Parasitol Res (2008) 103:11–20
DOI 10.1007/s00436-008-0877-8

ORIGINAL PAPER

Limited sequence variation in the major sperm protein 1 (MSP) gene within populations and species of the genus *Dictyocaulus* (Nematoda)

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Received: 9 October 2007 / Accepted: 3 January 2008 / Published online: 9 April 2008

Abstract Populations of the bovine lungworm, *Dictyocaulus viviparus*, are genetically structured based on variation in mtDNA and AFLP data. Our aim was to investigate if this genetic variability also is reflected in a protein recognized by the host immune system. We focused on the major sperm protein (MSP), a small and abundant protein used in diagnostic immunoassays, which has been shown to be variable in some nematodes but not others. MSP was sequenced using worm DNA from eight adult worms from each of nine populations whose genetic structure previously had been quantified. For comparison, we also analyzed MSP sequences of the closely related *Dictyocaulus eckerti* and *Dictyocaulus capreolus* and from nematodes with sequences deposited in GenBank. In contrast to previous results, this study shows that the MSP of *D. viviparus* is similar to that of other nematodes. Almost no sequence variation, and thus no antigenic diversity, was detected in MSP between worms from different sub-populations or in the other *Dictyocaulus* species investigated. A functional test of a recombinant variant of the MSP showed that the expressed protein was recognized by antibodies in sera from infected cattle. This has practical implications for the development of species-specific markers, recombinant vaccines, and immunodiagnostics.

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Introduction

The bovine lungworm, *Dictyocaulus viviparus*, is a pathogenic, pasture-borne nematode that causes parasitic bronchitis in cattle (Anderson 1992). Infective third-stage larvae migrate from the intestine to the lungs, where the adults mature and induce a severe inflammatory response (Urquhart et al. 1996). Although there are sporadic clinical outbreaks of dictyocaulosis causing severe illness and death, sub-clinical morbidity has an impact on production losses both in dairy and beef herds throughout Europe. The cost of control of a moderate outbreak in a dairy herd with 100 cows in the U.K. has been calculated at 30,000–35,000 € (Woolley 1997).

In a recent serological survey, we showed that 40% of the herds in Sweden were infected with *D. viviparus* (Höglund et al. 2004b). We have also demonstrated that there is a high degree of genetic structure between isolates of *D. viviparus* in Sweden, based on both amplified fragment-length polymorphisms (AFLP) data and mtDNA sequences (Höglund et al. 2006). The emerging pattern shows that there is limited genetic interchange between different isolates, with structured genetic variation at several loci. If a similar genetic structure also is observed among immunogenic proteins, this could have implications for the development of immunity to the parasite. Antigenic variation in immunogenic proteins is also of practical relevance for the development of subunit vaccines and for diagnostic assays based on recombinant antigens.

The major sperm protein 1 (MSP), also known in *D. viviparus* as Dv3–14, has been employed in serological assays (Schnieder 1992b, 1993a). It is also potentially an antigen candidate for vaccine development (Schnieder 1993b), and the immunogenic and protective properties have recently been investigated in immunized calves (von Holtum 2006). The name MSP derives from the abundance

and cellular specificity of the protein. In *Caenorhabditis elegans*, MSPs make up 15% of the total protein content of the sperm, where they are involved in both extracellular signaling and cytoskeletal functions during reproduction (Burke and Ward 1983). Structural and biochemical studies have shown that MSP genes are expressed in late primary spermatocytes, and that they are a relatively small basic protein and a core element of the motility machinery that drives amoeboid movement of nematode sperm cells (Miller et al. 2001; Schormann et al. 2004; Smith and Ward 1998). Overall, this makes MSP an interesting experimental model to investigate whether the overall genetic variability and structure observed in AFLP data and mtDNA sequences are reflected in immunogenic proteins.

MSP-like alleles have been identified from parasitic nematodes such as the pig roundworm *Ascaris suum* (Tarr and Scott 2005), the nodular worm of pigs *Oesophagostomum dentatum* (Cottee et al. 2004), and in the human filarial parasites *Onchocerca volvulus* and *Mansonella ozzardi* (Morales et al. 2000), where intraspecific variation has been shown sometimes to exist. For example, two distinct groups of MSP gene variants were noted in *O. volvulus* (Morales et al. 2000), and up to seven MSP-like allelic forms were found in *A. suum* (Tarr and Scott 2004). Conversely, no variation was found in the MSP genes of *M. ozzardi* (Morales et al. 2000).

In this study, we have sampled the major sperm protein 1 (MSP) gene across a subset of isolates to identify any possible antigen variation in *D. viviparus*. We also analyzed a recombinant version of the protein with sera from infected cattle, because there are some inconsistencies among previously published data on MSP from *D. viviparus* (Schnieder 1993b; Setterquist and Fox 1995).

If strain variation exists, then this may have important implications for immune recognition. To test this, eight of the isolates examined were from cattle in geographically separated farms in Sweden, and one was a laboratory strain that has been maintained in experimentally infected calves for almost four decades in the Netherlands. For comparative purposes, we also obtained MSP sequences of the closely related *Dictyocaulus eckerti* and *Dictyocaulus capreolus* from Sweden, and included other nematodes with sequences deposited in GenBank.

Materials and methods

DNA for sequence analysis

A total of 72 worms of bovine origin was sampled, representing eight worms from each of eight geographically separated farms from south-central Sweden plus one isolate that has been maintained in the laboratory for several decades, as described by Höglund et al. (2004a, 2006). In addition, we included eight to 11 worms from the closely related *D. eckerti* from both moose and red deer, and *D. capreolus* from both moose and roe deer, which had been identified earlier based on differences in the 18S rRNA (SSU) and the internal transcribed spacer 1 (ITS1; Divina et al. 2000; Höglund et al. 2003b).

Genomic DNA was isolated mainly using the QIAamp DNA Mini kit (Qiagen). The MSP gene was amplified by polymerase chain reaction (PCR) in 25-μL reactions with primer OP433 (CAA ACA ATG GCG TCA GTT CCC) and primer OP434 (GCA TAG TGT ATA ACC ACC ATG C). The reaction mixtures contained 10 pmol of each primer, 0.2 mM of each dNTP, 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 U AmpliTaq polymerase, and 3 μL of DNA template. The reactions were run on a PTC-200 DNA engine (MJ Research) at 94°C for 2 min, 30×[94°C for 45 s, 45°C for 45 s, 72°C for 1 min], and 72°C for 3 min. Before cleanup, 3 μL of the PCR product was loaded onto a 1% agarose gel containing ethidium bromide to control the PCR reactions. The PCR products were then purified using Montage PCR Cleanup Kit (Millipore) according to the manual and resuspended in 30 μL of nuclease-free water. The amplicons were then sequenced with BigDye-chemistry (Applied Biosystems) before analysis on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Total RNA, expression vector, and *Escherichia coli* strains

Adult *D. viviparus* worms were recovered from a naturally infected cow and individually transferred to −70°C. Five male nematodes were thawed on ice and pooled, before total RNA was isolated with the TRIZOL-reagent (Invitrogen). The T7-promotor driven vector pET-14b (Novagen) was used for recombinant protein expression. *Escherichia coli* strain XL-1 Blue MRF' cells (Stratagene) was used for cloning and preparation of expression plasmids, whereas *E. coli* strain BL21(DE3) was used for high-level expression.

Cloning of the MSP cDNA

Total RNA (1 μg) was mixed with 1 μL oligo dT (500 μg/mL), 18 μL of DEPC-treated H_2O, and 4 μL dNTP 10 mM (GE Healthcare) and incubated at 65°C for 5 min. To the annealing mix, 8 μL of 5× first strand buffer (250 mM Tris–HCl, pH 8.3 at room temperature (RT); 375 mM KCl; 15 mM $MgCl_2$), 4 μL DTT (0.1 M), and 2 μL RNaseOUT (40 units/μL). After incubation at 42°C for 2 min, 2 μL of SuperScript II Reverse Transcriptase was added to the mix, and the incubation was continued for an additional 50 min at 42°C before termination at 75°C for 15 min. The components

used for the cDNA-synthesis were from Invitrogen life technologies except dNTP.

For the open reading frame sub-cloning of the MSP into pET14b vector, the cDNA was amplified with the forward primer OP557 (5′-GGA ATT CCA TAT GAT GGC GTC AGT TCC CCC T-3′) and the reverse primer OP558 (5′-CGG GAT CCT CAT GGA TTG TAC TCG AT-3′). To facilitate the cloning, the primers were designed with restriction sites for *Nde*I and *Bam*HI, respectively. The cycle conditions were: 93°C for 2 min, (93°C for 30 s, 60°C for 30 s, 72°C 1 min)×30 cycles, and 72°C for 10 min. After purification of the amplicons using standard techniques, they were digested with the appropriate restriction enzyme and ligated into pET14b. Positive clones were identified by colony PCR with primers directed against vector sequences outside the multi-cloning site. MSP positive expression plasmids were sequenced as above.

Protein expression and purification

For high-level expression, pPU113 was freshly transformed into *E. coli* strain BL21(DE3). A single colony was inoculated into 20 mL of LB broth with ampicillin (LB-amp) at a concentration of 50 μg/mL and incubated on a shaker at 37°C overnight. The next day, 10 mL of the overnight culture was inoculated in 0.5 L fresh LB-amp. Cultures were grown on a shaker at 37°C until the OD_{600} reached ~0.5. The recombinant gene expression was induced by the addition of isopropylthiogalactoside to a final concentration of 0.5 mM. Induced cells were transferred to a shaker and incubated at 37°C for 2 h. After incubation, cells were collected by centrifugation (4,000×*g*, 4°C, 10 min). The cells were resuspended in HiTrap Start buffer (1× phosphate buffer pH 7.4, 10 mM imidazole), and to prevent protein degradation, the buffer was supplemented with Complete Protease Inhibitor (Roche Molecular) according to the manufacturer's instructions.

Harvested cells from the high-level expression were lysed by sonication. Cell debris was collected by centrifugation (9,000×*g*, 4°C, 30 min), and the resulting supernatant was filtered through a 0.45-μm filter. The recombinant MSPs were purified by affinity chromatography with 1 mL HiTrap chelating HP columns (GE Healthcare) according to manufacturer's instructions on a FPLC system (GE Healthcare).

All protein expressions and purification steps were checked by SDS-PAGE and stained in 0.1% Coomassie Brilliant Blue solution. Final protein concentrations were estimated spectrophotometrically using a NanoDrop ND-100.

Sequence analyses

The sequence data were processed using ContigExpress in the Vector NTI Advance™ 9.0 (Informax™) suite of programs, aligned with MSP sequences of *D. viviparus* from GenBank using the default options of ClustalX version 1.83 (Thompson et al. 1997), and then manually edited using MacClade version 4.06 (Maddison and Maddison 1989). Limits of the intron were determined by comparison with our cDNA sequence and the German MSP gene and cDNA sequences (GenBank accession numbers EF012201 and DQ999999) and by comparison with MSP sequences from other nematodes (see below).

Descriptive statistics for sequence polymorphism and divergence within and between lungworm species and populations were calculated using DnaSP version 4.10.2 (Rozas and Rozas 1999). Using the same software, we also calculated the number of haplotypes (h) and estimated the nucleotide diversity (P_i) using all of the sequences from all species combined.

Phylogenetic analyses

Our sequences were compared with those of other related nematodes. Several MSP sequences from various Nematoda available in GenBank (non-redundant nucleotide and protein databases) were thus accessed and aligned based on their translated amino acid sequences. The final alignment included MSP nucleotide sequences of the three *Dictyocaulus* taxa generated in the present study, plus *Ancylostoma ceylanicum* (CB176395), *A. suum* (CB014916), *Haemonchus contortus* (CB191457), *M. ozzardi* (AJ404209–25), *Nippostrongylus brasiliensis* (BU493394, BQ529557), *O. dentatum* (AJ627869, AJ627870, AJ627872, AJ627873), *O. volvulus* (J04662), *Teladorsagia circumcincta* (CB039111, CB037657), and *C. elegans* (NM069321, NM062571, NM069380). Additional MSP nucleotide sequences are available that were either identical to the included sequences, or, as with the sequences of *Trichuris* spp. (CB013663 and CB188941), very divergent and therefore rejected as not being homologous.

The final data set, introns excluded, contained 384 aligned nucleotide positions, of which 151 were parsimony informative. Phylogenetic trees were produced using both maximum-parsimony and maximum-likelihood algorithms using the PAUP* version 4.0b10 package (Swofford 2002). Maximum-parsimony heuristic searches used 100 random-addition sequences of tree bisection and reconnection (TBR) branch swapping, as well as 20 replications of the parsimony ratchet (Nixon 1999) based on 200 iterations of TBR branch swapping. Maximum-likelihood heuristic searches used the ratchet (Morrison 2007) strategy based on 100 iterations of TBR branch swapping. The SYM+G substitution model was used, determined after preliminary testing with the ModelTest version 3.7 program (Posada and Crandall 1998), and the parameter values (fixed during the searches) were estimated using successive approximations (Sullivan et al. 2005).

Support for the phylogenies was measured by bootstrapping. For the maximum-parsimony analyses, this was based on 1,000 pseudoreplicates, each with 100 random-addition sequences of TBR branch swapping, while for the maximum-likelihood analyses, it was based on 100 pseudoreplicates, each with one random-addition sequence of TBR branch swapping. The final trees were drawn using TreeView version 1.6.6 (Page 1996).

Western blot analysis

Protein fractions prepared by HisTrap purification were separated on 12% SDS-PAGE and then transferred onto a nitrocellulose (NC) membrane at 300 mA for 45 min with a mini-Trans-Blot transfer cell (BioRad, Hercules, CA, USA). The membrane was blocked in Tris buffered saline (TBS: 100 mM Tris–HCl, pH 7.5, 0.9% NaCl) with 5% non-fat dried milk for 1 h at RT or overnight at 4°C. The membranes were cut into strips and incubated for 60 min at RT. All tested sera were diluted 1:50. The strips were washed with T-TBS (0.1% Tween in TBS) and then incubated with polyclonal rabbit anti-cow Ig conjugated with horseradish peroxidase (Dako Cytomation), diluted 1:500. After a 1-h incubation at RT, the strips were washed with T-TBS, and the bound secondary antibodies were visualized by the ECL fluorescent detection reagents on Hyper film according to the manufacturer's instructions (GE Healthcare).

Results

Polymorphic sites in *Dictyocaulus*

The complete *Dictyocaulus* dataset generated in this study consisted of 100 sequences. These comprised of 72 sequences from *D. viviparus* (all from cattle), 11 from *D. capreolus* (nine from roe deer and two from moose, respectively), and 17 from *D. eckerti* (nine from moose and eight from red deer, respectively). Attempts to use a primer targeting the 5′ UTR of the previously published gene sequences were unsuccessful. Consequently, we moved our forward primer into the coding sequence of the MSP gene of *D. viviparus.* A revisit to the GenBank entry (EF012201) showed that the sequence was updated after our sequencing begun, and that two nucleotides in the region corresponding to the OP433 primer had been changed. Our MSP sequences have been deposited in GenBank with accession numbers EU606052–EU606151.

In *D. viviparus*, a total of 478 nucleotide sites were identified, of which 469 bp were parsimony informative when alignment gaps were excluded. Gene arrangement consists of two exons flanking one intron, which starts at position 248 (all positions numbers include 6 unsequenced bases at the beginning of the gene). Only a low level of polymorphism was detected between the bovine lungworm MSP sequences, and the intraspecific nucleotide variation observed was as a result of double peaks in isolated positions in single worms in positions 111, 234, 252, 260, 261, 263, 362, and 425.

When the bovine sequences were compared with those of *D. capreolus* and *D. eckerti*, a T-rich insert was noted at the beginning of the intron between positions 262 and 267. Consistent or fixed differences were noted at three sites in this region in one of the Swedish field isolates, which has a G instead of T at position 263 and A instead of T at both positions 264 and 268. Exactly the same mutations were noted in seven out of the eight *D. viviparus* worms representing this isolate, whereas in the remaining worms, there were double peaks instead. The worms with the G and A mutations also differed at position 365 with a G instead of a C. Likewise, the sequences from three other *D. viviparus* worms, of which one belonged to the Dutch laboratory strain, had either a C at position 264 or an A at position 266, with the other worms having double peaks at these positions. Thus, these mutations were uninformative, and accordingly, no nucleotide diversity was observed between the individual bovine lungworms.

In *D. capreolus* and *D. eckerti*, 472 and 469 nucleotides out of the 478 nucleotides in the complete dataset were informative, respectively. Similar to the MSP of *D. viviparus*, almost no polymorphism was found within each species. Intraspecies variation was observed only at position 60, where five out of the eight *D. eckerti* MSP sequences from red deer had a double peak instead of a C, and there was either a double peak or a C instead of a T at position 255 in 15 of the *D. eckerti* sequences from both hosts, and a double peak or a T instead of a C at position 298 in five lungworms from red deer and in two from moose.

When all 100 sequences were compared between the three *Dictyocaulus* species, eleven variable or polymorphic (segregating) parsimony-informative sites were found (Table 1). Exactly the same pattern was noted for each species at the positions shown, and this was so irrespective of the host origin of the different lungworm species. Six out of the eleven substitutions were non-synonymous in the third codon position (Table 2).

Although the first six coding nucleotides are excluded from our dataset, it was evident that the MSP gene in *Dictyocaulus* carries an intron at positions 250–317. Interestingly, in *D. capreolus* and *D. eckerti*, there is a 6-bp gap between positions 259 and 264, and the intron of *D. viviparus* is therefore slightly longer. Most of the intraspecific sequence variation we observed was in the intron, as six out of the eleven parsimony-informative sites were located in this untranslated region of the MSP gene (Table 1).

Table 1 Parsimony-informative sites in 100 unique MSP1 sequences from *D. capreolus* from moose and roe deer, *D. eckerti* from moose and red deer, and *D. viviparus* from 8 different isolates of cattle

Species/position	174*	201*	216*	*257*	*265*	*273*	*275*	*287*	*309*	447*	448
D. capreolus (*n*=11)	T	C	A	G	T	G	A	A	T	T	C
D. eckerti (*n*=17)	T	C	A	A	A	G	G	G	T	A	G
D. viviparus (*n*=72)	C	T	G	A	A	T	G	G	C	A	C

The base positions are given relative to the first base of the start codon of the protein-coding region and with the third triplet that was observed in some species excluded. The number of parasites with these alleles is also shown. Mutations that occur in the third codon position have been indicated with an asterisk. Codon positions situated in the intron are in italics.

Phylogenetic analysis of MSP sequences

The phylogenetic analyses produced a single maximum-likelihood tree (Fig. 1) and 165 maximum-parsimony trees. The majority-rule consensus of the maximum-parsimony trees differed from the maximum-likelihood tree only in being less resolved and in the placement of two terminal branches; and the bootstrap support for the relevant branches was also approximately the same between the analyses.

These phylogenetic analyses revealed three well-supported clades and one clade that were only partially supported. These clades appeared in most of the analyses and irrespective of the evolutionary model or tree-building method used, but the relationships among them varied somewhat depending on the particular analysis. Where available, these clades grouped the multiple sequences from each species. More importantly, there was clearly less genetic variation among the MSP sequences from the three *Dictyocaulus* species than there was among the sequences within species such as *N. brasiliensis*, *T. circumcincta*, *O. dentatum*, and *C. elegans*. Only the (partial) *M. ozzardi* sequences showed little within-species variation.

MSP nucleotide diversity and species structure

One haplotype was found per lungworm species, with a haplotype (gene) diversity and nucleotide (P_i) diversity per site of 0.445 and 0.000753, respectively. The base compo-

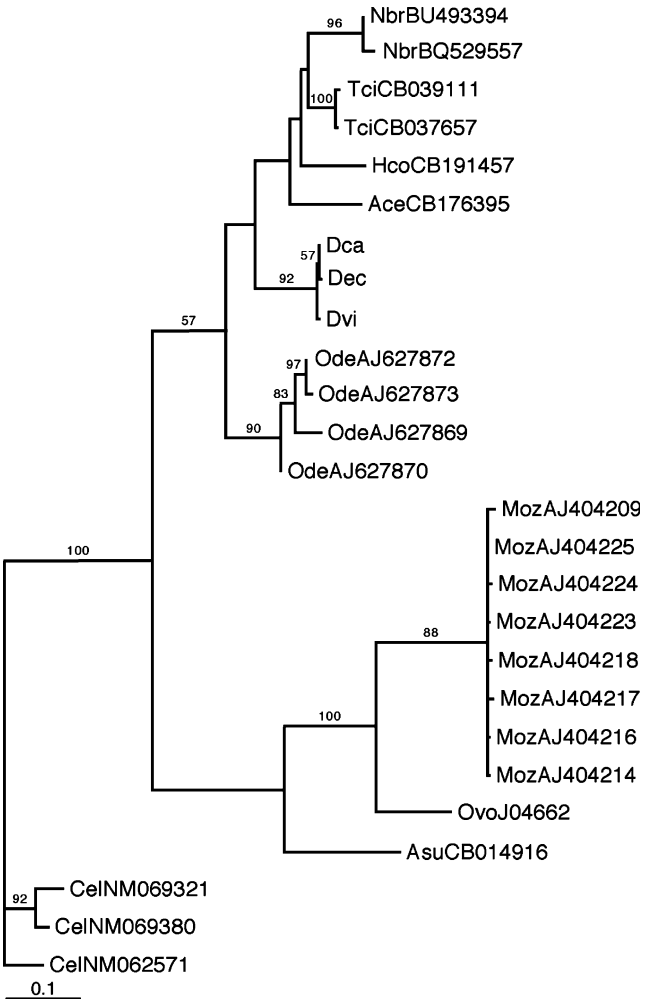

Fig. 1 Maximum-likelihood tree for MSP gene sequences from the exon of various animal parasitic nematodes plus three sequences of *C. elegans*, which was used as the outgroup. *Branch lengths* are proportional to the inferred number of nucleotide changes, and bootstrap values are given as percentages for each node. The evolutionary model used was the general time-reversible model with equal base frequencies (SYM+G): A↔C=0.7611, A↔G=2.9839, A↔T=2.2588, C↔G=0.6376, C↔T=8.2627, G↔T=1.0000; gamma distribution for variable sites with shape parameter: 0.3405. For abbreviations in the tree, see Table 2

Table 2 DNA polymorphism analysis of MSP sequences showing the genetic divergence between three lungworm species

Sites (k)/P_i(t)	*D. capreolus*	*D. eckerti*	*D. viviparus*
D. capreolus (*n*=11)		0.00738	0.00400
D. eckerti (*n*=17)	7 (3.463)		0.00540
D. viviparus (*n*=72)	8 (1.862)	8 (2.501)	

The total number of polymorphic sites and the average number of nucleotide differences (k) are shown in the lower left corner, whereas the nucleotide diversity P_i(t) is displayed in the upper right corner.

Table 3 Comparison of amino acid sequences among representative MSPs of Ace *Ancylostoma ceylanicum* (GenBank CB176395), Asu *Ascaris suum* (CB014916), Ce1 *Caenorhabditis elegans* (NM069321), Ce2 *Caenorhabditis elegans* (NM062571.5), Ce3 *Caenorhabditis elegans* (NM069380.2), Dca *Dictyocaulus capreolus*, Dec *Dictyocaulus eckerti*, Dvi *Dictyocaulus viviparus*, Hco *Haemonchus contortus*, (CB191457), Moz *Manzonella ozzardi* (AJ404225), Nbr1 *Nippostrongylus brasiliensis* (BU493394), Nbr2 *Nippostrongylus brasiliensis* (BQ529557), Ode1 *Oesophagostomum dentatum* (AJ627869), Ode2 *Oesophagostomum dentatum* (AJ627870), Ode3 *Oesophagostomum dentatum* (AJ627872), Ode4 *Oesophagostomum dentatum* (AJ627873), Ovo *Onchocerca volvulus* (J04662), Tci1 *Teladorsagia circumcincta* (CB037657), Tci2 *Teladorsagia circumcincta* (CB039111), Tmu *Trichuris muris* (CB013663), Tvu *Trichuris vulpis* (CB188941)

	5	10	15	20	25	30	35	40	45	50	55	60
Ace	MA-SV	PPGDI	NTQPN	SKIVF	NAPYD	DKHTY	HIKIT	NASGR	RIGWA	IKTTN	MRRLG	VDPAC
Asu	..Q..	….	..Q.G	….	….	….	….	..G..	….	….	….	….
Ce1	..Q..	….	Q…G	….	….	….	…VI	.S…	…YG	….	.K…	….
Ce2	..Q..	….	Q…G	….	….	….	…VI	.S…	…YG	….	.K…	….
Ce3	..Q..	….	Q…G	….	….	….	…VI	.S…	…YG	….	.K…	….
Dca	??-..	….	….	….	….	….	….I	….	….	….	.K…	…A.
Dec	??-..	….	….	….	….	….	….I	….	….	….	.K…	…A.
Dvi	??-..	….	….	….	….	….	….I	….	….	….	.K…	…A.
Hco	..-..	….	….	….	….	….	….I	….	….	….	….	…A.
Moz	?????	??…	H..QG	….	….	….	….	..G..	….	….	….	…P.
Nbr1	..-T.	….	….	A….	….	….	….I	….	….	….	.K…	…A.
Nbr2	..-T.	….	….	A.VN.	….	….	….I	….	….	….	.K…	…A.
Ode1	..-T.	….	H..QG	T….	….	….	….	….	….	….	….	…A.
Ode2	..-T.	….	H..QG	….	….	….	….	….	….	….	….	…A.
Ode3	..-T.	….	H..QG	T….	….	….	….	….	….	….	….	…A.
Ode4	..-T.	….	H..QG	T….	….	….	….	….	….	….	….	…A.
Ovo	..Q..	….	H..QG	….	….	….	..N..	..G..	….	….	.K…	….
Tci1	..-..	….	….	….	….	….	….I	….	….	….	….	…A.
Tci2	..-..	….	….	….	….	….	….I	….	….	….	….	…A.
Tmu	.S—	L.A..	A.D.G	.ELWL	.G..L	TDQ.T	A..L.	..GDK	L…R	VNPSV	ETRYV	MN.KE
Tvu	.S—	L.A..	A.D.G	.ELWL	.G..L	TDQ.T	A..L.	..GDK	L…R	VNPSV	ETRYV	MN.KE

The intron in the 3 *Dictyocaulus* species is situated after amino acid position 84, whereas in *M. ozzardi* and in *O. volvulus*, it is situated after position 33.

sition was balanced, with a G+C content of 0.495. Pairwise comparisons of DNA divergence between species are shown in Table 3. Between the species included in the analyses, seven to eight polymorphic sites were noted. Interestingly, the differences between the lungworms from the wild ruminants, *D. capreolus* and *D. eckerti*, were of the same magnitude as the differences to the lungworm of domesticated cattle, *D. viviparus*.

Characterization of MSP cDNAs and predicted proteins

The cDNA sequences from independent clones were confirmed to be MSP. After a pilot experiment, a single clone was used to purify the recombinant MSP. In Western blot (WB) analysis (Fig. 2), purified MSP were recognized by antibodies in sera both from calves experimentally (lanes A1–6) and naturally (lanes B1–6 and C1–2) infected with *D. viviparus*; for details of the experimental sera, see Höglund et al. (2003a). In the WB, a strong ~17-kDa band was observed in all lanes on the NC membrane incubated with lungworm-positive sera. As expected, there was an increasing intensity dependent on the antibody levels in the sera that were used to probe the NC membrane (Fig. 2).

Discussion

In this study, we investigated the sequence variation in the major sperm protein 1 (MSP) gene of the lungworm *D. viviparus*. Although both a high degree of intra-population genetic variation and little genetic interchange between populations have been demonstrated for this parasite using AFLP markers and mtDNA data, no or only limited sequence variation was observed in the MSP gene of the same bovine isolates. This was so despite comparison of one isolate of a laboratory-maintained strain from the Netherlands with eight Swedish field isolates. As no isolate-associated antigenic diversity or size variation of MSP was detected, it is likely that the immunodominant regions of this antigen also are conserved among more geographically distinct strains.

On the other hand, when MSP sequences from the bovine lungworm were compared with those of two other lungworm species from wildlife ruminants, *D. capreolus* and *D. eckerti*, minor but consistent parsimony-informative sequence differences were observed. As the variability between species was limited, the capture MSP antigen used in existing immunodiagnostic tools can probably also be

65	70	75	80	85	90	95	100	105	110	115	120	125	
GVLDP	KESTL	MAVSC	DVFDY	GREDT	NNDRI	TVEWC	NTPDG	AAKQF	RREWF	QGDGM	VRRKN	LPIEY	NP
…..	…..	…..	.T.NA	ATEDL	…..	.I..T	…..	…..	…..	…..	…..	…..	..
…..	..AV.	L….	.A.AF	.Q…	…..	….T	…..	…..	…..	…..	…..	…..	..
…..	..AV.	L….	.A.AF	.Q…	…..	….T	…..	…..	…..	…..	…..	…..	..
…..	..AV.	L….	.A.AF	.Q…	…..	….T	…..	…..	…..	…..	…..	…..	..
…..	..AT.	…..	.T.E.	…V.	…..	…..	…..	…..	…..	…..	…..	…..	..
…..	..AT.	…..	.T.E.	…V.	…..	…..	…..	…..	…..	…..	…..	…..	..
…..	..AT.	…..	.T.E.	…V.	…..	…..	…..	…..	…..	…..	…..	…..	..
…..	..AT.	…..	…..	…..	…..	…..	…E.	…..	…..	…..	…..	…..	..
…..	..NV.	…..	.T..A	TK..I	…..	.I..T	…..	…..	…..	….?			
…..	..AT.	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	..
…..	..AT.	…..	…..	.H…	…..	…..	…..	…..	…..	…..	…..	…..	..
…..	..AT.	…..	.T…	…..	…..	…..	…E.	…..	…..	…..	…..	…..	..
…..	..T..	…..	.T…	…..	…..	…..	…E.	…..	…..	…..	…..	…..	..
…..	..T..	…..	.T…	…..	…..	…..	…E.	…..	…..	…..	…..	…..	..
…..	..T..	…..	.T…	…..	…..	…..	…E.	…L.	…..	…..	…..	…..	..
…..	..VN.	…..	….A	T…T	…..	.I..T	…..	…..	…..	…..	…..	…..	..
…..	..AT.	…..	.T…	…..	.H…	…..	…..	…..	…..	…..	…..	…..	..
…..	..AT.	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	..
.A.PG	RGTIM	…..	VGIVC	KPDAP	.I.TI	.I…	EA.E.	QTV-Y	DA…	AKGLV	….		
.A.PG	RGTIM	…..	VGIVC	KPDAP	.I.TI	.I…	EA.E.	QTV-Y	DA…	AKGLV	….		

employed for other lungworm–host interactions, as for example, in red deer where *D. eckerti* poses a problem (Johnson et al. 2001). By taking advantage of a secondary antibody that will recognize cervid immunoglobulins, recombinant bovine lungworm MSP can probably also be used as the capture antigen in an enzyme-linked immunosorbent assays (ELISA) specifically designed for the detection of lungworm infection in red deer. Furthermore, from the phylogenetic analysis, it is clear that the sequence differences that were observed between different lungworm taxa could also be useful as an additional genetic marker for species delimitation within the genus *Dictyocaulus* and to further investigate how it relates to other genera and more closely related species (see below).

It has been considered important to look at the polymorphism in the materials that parasites expose to their hosts not only to understand the mutual adaptations undergone between them but also to study how this may affect the hosts' variability in responsiveness and immunopathology (Wakelin 1992). In the current study, the term antigenic variation or variability refers to the genetic diversity by which pathogens may alter their immunogenic

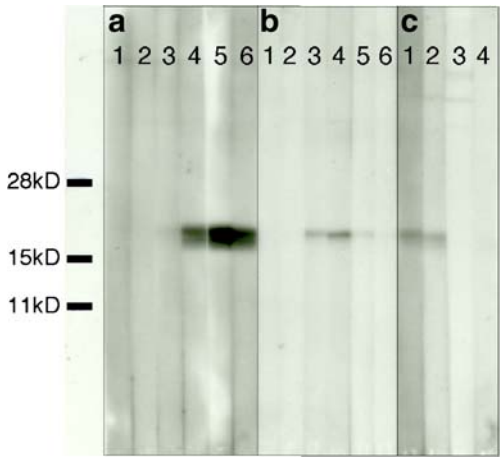

Fig. 2 Western blotting of recombinant MSP from *D. viviparus* incubated with calf sera from **a** an experimentally infected animal sampled every fortnight, lanes *A 1–6*; **b** an animal infected on pasture that was sampled at 4-week intervals, lanes *B 1–6*; and **c** from two calves that were positive (*C1* and *C2*) and negative (*C4* and *C4*) using the Ceditest® lungworm ELISA (for details, see Cornelissen et al. 1997). Molecular weight standards are shown in the *left*

proteins, for example to be recognized by the host immune system or evade a host immune response. Although antigenic variation may arise through different genetic processes in parasitic nematodes, there are few examples to support this view. This is in sharp contrast to the fine detail with which helminth species have been delineated by morphological techniques (for a review, see Gasser and Newton 2000).

Previous results suggest that the effect of molecular variation in antigens from geographically and genetically separated parasite populations may or may not exist (Currie et al. 1998; Jasmer et al. 2004; Qiang et al. 2000). Thus, this must be considered during development of immunodiagnostic tools and subunit vaccines. Until now, no study has examined whether lungworms exhibit antigenic diversity or variation. This is so despite the fact that *D. viviparus* is one of the few parasitic nematodes where a commercial vaccine that contains irradiated infective larvae is commercially available (Ploeger 2002). In addition, the bovine lungworm is one of the few nematode parasites where there is access to diagnostic tests based on immunological tools such as the ELISA (Cornelissen et al. 1997; Schnieder 1992a, b). The patterns of variation shown here further suggest that MSP is suitable as the capture antigen in such diagnostic tests.

Several *D. viviparus* immunogens have been identified, of which some have shown promising results either as subunit vaccine candidates or as immunodiagnostic tools (Britton et al. 1993b; Britton et al. 1994; Britton et al. 1995; Gilleard et al. 1995a; Haslam et al. 2000; Schnieder 1993b). The list includes two secreted or neuronal acetylcholinesterases (ACE; Matthews et al. 2001), one nematode polyprotein allergen (Dv-NPA; Britton et al. 1995), a major sperm protein (MSP) (Schnieder 1993b), a cytosolic and an extracellular superoxide dismutase (cSOD and ecSOD) (Britton et al. 1994), and an immunodominant antigen localized in the sheath of infective larvae (L3-ShA; Britton et al. 1993a; Gilleard et al. 1995b). Some of these substances have been demonstrated to be associated with the induction of immune responses either in laboratory animals or in the definitive natural host, notably the secreted ACEs and MSP (Matthews et al. 2001; Schnieder 1992a). These proteins may have important external roles in the life-cycle, such as immunomodulation or, in the case of proteases, tissue penetration and feeding. These are functional necessities for the parasite, for which selection pressure would be intense. Other proteins could be viewed as an incidental excretion. Therefore, although immunogenic, the resultant immune recognition may not threaten parasite survival within the host, and hence apply no selection pressure. MSP is released from the worm during infection and has been shown to be associated with a rise in systemic antibody levels and a reduction in larval shedding in experimentally immunized calves (von Holtum 2006). It therefore seems likely that it is not an incidental excretion.

Still, none of these substances have previously been analyzed in any depth when it comes to their genetic polymorphism. At the same time, it has become increasingly clear that lungworms are genetically heterogeneous, and sub-populations on different farms have been found, between which gene flow rates are low (Höglund et al. 2004a; Höglund et al. 2006). Hence, we considered it to be of utmost importance to address fundamental questions pertaining to the population structure of *D. viviparus* in relation to vaccine development and to investigate the polymorphism in potential vaccine candidates from different geographic isolates of the parasite under study.

Although we sequenced MSP from eight bovine lungworms of each of eight field populations, only minor sequence variation was observed. Furthermore, another isolate was from a Dutch laboratory-maintained population. Thus, in contrast to similar studies on other immunogenic proteins in nematode parasites (Currie et al. 1998; Jasmer et al. 2004; Qiang et al. 2000), MSP of *D. viviparus* can be considered to be a evolutionarily stable protein with no or limited genetic variation across isolates that show a high degree of genetic variability and structures based on AFLP and mtDNA markers (Höglund et al. 2004a; Höglund et al. 2006).

Although this result is in agreement with the view that MSPs belong to a family of evolutionarily highly conserved genes that are exclusively found in nematodes, earlier studies have revealed that various regional variants may exist. For example, Morales et al. (2000) noted two distinct groups of MSP gene variants in the filaroid *O. volvulus*, whereas the MSP genes of *M. ozzardi* were similar to each other. Furthermore, intraspecific differences in MSP sequences have also been detected in the nodular worm of pigs, *O. dentamum* (Cottee et al. 2004), and up to seven MSP-like allelic forms in the pig roundworm *A. suum* (Tarr and Scott 2004).

When MSP of the three *Dictyocaulus* lungworm species in this study were compared, consistent parsimony-informative differences were found, indicating that the sequence variation in this protein can be used as a diagnostic tool for species delineation within this genus at a wider scale. This finding further substantiates the existence of *D. capreolus*, which is a rather novel *Dictyocaulus* species that is different from both *D. eckerti* and *D. viviparus* using other genetic markers (Divina et al. 2000; Höglund et al. 2003b; Höglund et al. 1999). This finding is in agreement with Morales et al. (2000) who demonstrated that two cryptic filarial parasites of humans could be identified by fixed sequence differences in their MSP genes. The possible usefulness of sequences from highly conserved genes such as MSP to differentiate between nematode species in taxonomic studies was also suggested by Scott et al. (1989).

The MSP gene of *Dictyocaulus* was more conserved than those of other parasitic nematodes, at least compared to the limited number of other species from which multiple sequences are available. Furthermore, as can be observed in our phylogram, variable sequences from the same and or related genera group together in well-supported clades. Although the information available is still limited, this indicates that the variation in MSP genes has evolved after major events of species evolution (although the existence of MSP isoforms has been suggested). In fact, our two clades are compared to clades III and V of Blaxter et al. (1998), indicating that the MSP gene contains useful phylogenetic information at a more inclusive level. Before MSP sequences can be more widely employed as a diagnostic tool, investigation of gene organization and the search for universal primers that can be used on a higher taxonomical level must be intensified.

Acknowledgment The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS: 22.0/2004-0432) funded this study.

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