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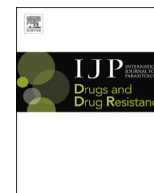

ABC-B transporter genes in *Dirofilaria immitis*

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ABSTRACT

Dirofilaria immitis is a filarial nematode causing infection and heartworm disease in dogs and other canids, cats, and occasionally in humans. Prevention with macrocyclic lactones (ML) is recommended during the mosquito transmission season. Recently, ML resistance has been reported. ABC-B transporter genes are thought to be involved in the mechanism of ML resistance in other nematodes. This study aimed to identify all the ABC-B transporter genes in *D. immitis* using as a reference the nDi.2.2 *D. immitis* whole genome, which is not completely annotated. Using bioinformatic tools and PCR amplification on pooled *D. immitis* genomic DNA and on pooled cDNA, nine ABC transporter genes including one pseudogene were characterized. Bioinformatic and phylogenetic analyses allowed identification of three P-glycoproteins (Pgps) (Dim-*pgp-3* Dim-*pgp-10*, Dim-*pgp-11*), of two ABC-B half transporter genes (one ortholog of *Cel-haf-4* and *Cel-haf-9*; and one ortholog of Cel-*haf-1* and Cel-*haf-3*), of one ABC half transporter gene (ortholog of Cel-*haf-5*) that contained an ABC-C motif, and of one additional half transporter that would require functional study for characterization. The number of ABC-B transporter genes identified was lower than in *Caenorhabditis elegans* and *Haemonchus contortus*. Further studies are needed to understand their possible role in ML resistance in *D. immitis*. These ABC transporters constitute a base for ML resistance investigation in *D. immitis* and advance our understanding of the molecular biology of this parasite.

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1. Introduction

Dirofilaria immitis is a filarial nematode infecting and sometimes causing heartworm disease in dogs and other canids, cats, and occasionally in humans (Lee et al., 2010; Damle et al., 2014). The parasite is transmitted to the host through infected mosquitoes. Chronic heartworm disease includes symptoms such as lethargy, exercise intolerance, loss of appetite, weight loss, coughing, cardiac insufficiency and difficulty breathing. In the host, *D. immitis* juvenile worms migrate to pulmonary arteries and mature, sometimes leading to the arteries becoming blocked. In heavy infections, adult worms may be present in the heart chambers. These conditions can lead to death of the animal in the most severe cases. Heartworm is distributed worldwide (Morchon et al., 2012; Simon et al., 2012).

Melarsomine, an arsenic-based drug, is used to kill adult worms and worms as young as 4 months old (Raynaud, 1992). Prevention with macrocyclic lactones (ML) is recommended all year-round in USA, or during the mosquito transmission season in other regions. In 2005, a first report on ML loss of efficacy (LOE) in the USA, was published (Hampshire, 2005). Then, other studies on ML LOE and ML resistance in *D. immitis* have been reported (Bourguinat et al., 2011a; Bourguinat et al., 2011b; Bourguinat et al., 2015; Pulaski et al., 2014).

The mechanism of ML resistance in nematodes is still not well understood. However, several studies reported that ML resistance may be polygenic (Prichard, 2001; Vercruysse and Rew, 2002; McCavera et al., 2007; Sutherland and Scott, 2009). P-glycoproteins (Pgp) have been shown to be implicated in several drug resistance processes such as chemotherapy resistance in tumour cells in humans (Lespine et al., 2012) and also to be associated with ML resistance in *C. elegans* and in parasitic nematodes (Blackhall et al., 1998; Xu et al., 1998; Ardelli et al., 2005, 2006; Ardelli and Prichard, 2007; Prichard and Roulet, 2007; James and Davey, 2009; Lespine et al., 2012; Ardelli, 2013; Janssen et al., 2013a). A correlation between loss of efficacy of ML heartworm preventives and Pgp genotype was reported in *D. immitis* (Bourguinat et al., 2011b). Pgps are members of the ATP binding cassette family of

Abbreviations: ML, macrocyclic lactones; LOE, loss of efficacy; Kb, kilobase; Tm, melting temperature; TM, transmembrane domain; Pgp, P-glycoprotein; ATP, adenosine triphosphate; IVM, ivermectin.

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proteins also called ABC transporters (from A to H) (Ambudkar et al., 2003). Pgps belong to the ABC-B group of transporters. Pgps are full size ABC-B proteins composed of two transmembrane (TM) domains that each contains six TM helices. Each TM domain is followed by a nucleotide binding domain (Ambudkar et al., 2003). The two sections of the protein are connected by a linker region. However, half size ABC-B transporter proteins are composed of one TM, including six TM fragments, and one nucleotide binding domain (Sheps et al., 2004). Pgps play the role of pumps that can enable a substrate to be transported outside the cell using ATP as energy. Substrates of Pgp are neutral and cationic hydrophobic compounds. Ivermectin (IVM) (Lespine et al., 2007; Kerboeuf and Guegnard, 2011) and selamectin (Griffin et al., 2005) are reported to be good substrates for Pgps, with moxidectin being less transported by Pgps (Cobb and Boeckh, 2009; Godoy et al., 2015). So far, 15 Pgps have been reported in *C. elegans* (Sheps et al., 2004) including a pseudogene (Lespine et al., 2008) and 10 in *H. contortus* (Laing et al., 2013). Based on their respective genomes, the filarial worms, *Brugia malayi*, *Onchocerca volvulus* and *Loa loa* appear to have fewer Pgps than *C. elegans* or *H. contortus*. It is not clear how many Pgps are presents in *D. immitis*. The purpose of this study was to identify all the ABC-B transporters in heartworm, using the D. *immitis* whole genome that is available (Godel et al., 2012) but not yet completely annotated. The corresponding information obtained is valuable to improve knowledge of the structure of ABC-B transporters genes in *D. immitis* which may be useful for further investigation of ML resistance mechanisms in *D. immitis*. Also this information could be used to identify potential additional genetic marker that could predict ML resistance in *D. immitis*, and adds to our knowledge of the molecular biology of this parasite.

2. Material and methods

2.1. Bioinformatic identification of D. immitis ABC-B transporter genes

All ABC-B transporter genes reported (Sheps et al., 2004; Lespine et al., 2008) in the nematode model organism *C. elegans* (25 genes) were collected from WormBase (http://www.wormbase.org) and from GenBank NCBI (http://www.ncbi.nlm.nih.gov/). Also, based on the analysis of 15 genomic sequences that could predict ATP-binding cassette (ABC) systems in *B. malayi* (Ardelli et al., 2010), Pgp genes were identified using NCBI Blast tool, BLASTN 2.3.1+ (Zhang et al., 2000) and BLASTX 2.3.1+ (Altschul et al., 1997) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Additional Blast analysis on these 15 sequences was performed with the Broad Institute Blast tools (http://www.broadinstitute.org/), to identify additional sequences in filarial nematodes. In total, 69 sequences were collected from different organisms (Supplementary data S1): 25 in *C. elegans*, 9 in *Caenorhabditis remanei*, 9 in *Loa. loa*, 15 in *B. malayi*, 1 in *D. immitis*, 5 in *Wuchereria bancrofti* and 5 in *O. volvulus*. These 69 sequences were used to interrogate the *D. immitis* genome nDi.2.2 (http://xyala.cap.ed.ac.uk/downloads/959nematodegenomes/blast/filareu.php) with BLASTN 2.2.25 and TBLASTN 2.2.25 (Altschul et al., 1997). This allowed location of ABC-B transporter genes on *D. immitis* scaffolds. Additionally, sequences annotated as nematode orthologs of Pgps in the nDi.2.2 genome browser (http://salmo.bio.ed.ac.uk/cgi-bin/gbrowse/gbrowse/nDi.2.2.2/) were collected.

2.2. Confirmation sequencing of ABC-B transporter genes

Confirmation of sequences was performed from pooled *D. immitis* samples shipped from TRS Labs (TRS Labs Inc., Athens, GA, USA) on dry ice. Worms were thawed in petri dishes containing RNAlater™ RNA Stabilization Reagent (Qiagen Inc., Toronto, ON, Canada). The worms were sectioned: one third of each worm was used for gDNA isolation using DNeasy®Blood and Tissue kit (Qiagen). Two third of each worm was used for RNA isolation, using TRIzol®Reagent (Ambion®, Life technologies™ Inc., Burlington, ON, Canada). QuantiTect Reverse Transcription kit (Qiagen) was used to prepare cDNA.

Several specific primers per gene (Supplementary data S2) were designed, based on the genomic DNA of the nDi.2.2 *D. immitis* genome, from the 7 scaffolds identified from Blast analysis and from 1 scaffold identified from the genome browser, to amplify full length genomic DNA sequences of full and half *ABC-B* transporter genes. Simultaneously, open reading frame (ORF) finder from Geneious Pro.5.6.3 software (http://www.geneious.com/; Kearse et al., 2012) was used to predict cDNA sequences. Forward splice leader SL1 (Blaxter and Liu, 1996) primer and reverse specific primers (Supplementary data S2) in the 3′UTR region were used to amplify full cDNA sequences of *ABC-B* transporter genes.

One microliter of 10 μM forward and reverse primers, 2 μl of 50 mM $MgSO_4$ and 1 μl of 10 mM dNTPs were used with 0.3 μl High Fidelity Platinium® Taq DNA polymerase (Invitrogen Inc., Burlington, ON, Canada) in 50 μl reaction for all PCR amplifications. DNA template was adjusted to 80 ng. PCR amplifications were performed on a MJ Research PTC-200 thermal cycler following the cycling parameters: an initial denaturation step at 94 °C for 2 min followed by 36 amplification cycles, with each cycle including a denaturation step at 94 °C for 30 s, an annealing step at Tm°C, depending on the primer set (Tm included in Supplementary data S2) for 30 s and an extension step at 68 °C for 1 min/kb. A final extension was performed at 68 °C for 10 min.

PCR products were examined by agarose gel electrophoresis. When multiple amplicons were observed on agarose gels, bands were cut, DNA purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation, CA, USA) and Sanger sequenced (Sanger and Coulson, 1975; Sanger et al., 1977) using a 3730XL DNA Analyzer system at McGill University/Génome Québec Innovation Centre. When only one band was observed on agarose gels, PCR products were directly sent for Sanger sequencing at McGill University/Génome Québec Innovation Centre. All the sequences obtained from the PCR amplicons were assembled and analysed using Geneious Pro.5.6.3, Sequencher® version 4.10 software (Gene Codes Corporation, Ann Arbor, MI, USA) and the nuclear genome assembly nDi.2.2 as *D. immitis* reference genome (http://nematodes.org/genomes/dirofilaria_immitis/).

2.3. Identification of ABC transporter protein features

Amino acids were predicted from cDNA sequences using Geneious Pro.5.6.3 software translation tool. Bioinformatics Resource Portal called ScanProsite tool (released 20.123; http://prosite.expasy.org/scanprosite/) was used to identify the number and positions of transmembrane domains, ATP-binding cassette domains and ABC signature motifs of each predicted protein sequence from cDNA sequences. Based on the location of the predicted TM, the predicted number of transmembrane helices within the TMs were estimated using Transmembrane Hidden Markov models (TMHMM) (v2.0) (Sonnhammer et al., 1998) in Geneious Pro.5.6.3 software. Conserved Walker A and Walker B sites were located with NCBI server using BLASTP 2.3.1+ (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997).

2.4. Phylogenetic analysis

ABC-B transporter amino acid sequences from *C. elegans*, *B. malayi*, *O. volvulus* and *L. loa*, listed in Table 1, were included in the phylogenetic analysis with the different predicted protein

sequences from *D. immitis*. Only complete amino acid sequences were included in the analyses. The different isoforms of Cel-PGP-2, Cel-PGP-5 and Cel-PGP-6 were also included in this analysis. The two ClustalW alignments of full length amino acid sequences for ABC–B full and half transporters were performed with Geneious Pro.5.6.3 software using Matrix Blosum. The two alignments available in FASTA format were trimmed as indicated in the Supplementary data S3 for the full transporters and in Supplementary data S4 for the half transporters. The criterion for trimming was to work on a common region, for each of the amino acid sequences, that started and finished with a conserved region. An exception was made for Cel-PGP-5b and Cel-PGP-6b as they had shorter complete amino acid sequences compared to the others. Subsequently, with Geneious Pro version 5.6.3, the corresponding two un-rooted phylogenetic trees were built from the trimmed alignments using PHYML, an appropriate and accurate maximum likelihood method (Guindon and Gascuel, 2003) with Blosum 62, and using Bootstrapping. Bootstrap for higher confidence was performed at the level of 1000. Bootstrap proportions are indicated on the tree branches.

3. Results and discussion

3.1. Bioinformatic identification and confirmation of sequences of D. immitis ABC-B transporter genes

All the cDNA sequences discussed contained the SL1 sequence (Blaxter and Liu, 1996) and a stop codon. The gene, annotated as being an ortholog of *Cre-pgp-13* in nDi.2.2.scaf00049:93378..99486, was amplified by PCR. From the two amplicons identified, one sequence had a repeat region of 49bp similar to the sequence from nDi.2.2.scaf00049 and one sequence did not carry the extra repeated 49bp. Each amplicon was approximately 5 kb. Additional Blast analysis did not allow identification of the ABC signature. In nDi.2.2.scaf00049, the region from 93378 to 99486 hit protein Bm360 from *B. malayi*. Additional investigations and analysis in this scaffold and the amplified sequences did not reveal any additional evidence of a full-length ABC transporter gene for this previously annotated sequence and raises a question about this annotation. Thus, it was not included in the subsequent work.

Blast analysis in *D. immitis* nDi.2.2 with Pgp sequences from *C. elegans*, *C. remanei*, *B. malayi*, *W. bancrofti*, *D. immitis*, *O. volvulus*, *and L. loa* allowed 7 genes, that contained ABC-B motifs, to be identified in the following scaffolds (nDi.2.2.scaf00004, nDi.2.2.scaf00023, nDi.2.2.scaf00041, nDi.2.2.scaf00046, nDi.2.2.scaf00048, nDi.2.2.scaf00101 and nDi.2.2.scaf00496) (Supplementary data S5). In total, 7 complete gDNA sequences and 12 cDNA sequences (all including SL1 sequence (Blaxter and Liu, 1996) were successfully amplified, sequenced and confirmed. GenBank accession numbers of these sequences are available in Table 2. NCBI BLASTX 2.3.1+ results of the ABC-B transporter gene located in nDi.2.2.scaf00041 identified three fragments, nDi.2.2.scaf00041:17947..19267 (1321bp), corresponding to hypothetical protein LOAG_07988, nDi.2.2.scaf00041:19268..20612 corresponding to hypothetical protein Bm1_15040, and nDi.2.2.scaf00041:20612..27954 (7343bp) corresponding to hypothetical protein LOAG_07989 and Cbr-PGP-4 protein. Predicted cDNA sequences showed more similarity to *pgp-16* from *Haemonchus contortus* (Supplementary data S5). Subsequent analysis on the consensus sequence generated from nDi.2.2.scaf00041, revealed that the gDNA sequence contained 7 stop codons located in predicted exon regions (Supplementary data S6). Two stop codons

Table 1
Accession numbers of amino acid sequences used for the phylogenetic analysis. The sequences were either collected on WormBase (http://www.wormbase.org/#01-23-6) or GenBank NCBI (http://www.ncbi.nlm.nih.gov/genbank/).

	Genes	Accession numbers	Genes	Accession numbers
Full ABC-B transporter	*C. elegans*		*B. malayi*	
	Cel-PGP-1	WP:CE11932	Bm1_43165	XP_001900095.1
	Cel-PGP-2a	WP:CE41207		
	Cel-PGP-2b	WP:CE50744	*O. volvulus*	
	Cel-PGP-2c	WP:CE08576	Ovo-PGP-10	WP:OVP13603
	Cel-PGP-2d	WP:CE29212	Ovo-PGP-11	WP:OVP14503
	Cel-PGP-3	WP:CE03818	AAX82635.1	AAX82635.1
	Cel-PGP-C	CAA46191.1	AAD49436.1	AAD49436.1
	Cel-PGP-4a	WP:CE44238		
	Cel-PGP-4b	WP:CE03308	*L. loa*	
	Cel-PGP-5a	WP:CE43003	LOAG_00381	EFO28095.2
	Cel-PGP-5b	WP:CE43182	LOAG_03722	XP_003139307
	Cel-PGP-6a	WP:CE40818		
	Cel-PGP-6b	WP:CE40819	*D. immitis*	
	Cel-PGP-6c	WP:CE46295	“nDi2.2.scaf00004″	Genbank: KP296255
	Cel-PGP-7	WP:CE36668	“nDi2.2.scaf00046″	Genbank: KP296249
	Cel-PGP-8	WP:CE31624	“nDi2.2.scaf00048″	Genbank: KP296245
	Cel-PGP-9	WP:CE15714		
	Cel-PGP-10	WP:CE40807		
	Cel-PGP-11	WP:CE34788		
	Cel-PGP-12	WP:CE03260		
	Cel-PGP-13	WP:CE40253		
	Cel-PGP-14	WP:CE03262		
Half ABC transporter	*C. elegans*		*B. malayi*	
	Cel-HAF-1	WP:CE39331	Bm1_15490	XP_001894558.1
	Cel-HAF-2	WP:CE07240	Bm1_30435	XP_001897525.1
	Cel-HAF-3	WP:CE41666	Bm1_50255	XP_001901519.1
	Cel-HAF-4	WP:CE28355		
	Cel-HAF-5/HMT-1	WP:CE31731	*D. immitis*	
	Cel-HAF-6	WP:CE39850	“nDi2.2.scaf00023″	Genbank: KP296251
	Cel-HAF-7	WP:CE24404	“nDi2.2.scaf00101″	Genbank: KP296253
	Cel-HAF-8	WP:CE14926	“nDi2.2.scaf00496.1″	Genbank: KP296257
	Cel-HAF-9	WP:CE27353	“nDi2.2.scaf00496.2″	Genbank: KP296258

Table 2
GenBank accession numbers of *the D. immitis* ABC-B transporters genes and cDNA newly identified.

Name	Gene/cDNA	GenBank Accession number
Pseudogene	gene	KP296247
Dim-*pgp-3*	gene	KP296248
Dim-*pgp-3*	cDNA	KP296249
Dim-*pgp-10*	cDNA	KP296245
Dim-*pgp-10*	gene	KP296246
Dim-*pgp-11*	gene	KP296254
Dim-*pgp-11*	cDNA	KP296255
Dim-*haf-1*	gene	KP296250
Dim-*haf-1*	cDNA	KP296251
Dim-*haf-4*	gene	KP296252
Dim-*haf-4*	cDNA	KP296253
Dim-*haf-5*	gene	KP296256
Dim-*haf-5.1*	cDNA isoform 1	KP296257
Dim-*haf-5.2*	cDNA isoform 2	KP296258

were located in exon 25, which carries the ABC signature motif. The sequence could be considered as a pseudogene (Vanin, 1985) as the gene contains multiple genetic lesions that would prevent the translation of a functional protein. In this regard, cDNA could not be translated to a predicted amino acid sequence and thus could not be included in the phylogenetic analysis. No specific name was attributed to this pseudogene. Interestingly, in *C. elegans*, Cel-*pgp-15* was reported to be a pseudogene (Lespine et al., 2008). In *Entamoeba histolytica*, two Pgps have been reported with a frame shift and stop codons within their ATP binding domain (Descoteaux et al., 1992). However, even though the corresponding protein will not be functional, polymorphism in pseudogenes may lead to important physiological changes (Macphee et al., 2002). Alignments between gDNA sequences and cDNA allowed the observation that the ABC-B transporter transcript frame, from nDi.2.2.scaf00004, nDi.2.2.scaf00023, nDi.2.2.scaf00046, nDi.2.2.scaf00048, nDi.2.2.scaf00101 and nDi.2.2.scaf00496, contained 24, 14, 26, 29, 10, 13 introns and 25, 15, 27, 30, 11, 14 exons, respectively (Supplementary data S7, one worksheet for each scaffold).

In this study, cDNA translation to amino acid sequences allowed identification of the structure of ABC transporter proteins (Table 3). From the cDNA amplification related to nDi.2.2.scaf00048, five amplicons with different sizes were obtained (Table 3). Based on TM prediction and subsequent transmembrane helix prediction in TM domains from the translated nucleotides, the amino acid sequence SEQ-1 did not carry the first TM, and SEQ-2 contained, from the first methionine, a total of 2 transmembrane helices in the first TM domain. The rest of the sequences were both followed by an ATP binding cassette domain, a complete TM containing 6 transmembrane helices, and a second ATP binding cassette domain. SEQ-1 and SEQ-2 from nDi.2.2.scaf00048 were not considered complete and were not investigated further. The amino acid sequence from the translated nucleotides of SEQ-3 contained 2 TM domains including 6 transmembrane helices and 2 ATP binding cassette domains. SEQ-3 from nDi.2.2.scaf00048 was included in the phylogenetic analysis as a full ABC-B transporter. Additionally, the amino acid sequences from the translated nucleotides of SEQ-4 and SEQ-5 contained, from their first methionine, respectively 2 and 6 transmembrane helices in their TM and one ATP binding cassette domain. Only SEQ-5 from nDi.2.2.scaf00048 was included in the phylogenetic analysis and called in the tree "nDi.2.2.scaf00048.5". As a general observation for SEQ-1 to SEQ-5 from nDi.2.2.scaf00048, the difference between the sequences was only the length. Beside this, the sequences were identical. In this regard, the first methionine of SEQ-5 was located at position 708 of the amino acid sequence SEQ-3 related to nDi.2.2.scaf00048 or at position 2122 in nucleotide cDNA sequence of SEQ-3.

For cDNA amplification related to nDi.2.2.scaf00004, one amplicon was observed and sequenced. Based on TM prediction and subsequent transmembrane helix predictions in the TM, from the translated nucleotides, the amino acid sequence contained 2 complete sets of 6 transmembrane helices each in a TM domain, and two nucleotide binding domains as illustrated in Table 3. The amino acid sequence was inserted in the phylogenetic analysis of the full ABC-B transporters.

From the cDNA amplification related to nDi.2.2.scaf00046, two amplicons with different sizes were obtained (Table 3). Based on TM domain prediction and subsequent transmembrane helix predictions in the TM, from the translated nucleotides, the amino acid sequence SEQ-1 from nDi.2.2.scaf00046 contained, from the first methionine, an incomplete TM carrying 3 predicted transmembrane helices followed by one ATP binding cassette domain. The amino acid sequence SEQ-2 from nDi.2.2.scaf00046 contained 2 complete TM domains, each of which included 6 predicted transmembrane helices, and two ATP binding cassette domains as illustrated in Table 3. Only the amino acid sequence SEQ-2 from nDi.2.2.scaf00046 was inserted in the phylogenetic analysis of the full ABC-B transporters. As a general observation for SEQ-1 to SEQ-2 from nDi.2.2.scaf00046, the difference between the sequences was only the length. Beside this, the sequences were identical. Interestingly, in the *D. immitis* genome nuclear browser nDi.2.2.2 (http://salmo.bio.ed.ac.uk/cgi-bin/gbrowse/gbrowse/nDi.2.2.2/), a transcript was annotated as being an ortholog of Cre-PGP-4 in nDi.2.2.scaf00046 at position 226523..238764. The SEQ-2 cDNA sequence that we amplified from this scaffold started at position 226523 also in nDi.2.2.scaf00046. However, a 1664 bp difference was observed at the end of the amplicons between the annotated sequence from nDi.2.2 genome and SEQ-2 amplified in this study.

From the cDNA amplification related to nDi.2.2.scaf00023, nDi.2.2.scaf00101, nDi.2.2.scaf00496, one, one and two amplicons were observed, respectively and each sequenced. Based on TM predictions and subsequent transmembrane helix predictions in the TM domain from the translated nucleotides, the amino acid sequences contained one TM domain including 4 transmembrane helices followed by 1 ATP binding cassette domain.

All 4 amino acid sequences related to nDi.2.2.scaf00023, nDi.2.2.scaf00101, and nDi.2.2.scaf00496 were included in the phylogenetic analysis of the half transporters. The two sequences from nDi.2.2.scaf00496 were called "nDi.2.2.scaf00496.1" and "nDi.2.2.scaf00496.2". Between these last 2 sequences, only the beginning of the sequences differed.

As a summary, one pseudogene, three potential full, and five potential half ABC-B transporters were identified.

3.2. Phylogenetic analysis

Two phylogenetic trees have been generated allowing orthology identification of 8 out of the 9 amino acid sequences (pseudogene not included). The phylogenetic tree of the full ABC–B transporters is presented in Fig. 1. Even though the amino acid sequences from Cel-PGP-5b and Cel-PGP-6b were shorter than the others in the ClustalW alignment, in the phylogenetic tree, Cel-PGP-5b and Cel-PGP-6b grouped respectively with their isoforms Cel-PGP-5a and Cel-PGP-6a. The nucleotide translated sequence related to nDi.2.2.scaf00004 was an ortholog of Cel-PGP-11. Based on Beech et *al.* (Beech et al., 2010), the gene should be named Dim-*pgp-11*.The nucleotide translated sequence related to nDi.2.2.scaf00046 was an ortholog of Cel-PGP-3, of its spliced variant Cel-PGP-C, of Cel-PGP-4a and of Cel-PGP-4b. The corresponding gene is called Dim-*pgp-3*. The nucleotide translated sequence related to nDi.2.2.scaf00048 was an ortholog of Cel-PGP-10. The corresponding gene is called Dim-*pgp-10*.

Table 3
Analysis of potential ABC-B transporter cDNA amplicons and predicted structural organization of the amino acid sequences *D. immitis*.* indicates new sequences submitted to GenBank. TM and ABC refer to transmembrane domain and ATP-binding cassette domain, respectively. Nucleotide translation was performed with the translation tool of Geneious Pro.5.6.3 software. Predictions of transmembrane domain, ATP-binding cassette, and of ABC transporter family signature, were performed using ScanProsite (released 20.123; http://prosite.expasy.org/scanprosite/). Prediction of transmembrane helices was done with TMHMM (v.2.0) in Geneious Pro.5.6.3 software.

Location on nDi.2.2 genome	Variants	CDNA length (bp)	Predicted Amino Acid (AA) sequences	Positions based on the AA sequence			Predicted motif in AA sequences
				Predicted TM	Predicted ABC domain	ABC transporters family Signature	
scaf00048: 79265..63901	SEQ-1	3111	1036	476–761	1: 108–344 2: 796–1034	247–261: LSGGQKQRVAIARAV 937–951: LSGGQKQRIAIARAI	ABC – TM – ABC
	SEQ-2	3336	1111	1: 10–129 2: 551–836	1: 183–419 2: 871–1109	322–336: LSGGQKQRVAIARAV 1012–1026: LSGGQKQRIAIARAI	TM – ABC – TM – ABC
	SEQ-3	3978*	1325	1: 29–343 2: 765–1050	1: 397–633 2:1085–1323	536–550: LSGGQKQRVAIARAV 1226–1240: LSGGQKQRIAIARAI	TM – ABC – TM – ABC
	SEQ-4	1173	390	1–115	150–388	291–305: LSGGQKQRIAIARAI	TM – ABC
	SEQ-5	1857	618	58–343	378–616	519–533: LSGGQKQRIAIARAI	TM – ABC
scaf00004: 88673..79181		3861*	1286	1: 64–354 2: 726–1012	1: 389–625 2:1045–1281	528–542: LSGGQKQRIAIARTI 1184–1198: LSGGQKQRIAIARAL	TM – ABC – TM – ABC
scaf00046: 226523..237100	SEQ-1	1317	438	1–164	198–434	337–351: LSGGQKQRIAIARAI	TM – ABC
	SEQ-2	3759*	1252	1: 41–333 2: 691–978	1: 368–604 2:1012–1248	507–521: LSGGQKQRIAIARIL 1151–1165: LSGGQKQRIAIARAI	TM – ABC – TM – ABC
scaf00023: 388438..395064		2040*	679	100–399	432–672	573–587: LSGGQRQRIAIARAL	TM – ABC
scaf00101: 45240..51224		2277*	758	199–479	511–747	650–664: MSGGQKQRIAIARAL	TM – ABC
scaf00496: 20854..25667	SEQ-1	2142*	713	102–397	433–667	570–584: LSGGEKQRVAIARAL	TM – ABC
	SEQ-2	1896*	631	20–315	351–585	488–502: LSGGEKQRVAIARAL	TM – ABC

The phylogenetic tree of the half ABC–B transporters is presented in Fig. 2. The amino acid sequence corresponding to SEQ-5 related to nDi.2.2.scaf00048 and called "nDi.2.2.scaf00048.5" in Fig. 2 does not show clear orthology with other *C. elegans* half transporter. An additional phylogenetic tree (Supplementary data S8) including all the full ABC-B transporters and half-ABC-B transporters was constructed with the same trimmed sequences used in Fig. 1 and Fig. 2. In Supplementary data S8, "nDi.2.2.scaf00048.5" is localized between the branches of the full ABC-B transporter and the branches of the half transporter. We did not have enough data on which to name this sequence. Functional analysis may be required to confirm if this potential half transporter is functional. However, as explained previously, this sequence shares the same characteristics as Dim-*pgp-10*, as the second part of *Dim-pgp-10* is the same as SEQ-5 ("nDi.2.2.scaf00048.5") and could represent a splice variant. The nucleotide translated sequence related to nDi.2.2.scaf00023 was an ortholog of Cel-HAF-1 and of Cel-HAF-3. The corresponding gene is called Dim-*haf-1*. The nucleotide translated sequence related to nDi.2.2.scaf00101 was an ortholog of Cel-HAF-4 and Cel-HAF-9. The gene is called Dim-*haf-4*. The nucleotide translated sequences related to nDi.2.2.scaf00496.1 and nDi.2.2.scaf00496.2 were orthologs of Cel-HAF-5 also called HMT-1. The corresponding genes in *D. immitis* are called Dim-*haf-5.1* and Dim-*haf-5.2*. With additional Blast analysis using BLASTP 2.3.1+ (NCBI), Dim-HAF-5.1 and Dim-HAF-5.2 carried ABC-C motifs, as does Cel-HAF-5. As it was predicted in the first part of the study, the TM related to Dim-HAF-1, Dim-HAF-4 and Dim-HAF-5.1, and Dim-HAF-5.2 carried only 4 transmembrane helices. We additionally analysed all the amino acid sequences of the *C. elegans* half transporter orthologs using the same procedure as for *D. immitis* with Geneious Pro.5.6.3 software. Interestingly, from the analysis of Cel-HAF-1, Cel-HAF-3, Cel-HAF-4, Cel-HAF-5 and Cel-HAF-9, all carried 4 predicted transmembrane helices in their TM as did the *D. immitis* half transporters. Consequently, the structure of the TM presented in Table 3 for Dim-HAF-1 (nDi.2.2.scaf00023), Dim-HAF-4 (nDi.2.2.scaf00101) and Dim-HAF-5.1, and Dim-HAF-5.2 (nDi.2.2.scaf00496) should be considered as complete. In both Figs. 1 and 2, Clade III (Filariae) and Clade V (*C. elegans*) (Blaxter et al., 1998) clearly separated with the branches. The sequences from *B. malayi*, *L. loa*, *O. volvulus* and *D. immitis* always grouped together. With 1 pseudogene, 3 full, and 2 half ABC-B transporters genes identified from the genome of *D. immitis*, this organism carries only 20% of the number of ABC-B transporters as *C. elegans* (Sheps et al., 2004) and one third the number found in *H. contortus* (Laing et al., 2013). *C. elegans* is a free living nematode, while *H. contortus* (also from Clade V (Blaxter et al., 1998)) and the different filarial nematodes are parasitic. The difference in number of Pgps between *H. contortus* and *D. immitis* remains interesting. They have

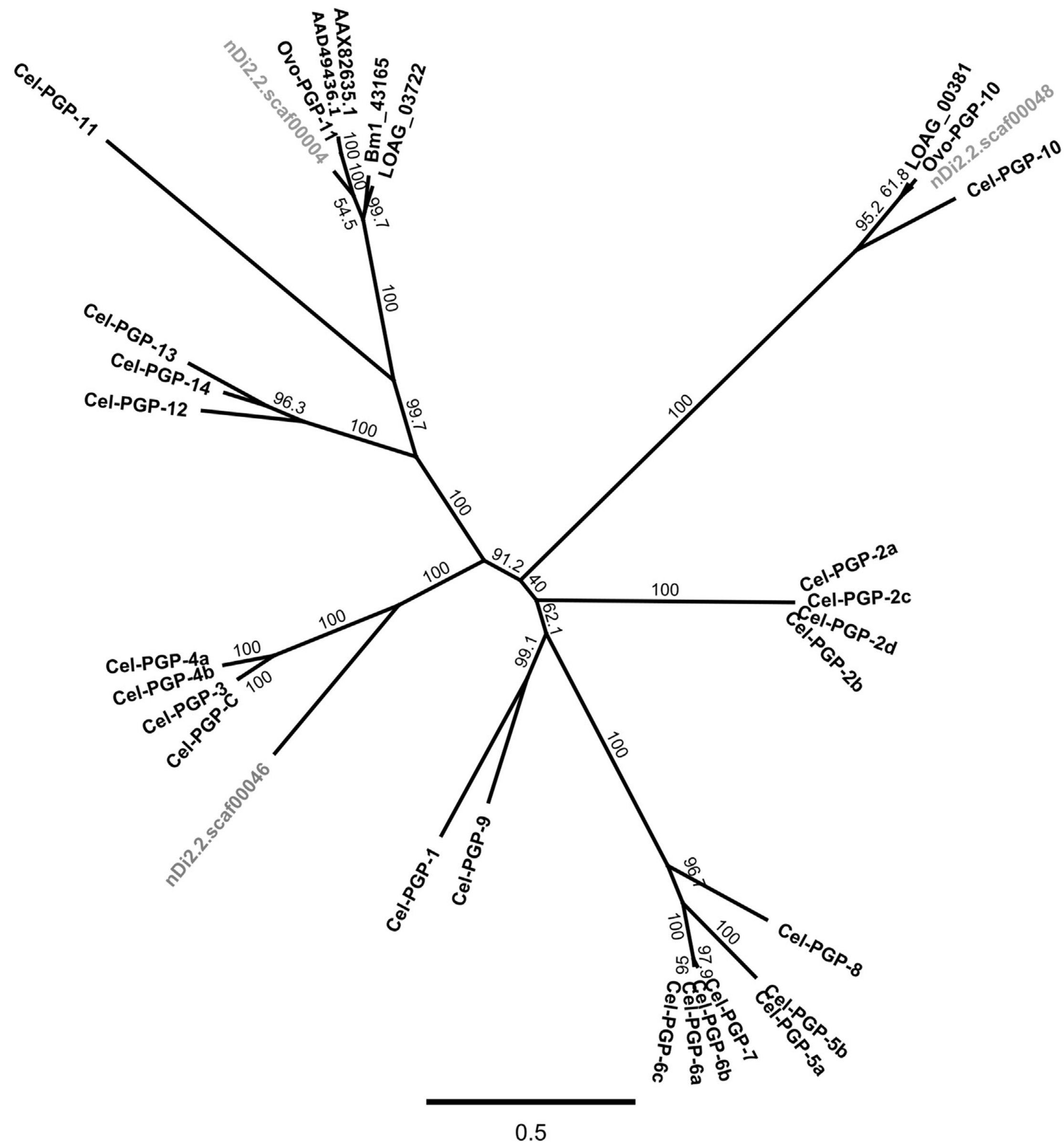

Fig. 1. Phylogenetic tree of full ABC-B transporter genes constructed from PHYML (Maximum likelihood) and Bootstrap 1000 with Geneious Pro.5.6.3 software. Numbers over the branches correspond to the percentage of bootstrap values (calculation based on 1000 pseudoreplicates). The three letter prefixes in Pgp gene names: Cel, Dim and Ovo refer to *C. elegans*, *D. immitis*, and *O. volvulus*. LOAG and Bm1 prefixes refer to *L. loa* and *B. malayi* sequences. AAX82635.1 and AAD49436.1 are GenBank accession numbers referring to *O. volvulus* Pgps. All the GenBank accession numbers for amino acid sequences are provided in Table 1 nDi.2.2.scaf00004, nDi.2.2.scaf00046 and nDi.2.2.scaf00048 labelling correspond to *D. immitis* amino acid sequences that were identified based on work on these scaffolds.

distinct life cycles with *D. immitis* being an obligate parasite at all stages of its life cycle, and requiring a mosquito vector (Grieve et al., 1983). Similar to *C. elegans*, *H. contortus* has several free living stages in its life cycle that may expose the parasite to considerable levels of environmental toxins, compared with *D. immitis* which only lives in mosquito or mammalian hosts. Such observation may explain the higher number of ABC-B transporter genes in Trichostrongyles and other Rhabditia, as they may need a high level of protection against natural toxins in the terrestrial environment (Broeks et al., 1995).

Besides differences observed between *C. elegans*, *H. contortus* and *D. immitis*, studies on different nematodes could be predictive in terms of the likely function and/or properties of ABC-B transporters in *D. immitis*. In *C. elegans*, Cel-HAF-1 was localized on the inner mitochondrial membrane and was reported to be necessary for peptide transport and for the mitochondrial unfolded protein response (UPRmt) signaling pathway (Haynes et al., 2010). Cel-HAF-4 and Cel-HAF-9 were localized on the membranes of intestinal granular organelles (Kawai et al., 2009). Cel-HAF-4 and Cel-HAF-9 were reported to form a heterodimer that was required for their own stabilization (Tanji et al., 2013). They both were reported to be necessary for a normal defecation cycle, for proper growth and for normal brood size (Kawai et al., 2009). Cel-*pgp-3* and Cel-*pgp-4* were located on the same chromosome X (Lincke et al., 1992). The expression of Cel-PGP-3 was identified in the apical membrane of the excretory and intestinal cells (Broeks et al., 1995; Ardelli and Prichard, 2013) while expression of Cel-PGP-4 was reported in the larval excretory cell (Zhao et al., 2004). Cel-PGP-3 expression was reported in developmental stages (Broeks et al., 1995). The deletion of Cel-*pgp-3* was not associated with an increased sensitivity to

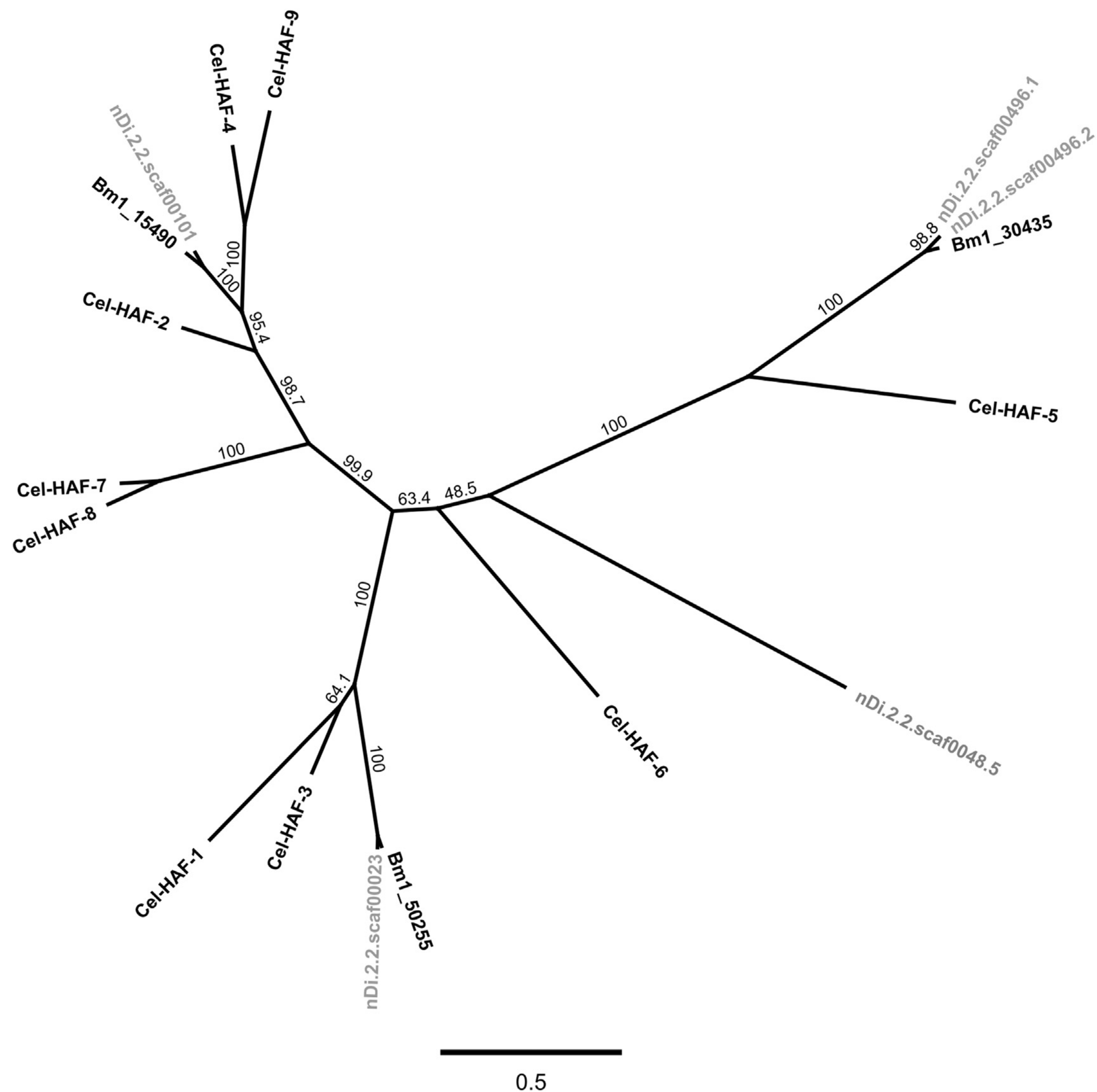

Fig. 2. Phylogenetic tree of half ABC transporter genes constructed from PHYML (Maximum likelihood) and Bootstrap 1000 with Geneious Pro.5.6.3 software. Numbers over the branches correspond to the percentage of bootstrap values (calculation based on 1000 pseudoreplicates). Cel, Dim, and Bm1 refer to *C. elegans*, *D. immitis*, and *B. malayi* sequences. All the GenBank accession numbers for amino acid sequences are provided in Table 1 nDi.2.2.scaf00023, nDi.2.2.scaf00101, nDi.2.2.scaf00496.1, nDi.2.2.scaf00496.2 and "nDi.2.2.scaf00048.5" (SEQ-5 from nDi.2.2.scaf00048) labelling correspond to *D. immitis* amino acid sequences that were identified, based on work with these scaffolds.

IVM. However, it was associated with increased sensitivity to colchicine and chloroquine (Broeks et al., 1995). In *C. elegans*, mRNA levels of Cel-*pgp-4*, Cel-*haf-1* and Cel-*haf-3* significantly increased in IVR10 (*C. elegans* able to survive at 10 ng/ml IVM) compared with the N2 wild-type strain (Yan et al., 2012). Also transcription down regulation of Cel-PGP-4, and Cel-HAF-9, induced by 15 or 20 ng/ml IVM, significantly increased the effect of IVM to reduce egg production (Yan et al., 2012). Additionally, down regulation of transcription of Cel-PGP-3, Cel-PGP-4 and Cel-HAF-9 increased motility at high IVM concentrations (Yan et al., 2012). Cel-PGP-10 was expressed in the worm's intestine as is Cel-PGP-3. Cel-PGP-3 showed a higher expression level after a short exposure of IVM in N2 wild-type strain (Ardelli and Prichard, 2013).

In *C. elegans*, an increase of IVM susceptibility was reported after loss-of-function of several Pgps including Cel-PGP-3 and Cel-PGP-11, while loss of function of Cel-PGP-10 had a limited effect (Janssen et al., 2013b). Interestingly, Cel-PGP-11 plays an important role in IVM detoxification (Janssen et al., 2013b). In ML resistant *Cooperia oncophora* adult worms and L3, an upregulation of the transcript level of Con-*pgp-11* was reported while it was not observed in ML susceptible parasites (De Graef et al., 2013). In *Parascaris equorum*, IVM resistance was correlated with an increase in the level of Peq-*pgp-11* mRNA and with the presence of 3 individual SNPs (Janssen et al., 2013a). In a motility assay, the expression of Peq-PGP-11 in *C. elegans* deficient with Cel-PGP-11 significantly reduced IVM susceptibility (Janssen et al., 2015). Interestingly, the "GG-GG" genotype which was associated with IVM response phenotype in *D. immitis* (Bourguinat et al., 2011b) is located in Dim-*pgp-11*.

In *H. contortus*, Hco-*pgp-3*, Hco-*pgp-4*, Hco-*pgp-10*, and Hco-*pgp-11* partial sequences had no significant change in their mRNA expression levels when pools of L3 larvae were compared between a rapidly selected IVM-resistant parasite isolate and its drug-sensitive parent (Williamson and Wolstenholme, 2012). However, there was great variability in these results which may have been due to the presence of larvae in different states of viability and development in the pools. Nine different *H. contortus* Pgp genes, including Hco-*pgp-3* and Hco-*pgp-11*, were specifically up-

regulated in parasitic life stages, which suggested a potential involvement of these Pgps in the detoxification of eosinophil granule products (Issouf et al., 2014). Also eosinophil granules had induced a dose dependent overexpression of Hco-*pgp-3*. It was hypothesized that some helminth Pgps could be involved in the detoxification of host products, and thus may help the worms to escape the host immune response (Issouf et al., 2014). In three subpopulations of *H. contortus* larvae, that were able to survive increasing concentrations of levamisole, Pgp gene expression levels were measured. Expression of Hco-*pgp-3*, Hco-*pgp-4* and Hco-*pgp-10* genes increased by 1.5–3 fold in the subpopulation surviving the lowest levamisole concentrations (Sarai et al., 2014).

4. Conclusions

This study identified, for the first time, 9 complete ABC transporter genes in *D. immitis*, including three full size Pgp-type sequences (Dim-*pgp-3*, Dim-*pgp-10*, Dim-*pgp-11*), and another pseudogene, two ABC-B half transporter genes (Dim-*haf-1* and Dim-*haf-4*), two ABC-C transporter genes (Dim-*haf-5.1* and Dim-*haf-5.2*) and one additional half transporter that may require additional characterization. Filarial nematodes seem to carry fewer ABC-B transporter genes than *C. elegans* and *H. contortus*. Thus, different ABC transporters may be involved in the development of ML resistance in the two clades. ABC-B transporters are considered important in ML resistance mechanisms. The ABC transporters identified may be used as tools to identify genetic markers for ML resistance prediction in *D. immitis*. Monitoring tools are urgently needed, particularly in USA where *D. immitis* resistance has developed in the Mississippi Delta region. Furthermore, this characterization of the ABC-B transporter genes in *D. immitis* should be a precursor for studies on the mechanism of ML resistance in this parasite.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2016.04.001.

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