Identification of ABC transporters in *Sarcoptes scabiei*

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SUMMARY

We have identified and partially sequenced 8 ABC transporters from an EST dataset of *Sarcoptes scabiei* var. *hominis*, the causative agent of scabies. Analysis confirmed that most of the known ABC subfamilies are represented in the EST dataset including several members of the multidrug resistance protein subfamily (ABC-C). Although P-glycoprotein (ABC-B) sequences were not found in the EST dataset, a partial P-glycoprotein sequence was subsequently obtained using a degenerate PCR strategy and library screening. Thus a total of 9 potential *S. scabiei* ABC transporters representing the subfamilies A, B, C, E, F and H have been identified. Ivermectin is currently used in the treatment of hyper-infested (crusted) scabies, and has also been identified as a potentially effective acaricide for mass treatment programmes in scabies-endemic communities. The observation of clinical and *in vitro* ivermectin resistance in 2 crusted scabies patients who received multiple treatments has raised serious concerns regarding the sustainability of such programmes. One possible mechanism for ivermectin resistance is through ABC transporters such as P-glycoprotein. This work forms an important foundation for further studies to elucidate the potential role of ABC transporters in ivermectin resistance of *S. scabiei*.

Key words: *Sarcoptes scabiei*, ivermectin resistance, ABC transporter.

INTRODUCTION

Scabies is a skin condition caused by infestation of the epidermis with the ectoparasitic mite *Sarcoptes scabiei*. The disease remains prevalent in the developing world, with an estimated 300 million people affected (Taplin *et al*. 1990). Scabies is endemic in northern and central Australian remote Aboriginal communities, with reported point prevalences of 25% in adults (Carapetis *et al*. 1997) and up to 50% in children (Currie, Connors and Krause, 1994). Secondary streptococcal infection of skin due to scabies lesions is a significant cause of morbidity, and has been linked to the extreme rates of post-streptococcal disease observed in these communities (Currie and Carapetis, 2000; McDonald, Currie and Carapetis, 2004). Mass treatment programmes using the pyrethroid acaricide 5% permethrin have been successful in reducing overall rates of scabies and streptococcal pyoderma (Carapetis *et al*. 1997). However, anecdotal reports of clinical failure and *in vitro* reports of tolerance to permethrin (Walton, Myerscough and Currie, 2000), unpublished observations) suggest the potential emergence of resistance, threatening the success of such programmes.

Ivermectin has been suggested as a suitable alternative for mass treatment programmes (Lawrence, Sheridan and Speare, 1994; Lawrence *et al*. 2005), and has recently been licensed for the treatment of ordinary scabies in France (Giudice, Chosidow and Caumes, 2003). Ivermectin was introduced for the treatment of crusted (severe) scabies in northern Australia in the early 1990s and has been used successfully in combination with topical acaricides. However, repeat infestations and recrudescence with ivermectin have been reported, with multiple doses required to achieve a cure (Huffam and Currie, 1998; Walton *et al*. 1999). Importantly, clinical and *in vitro* resistance to ivermectin has recently been documented in 2 crusted scabies patients who had previously received 30 and 58 doses of ivermectin over 4 and 4·5 years respectively. No *in vitro* resistance to topical acaricides was observed in these mites, however, and infestations were eventually cleared by increased topical permethrin and benzyl benzoate application (Currie *et al*. 2004). This raises serious concerns regarding the sustainability of ivermectin treatment for scabies, particularly if it is applied as a public health measure in a community setting. Ivermectin has been successfully used for the annual mass treatment of onchocerciasis in West Africa since 1987, with over 100 million doses distributed (Ardelli and Prichard, 2004). Ivermectin resistance has not yet been documented, although suboptimal responses of *Onchocerca volvulus* to ivermectin have recently been reported (Awadzi *et al*. 2004), suggesting resistance may be developing. After more than 20 years

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of intensive use, ivermectin resistance is widespread in veterinary helminths (Wolstenholme *et al.* 2004).

As information accumulates, it is becoming apparent that ivermectin resistance mechanisms are complex and probably multifactorial. However, several candidate mechanisms are well established. One proposal involves alterations to the Glutamate (GluCl) or GABA-gated chloride channels, which are members of the ligand-gated ion channel family and the physiological targets of ivermectin in invertebrates (Blackhall *et al.* 1998*a*; Feng *et al.* 2002; Blackhall, Prichard and Beech, 2003; Njue *et al.* 2004). Interestingly, selection at a beta-tubulin gene has recently been observed in ivermectin exposed *O. volvulus*, although the functional basis behind this remains uncertain (Eng and Prichard, 2005).

Another potential mechanism for ivermectin resistance is increased cellular export mediated by ATP-binding cassette (ABC) transporters. ABC transporters can confer multidrug resistance by transporting a broad range of substrates across membranes, thus leading to decreased intracellular accumulation. ABC transporters are presently grouped into 8 subfamilies according to sequence similarity and domain organization (Sheps *et al.* 2004). Several of these proteins have been associated with drug resistance, the most well known of which is the ABC-B transporter P-glycoprotein.

P-glycoprotein was first discovered to be overexpressed in multidrug-resistant cancer cells; hence the common referral of P-glycoprotein as the multidrug resistant gene (*mdr*). Increased expression of P-glycoprotein in these cells confers resistance to a wide range of hydrophobic drugs (reviewed by (Gottesman *et al.* 1995). Increased copy number of the P-glycoprotein homologue *pfmdr1* in *Plasmodium falciparum* was initially thought to be associated with chloroquine resistance in malaria parasites (Foote *et al.* 1990), but more recent evidence suggests a stronger link to mefloquine resistance (Price *et al.* 1999, 2004).

Ivermectin is known to be an excellent substrate for ABC transporters such as P-glycoprotein (Nobmann, Bauer and Fricker, 2001), with mammals deficient in P-glycoprotein displaying hypersensitivity to ivermectin (Schinkel *et al.* 1994; Roulet *et al.* 2002). Several molecular studies suggest the involvement of P-glycoprotein in ivermectin resistance, although a functional association has not yet been demonstrated. Early research found alterations to P-glycoprotein and increased mRNA levels in ivermectin resistant *Haemonchus contortus* (Xu *et al.* 1998). Selection at a P-glycoprotein allele following ivermectin treatment has been observed in *H. contortus* (Blackhall *et al.* 1998*b*), and more recently *Onchocerca volvulus* (Eng *et al.* 2005).

Thus it is possible that P-glycoproteins or other ABC transporters might play an important role in the development of ivermectin resistance in *S. scabiei*. Better monitoring tools will enable more accurate assessment of treatment failures and provide a more sensitive evaluation method, particularly in the management of emerging resistance in a remote community setting. To investigate mechanisms of ivermectin resistance, an important preliminary step is to identify and characterize candidate resistance genes. In this study, we utilized an EST library and database to identify potentially relevant ABC transporter genes from *S. scabiei*.

MATERIALS AND METHODS

Searching the Sarcoptes scabiei *var.* hominis *EST dataset*

A *Sarcoptes scabiei* var. *hominis* cDNA library was constructed using the shed skin of crusted scabies patients as previously described (Fischer *et al.* 2003*a*, *b*). An EST dataset of 43 776 sequences was established from the library (Holt *et al.* 2003). Putative ABC transporters were initially identified by comparing the EST dataset to GenBank using BLASTx (Altschul *et al.* 1990). Additional putative ABC transporters were identified by searching the EST dataset for homologues of the P-glycoprotein ABC transporters, Pgp-49 (Q00449) from *Drosophila melanogaster*, and Pgp-A (AAC38987) from *H. contortus*. Contigs identified as potential ABC transporters were further analysed using BLASTp.

Sequence extension of EST contigs

Sequences of the individual clones within contigs were completed by sequencing with vector and specific primers as required. In addition, a semi-nested PCR approach on the cDNA library using a combination of vector and nested specific primers was employed to try and extend the contig sequences. Primers used for clone sequence completion and contig extension are listed in Table 1. All primers were synthesized by Proligo (Lismore, NSW, Australia).

PCR reactions contained 1× PCR buffer, 0·2 mM dNTPs, 1·25 μM each of vector and sequence-specific primer, 1 U *Taq* polymerase (Qiagen, Brisbane, QLD, Australia) and 1 μl cDNA library template DNA. Reactions were cycled at 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 1 min and 30 sec for 35 cycles, followed by a final extension step of 72 °C for 10 min (annealing temperatures varied slightly according to primer Tm). First round PCR products were diluted 10-fold and 100-fold, and subjected to a second round of PCR using the sequence-specific nested primer.

PCR products were either excised from agarose gels prior to purification or directly purified using

Table 1. *Primers used for extension and sequencing of EST contigs*

Contig	Primer name	Primer sequence (5′–3′)
4012G02	G02F1	CGT CTC ATT GAC TTA TAT CTG G
	G02R1	TTC GAG ATC AAT AGC CGT A
	G02R3	CTA AAA TTC GAG TGC GAT CT
	G02R4	TTG GCG ACA AGA GAA TCA AC
8060B04	B04R1	GAC TGC TAA CCA ACG ATT AGC
	B04R2	GCT CTA ATA GTC GAT ACA CC
	B04R3	ATC GCT AAA GCA CCG ATC AC
	B04R4	ACT AAA ATT CGA GTG CGA TCT
	B04R5	TTG GCG ACA TGA GAA TCA AC
	B04R6	GTT GAA TGG ACT TCC GAA CA
	B04R7	GTA ACA TTC TGA ATC AGG GC
7001E12	E12R1	GTT TGA TGA TAT CTA GCA GAG
	E12R2	GTG GAA GCT TAA CAT ATT CCA
7008C03	C03F1	TAG TAT TGC ACA TGC ACC GA
	C03F2	GCA TCG GTA GAA TTT TGA TC G
	C03R1	CCA ACA TCT TCA ATG TGT CC
	C03R2	CAT CGA CCA TCG GTA GAA CA
9002G04	G04F1	ACG ATA GCA CAT CGA ATC CA
	G04R1	TCG GCG AAA ATT CTT ACA CA
7002E01	E01R1	ACT TTC TTG ATC GGT CAA CG
	E01R2	TCT AAT ACC GAC TCA ATA GC
mdr7-236	236F1	CAC AAT CAA ATT AAT CGA GCG
	236R1	TTG TAT CAT AAC CCA ACG GTA
Vector	T3-pBK-CMV	AAT TAA CCC TCA CTA AAG GG
	T7-pBK-CMV	GTA ATA CGA CTC ACT ATA GGG C
	M13f	CGC CAG GGT TTT CCC AGT CAC GAC
	M13r	TCA CAC AGG AAA CAG CTA TGA C

the MinElute PCR purification kit (Qiagen). PCR products were cloned using the pGEM-T Easy cloning kit according to the manufacturer's protocol (Promega, Annandale, NSW, Australia). Ligations were electroporated into *E. coli* XL1-Blue cells (Stratagene, LaJolla, CA, USA) and plasmid DNA was extracted using a miniprep kit according to the manufacturer's protocol (Bio-Rad, Regents Park, NSW, Australia). Plasmids were sent to the Bio-molecular Research Facility (Newcastle, NSW, Australia) for sequencing with M13 vector primers (Table 1).

Identification of a P-glycoprotein using degenerate PCR

To remove *E. coli* contamination, the cDNA libraries were treated with DNaseI to digest non-phage DNA. A fresh aliquot of the normalised cDNA library was mixed with 10 U DNaseI (Roche, Castle Hill, NSW, Australia) and 2·5 mM of MgCl₂ and incubated at 37 °C for 2 h. The reaction was heat inactivated for 10 min, cooled and purified using the QiaQuick reaction clean-up kit (Qiagen).

Degenerate primers for amplifying P-glycoprotein fragments were employed based on the highly conserved ATP-binding domains of P-glycoprotein (Table 2). This approach has been successfully used to isolate P-glycoprotein in several other studies (Kwa *et al.* 1998; Xu *et al.* 1998; Huang and Prichard, 1999; Sangster *et al.* 1999). *S. scabiei* var. *hominis* cDNA libraries (Fischer *et al.* 2003 *a*, *b*) and multi-mite genomic DNA preparations (Mounsey *et al.* 2005) were used as templates for nested PCR. First round primers were used in all 16 possible combinations. Cycling conditions were 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min and 30 sec, for 35 cycles followed by a final extension step of 72 °C for 10 min. PCR reactions contained 20–200 ng DNA, 0·2 mM dNTPs, 2 μM of each primer and 1·25 U of *Taq* polymerase. One μl of the first round PCR products was used as a template for the second round reaction with the above parameters employed.

PCR-based library screening for P-glycoprotein

The P-glycoprotein cDNA fragment obtained through degenerate PCR was used to probe the normalised cDNA library. A high-stringency PCR-based screening approach was employed, based on the method developed by Israel (1993). Primers corresponding to the sequence were designed to generate a 280 bp PCR product (236F1/R1, Table 1). All PCR reactions used for screening contained 1 μl of template DNA, 1× PCR buffer, 0·2 mM dNTPs, 1·25 μM primers and 1·25 U *Taq* polymerase. Reactions were cycled at 94 °C for 2 min, followed

Table 2. *Degenerate PCR primers based on ATP-binding domain of P-glycoprotein*

Direction	Peptide sequence	Name	Primer sequence (5′–3′)
Sense	SGCGKST	mdrF1	TCD GGI TGY GGN AAR TCD AC
		mdrF2	AGY GGI TGY GGN AAR TCD AC
		mdrF3	TCD GGI TGY GGN AAR AGY AC
		mdrF4	AGY GGI TGY GGN AAR AGY AC
Antisense 1	DEATSALD	mdrR1	TCI AGI GCI GAN GTN GCY TCR TC
		mdrR2	TCI AGI GCR CTN GTN GCY TCR TC
		mdrR3	TCY AAI GCI GAN GTN GCY TCR TC
		mdrR4	TCY AAI GCR CTN GTN GCY TCR TC
Antisense 2	GQKQRIAI	mdrR5	ATI GCD ATI CGY TGY TTY TGN CC
		mdrR6	ATI GCD ATY CTY TGY TTY TGN CC

by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 45 sec.

Library titration and a dilution series PCR on the normalised cDNA library determined the minimal starting amount of library phage DNA required to generate a PCR product. For the first round of screening, 10^6 pfu (in 1 ml of SM buffer) were used to infect 1 ml of *E. coli* XL1-Blue MRF' cells at OD_{590} 1.0 (Stratagene). Then 18 ml of LB broth were added and the mixture plated in an 8×8 matrix (100 μl/well) in a 96-well plate. The plate was sealed and incubated at 37 °C with shaking for 5 h. Rows and columns were pooled by taking a 20 μl aliquot from each well and diluting 1 : 1 with distilled water. Ten μl of chloroform were added to the pooled phage suspensions and reactions were centrifuged briefly prior to PCR. Single PCR-positive wells were selected and the above process repeated several times to enrich the number of positive clones for each round of screening.

After the 4th round of screening, approximately 2000 pfu from the PCR-positive well were plated onto NZY agar plates. Plaque lifts were performed using Hybond-XL membranes according to the manufacturers' protocol (Amersham, Castle Hill, NSW, Australia). A 280 bp probe was generated using the 236F1/R1 primers, purified and labelled with [α-^{32}P] dCTP using the Redivue random priming labelling kit and hybridization was performed at 55 °C overnight according to the manufacturer's protocol (Amersham). Following hybridization, filters were washed 3 times at 55 °C in 2× SSC, 0·1% SDS. Membranes were sealed, exposed to X-ray film at −80 °C overnight and developed.

Hybridization-positive plaques were cored from the agar and placed into 500 μl of SM buffer and 20 μl of chloroform, vortexed, and stored at 4 °C. PCR on the eluted phage stocks was performed as described above. Single-clone phagemid excision was performed on the hybridization and PCR-positive plaques according to the Stratagene Zap Express protocol. Plasmid DNA was extracted using the QiaPrep Spin Miniprep Kit (Qiagen), and sequenced as described above, using the bacteriophage T3 and T7 vector primers (Table 1).

Sequence analysis

Sequence chromatograms were viewed, edited and aligned using the Lasergene software package (DNAstar Inc, Madison, WI, USA). Edited DNA sequences were translated into amino acid sequences using Flip6 frames (Brossard, 1997), accessed via Biomanager (www.angis.org.au). Amino acid sequences were then compared to the conserved domain databases accessed via the NCBI server (http://www.ncbi.nlm.nih.gov/blast/) using BLASTp. Additionally, BLASTn searches were performed between the nucleotide sequences and *E.coli* genomic databases to assess potential contamination. Sequences reported in this paper are available in the GenBank™ database under the Accession numbers DQ146410-DQ146418.

Cluster analysis

Further analysis of the *S. scabiei* sequences was conducted to confirm the assignment of ABC-subgroups, and to examine the relationship of the *S. scabiei* ABC transporters to sequences from *Caenorhabditis elegans* and *Drosophila melanogaster* (Table 3). Peptide sequences corresponding to the conserved ATP-binding regions were aligned using ClustalW and bootstrapping for confidence determination was performed using Seqboot. Consensus dendrograms were constructed using the Parsimony (Protpars) algorithm. Because the contig 7008C03 did not appear to contain an ATP-binding motif it was excluded from this analysis.

RESULTS

Identification and extension of EST contigs with homology to ABC transporters

Preliminary analysis of a *S. scabiei* EST dataset of 43 776 sequences, identified 9 contigs with homology to ABC transporters. Contig extension using a

Table 3. Sequences used in cluster analysis of EST contigs

Subfamily	Species	Protein	Accession number
A	*Caenorhabditis elegans*	Y39D8C.1/Abt-4	AAC69223
	Drosophila melanogaster	CG1718	AAF50837
B	*Caenorhabditis elegans*	K08E7.9/Pgp-1	CAB01232
	Drosophila melanogaster	Mdr49	AAF58437
C	*Caenorhabditis elegans*	F57C12.5/Mrp-1	AAD31550
	Drosophila melanogaster	CG6214	AAF53223
D	*Caenorhabditis elegans*	C54G10.3	CAA99810
	Drosophila melanogaster	CG12703	AAF49018
E	*Caenorhabditis elegans*	Y39E4B.1	CAB54424
	Drosophila melanogaster	CG5651	AAF50342
F	*Caenorhabditis elegans*	T27E9.7/GCN20-2	CAB04880
	Drosophila melanogaster	CG9330	AAF49142
G	*Caenorhabditis elegans*	C05D10.3	AAA20989
	Drosophila melanogaster	CG3164	AAF51548
H	*Caenorhabditis elegans*	C56E6.1	AAA81093
	Drosophila melanogaster	CG9990-PA	AAF56807

semi-nested PCR approach on the cDNA libraries and on further sequencing of individual clones yielded additional sequence information. During this process, 2 of the previously separate contigs were found to overlap and were combined.

Sequence analysis of contigs

Four of the resulting 8 *S. scabiei* contigs displayed significant homology to the multidrug resistance protein (MRP/ABC-C) family of ABC transporters (Table 4). 8060B04, 9002G04 and 7001E12 all aligned over the C-terminal region of their MRP homologues, while 7008C03 aligned over the central region of the protein, possibly between ATP-binding domains. This was supported by the absence of ATPase consensus regions in the 7008C03 sequence. Although the contigs presented here were homologous to similar proteins, alignments between the contigs indicated they were sufficiently different from each other not to be considered duplicate or different isoforms of a single *S. scabiei* protein.

Contig 7067D09 shared high levels of homology (77%) with the conserved RNAse L inhibitor proteins from many organisms. These proteins belong to the ABC-E subfamily of ABC transporters. Two ATP-binding domains were identified, and a conserved domain associated with the RNAase L inhibitor ATPase was also detected (Table 4).

The deduced amino acid sequence of the contig 7002E01 was determined to have significant similarity to the ABC-F transporter GCN20 from several organisms (Table 4). From alignment with the other proteins in this family, the sequence of 7002E01 was determined to be complete. Conserved domains of ATPase components were detected in 2 regions of the sequence (Table 4). No transmembrane domains were identified in the protein, which is consistent with other ABC-F transporters.

Contig 4013B10 displayed significant similarity to the C-terminus of ABC-A proteins from various organisms (Table 4). The 7052B06 clone contig appeared to be chimeric, with BlastP detecting no homology over the first 150 bp. The remainder of the protein aligned with the N-terminal ATP binding domain of ABC-H proteins from *Anopheles gambiae* and *D. melanogaster*, and ABC-G proteins from other organisms (Table 4).

As none of these contigs appeared to be members of the ABC transporter subfamily B, which includes the P-glycoproteins implicated in drug resistance in some organisms, a further degenerate PCR approach was utilized in order to identify a member of this group.

Degenerate PCR

PCR of the DnaseI-treated cDNA library using degenerate primers designed to the highly conserved ATP-binding domains of P-glycoproteins, successfully yielded products of the expected size for 2 primer combinations–mdrF2/R3/R5 and mdrF2/R3/R6. The fragment from the latter (designated mdr7-236) was cloned and sequenced. The sequence was found to share significant homology to P-glycoprotein from several organisms. No significant similarity was detected from BlastN against the *E. coli* database indicating the sequence was not derived from *E. coli* contamination. Degenerate PCRs on genomic mite DNA preparations resulted in the cloning of 2 fragments. The first clone was amplified using the primer combination mdrF1/R4/R6. It was found to be identical to the mdr7-236 sequence but containing a 70 bp intron. The second genomic clone was amplified with the primer combination mdrF3/R3/R5. Although no obvious introns were present, there were multiple stop codons and thus the sequence could not be translated to a single

Table 4. *Sarcoptes scabiei* ABC transporters identified in this study

Contig[1]	Length (aa)	% of total[2]	BLASTp top match (Accession no.)	Description	No. ABC domains[3]	% id	e value	Sub family[4]
8060B04	807	51	*Danio rerio* (AAH56740.1)	MRP2/ABCC2	2	50	0	C
9002G04	402	26	*D. melanogaster* (AAS64699)	CG6214-PM (dMRP)	1	61	6e-125	C
7001E12	320	20	*Apis mellifera* (XP395679)	Similar to ENSANGP27587 (MRP like protein)	1	45	4e-67	C
7008C03	313	20	*Canis familiaris* (AAS91646)	MRP2	0	36	3e-36	C
7067D09	397	67	*Anopheles gambiae* (EAA45523)	ENSANGP22549 (RNAseL inhibitor)	2	78	9e-178	E
7002E01	715	100	*Xenopus laevis* (AAH84777)	LOC398565 protein (ABCF3)	2	49	0	F
4013B10	344	20	*Danio rerio* (XP_692776)	ABCA3	1	48	9e-82	A
7052B06	216	29	*Anopheles gambiae* (EAA08957)	ENSANGP19635	1	43	7e-38	H
mdr7-236	254	21	*Gallus gallus* (XP418636)	Mdr/Pgp-3	1	60	8e-83	B

[1] These sequences have been submitted to GenBank and were assigned the Accession numbers DQ146410–DQ146418.
[2] Based on comparison of the length of the contig with its top BLASTp match.
[3] Number of ATP binding domains determined by searching against the conserved domain database.
[4] As predicted by BLASTp and cluster analysis results.

open reading frame. tBLASTx was performed on the nucleotide sequence, with the highest match being the N-terminal ATP-binding domain of CG3879/mdr 49 from *Drosophila melanogaster* (63% ID, 2e-12). The presence of multiple stop codons suggested the sequence might represent a pseudogene so it was not included for further analysis.

PCR-based library screening

The cDNA was enriched for mdr7-236 phage, through several rounds of PCR-based screening and amplification. Hybridization of the plated enriched stock with a mdr7-236 probe, yielded 2 positive plaques. These phages were confirmed to be positive via PCR with mdr7-236 specific primers then excised to phagemids and sequenced. The clones were found to contain a 1094 bp insert which included the original mdr7-236 sequence. This extended mdr7-236 sequence showed homology to the C-terminal region of many P-glycoproteins, with the top BlastP match being P-glycoprotein 3 from *Gallus gallus* (Table 4).

Cluster analysis of ABC transporters from S. scabiei

The EST contigs from *S. scabiei* all grouped closely with their respective homologues from *D. melanogaster* and *C. elegans* (Fig. 1). The proteins clustered according to predicted ABC-subfamily. Although the designated ABC-H proteins from *S. scabiei* and *D. melanogaster* grouped together, the ABC-H from *C. elegans* instead grouped with ABC-G. The MRP-like proteins from *S. scabiei* all fell within the ABC-C group. 7001E12 sat apart from other members in this subgroup, supporting the BLASTp result which indicated that 7001E12 was more divergent, whereas 8060B04 and 9002G04 were more similar to MRP 1 and 2 from other organisms. ABC subfamilies B and C were closely related, with group B apparently derived from group C.

DISCUSSION

In this study we identified 9 ABC transporter genes from *Sarcoptes scabiei*. ABC-subfamilies A, C, E, F and H were represented in the EST database, with an ABC-B protein subsequently identified by further library screening. Cluster analysis found that most *S. scabiei* contigs clustered closely with their *D. melanogaster* homologue in each respective subgroup.

Contig 4013B10 was found to belong to the ABC-A subfamily. These are among the largest ABC transporters, averaging 1700aa or more. *Anopheles gambiae* has 6 ABC-A proteins, whereas
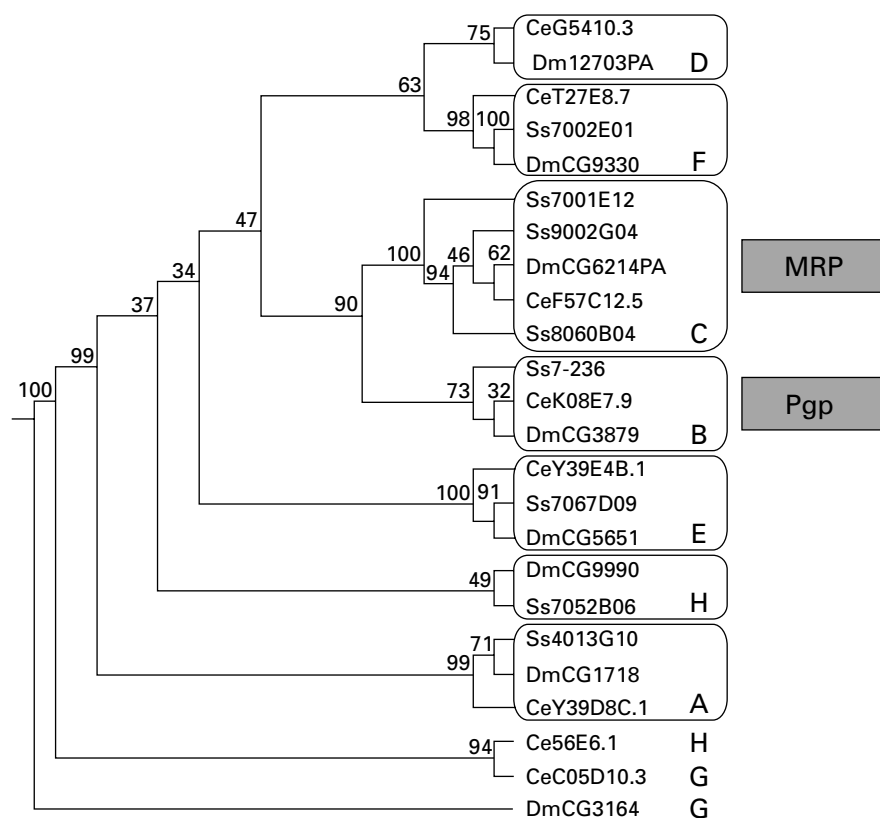
Fig. 1. Dendrogram of *Sarcoptes scabiei* (Ss) and selected *Drosophila melanogaster* (Dm) and *Caenorhabditis elegans* (Ce) ABC transporter ATP-binding domains. Accession numbers for the sequences used are listed in Tables 3 and 4.

Drosophila has 19 (Roth *et al.* 2003). The physiological roles of these proteins in invertebrates remain unexplored. In humans, ABC-A proteins may be involved in cholesterol transport and have also been implicated in drug resistance (Dean, Rzhetsky and Allikmets, 2001). ABC-A proteins are not present in the yeast chromosome, indicating they evolved following multicellularity (Sheps *et al.* 2004). Contig 7067D09 was found to encode the RNAse-L inhibitor protein, belonging to the ABC-E family. This gene is highly conserved and represented as a single copy across most genomes, which suggests an essential housekeeping function. In humans it is involved in the antiviral immune response and is implicated in mRNA turnover. Unlike other ABC transporters, this subfamily contains no trans-membrane domains, and apparently duplicated ATP-binding domains. It is consequently often classified as a 'non-transport' ABC protein. The *S. scabiei* 7002E01 sequence displayed homology to the ABC-F protein GCN-20. ABC-F proteins share a similar domain organization to ABC-E, with no transmembrane domains. GCN-20 is involved in translational regulation via the activation of eIF2α kinase (Marton *et al.* 1997). This group of proteins is well conserved between most genomes studied, with 3 members each.

An interesting discovery was the assignment of contig 7052B06 to the ABC-H subfamily. This recently discovered group of proteins has been identified in the *D. melanogaster* and *A. gambiae* genomes (Misra *et al.* 2002; Roth *et al.* 2003), but is apparently absent from higher eukaryotes. Although there are suggested ABC-H representatives in *C. elegans*, our results indicate they are phylogenetically distinct from the arthropod protein (Sheps *et al.* 2004). Nothing is known about the physiological function of this subfamily, but because of its apparent uniqueness to arthropods it has been earmarked as a potential insecticide target (Roth *et al.* 2003).

Four of the 9 *S. scabiei* ABC transporters identified in this study belonged to the ABC-C subfamily. ABC-Cs are well represented across other genomes, with *Homo sapiens* and *Drosophila* containing 12 members, and *Anopheles* having 14 members. ABC-C proteins have been extensively studied due to their implication in multidrug resistance, hence their alternate title of multidrug resistance proteins (MRP). They are closely related to P-glycoproteins with a broad, sometimes overlapping substrate profile. One of the main differences is that MRPs transport substances complexed with glutathione. Additionally, several MRPs are distinct from P-glycoproteins, in that they contain an additional N-terminal transmembrane domain of unknown function. Three *S. scabiei* contigs (8060B04, 9002G04 and 7008C03) were found to have

homology to MRPs 1 and 2 from mammals and *Drosophila*. This gene has received recent attention in both *Anopheles* and *Drosophila* genome annotation. Splice variants from a single MRP gene from *Anopheles* can encode 10 isoforms (Roth *et al.* 2003), whereas the *Drosophila* ortholog (CG6214/dMRP) can encode 14 isoforms (Grailles, Brey and Roth, 2003). The implication of the presence of multiple isoforms is not known, but since exon variation occurs in regions thought to be involved in substrate recognition, these proteins may have an even broader range of substrates, which has implications for drug resistance. Multiple sequence alignments of the *S. scabiei* ABC-C contigs suggest that they are independent proteins rather than isoforms of a single gene. However, further investigations are needed to fully characterize this important group of proteins in *S. scabiei*, and particularly to determine whether ivermectin is a substrate.

The identification of a P-glycoprotein (ABC-B) homologue from *S. scabiei* was highly significant for our search into ivermectin resistance candidates. Our initial survey of the *S. scabiei* EST database failed to identify P-glycoprotein sequences. However, the EST dataset represents only a small proportion of the library, and P-glycoproteins were subsequently identified from the cDNA library and genomic DNA via degenerate PCR and library screening. The resulting clone had over 50% identity to the C-terminal region of many P-glycoproteins across a range of organisms.

Homologues of several of the ABC transporters identified in this study have been implicated in drug resistance in other organisms and thus are of interest to future studies detailing mechanisms of ivermectin resistance in scabies mites. In humans, the ABC-A3 is found in association with MRP and is also thought to play a role in resistance to anti-cancer drugs (Klugbauer and Hofmann, 1996). Of the greatest significance is the identification of 2 *S. scabiei* ABC transporter groups strongly associated with drug resistance: P-glycoprotein (ABC-B) and multidrug resistance proteins (ABC-C).

An important aspect of future research will be to explore, compare and contrast the copy number and expression levels between MRP and P-glycoprotein, and importantly to determine any significant differences between ivermectin resistant and susceptible mites. Recent studies have shown evidence of ivermectin exerting selection pressure at a P-glycoprotein gene from *Onchocerca volvulus* (Ardelli, Guerriero and Prichard, 2005; Eng *et al.* 2005). It will be important to determine whether similar selection is observed in our mite populations subjected to multiple doses of ivermectin and presumably under extremely high selection pressure.

The ABC transporters identified in this study will facilitate more detailed investigations into potential mechanisms of ivermectin resistance in *S. scabiei*.

In light of *in vitro* and clinical evidence of emerging ivermectin resistance, careful monitoring and surveillance for further cases is critical to help avert the scenario of widespread ivermectin resistance observed in other parasites such as veterinary helminths. The development of a new class of acaricides is unlikely in the near future, and therefore prolonging the life of the limited available drugs is essential. Future applications of this work may be the development of more sensitive molecular-based methods for resistance monitoring, enabling strategies for early management of resistance.

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