coli ΔhemH strains were cultured overnight in LB medium supplemented with hemin (10 μg/ml). For the culture of the transformed ΔhemH, ampicillin was also added at a concentration of 50 μg/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 OD600 of 0.1 in hemin-containing (10 μg/ml) or hemin-free LB medium with (for the transformed ΔhemH) or without (for the untransformed ΔhemH) 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 S 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 SFeCH 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 S. 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 paraanaensis, 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 SFeCH protein sequence was used as a query for the BLAST analysis, two additional species were found to carry FeCH gene
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%)

Figure 5. Genetic complementation assay of ΔhemH E. coli. An untransformed ΔhemH strain of E. coli was grown in the absence (diamond) or presence 10 μg/ml hemin (square). In the same experiment, a transformed ΔhemH strain of E. coli either with SvFeCH gene expression vector (triangle) or with empty vector (x-mark) was cultured in the absence of hemin. OD₆₀₀ was measured every hour up to 20 h to monitor bacterial growth.
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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 (±) of biological triplicates. Real-time RT-PCR was performed in triplicate wells for each biological replicate.
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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].

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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 *Sv*FeCH 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 owczarzaki*, oomycetes, amoeboboa, 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 (2.0×10⁻⁷¹–2.0×10⁻³⁶).

**Chelatase Assay using Recombinant *Sv*FeCH**

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 *Sv*FeCH. We constructed an expression plasmid, pET-*Sv*FeCH, 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 *Sv*FeCH 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 *Sv*FeCH by pFLAG-CTC-*Sv*FeCH 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 *Sv*FeCH is an active enzyme that can function as FeCH.

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

The relative expression levels of *Sv*FeCH 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 *Sv*FeCH 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 (*Cyandioschyzon 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 filariae 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 filariae, 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 filariae 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^{2+} 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.

References