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# Genetic diversity assessed by amplified fragment length polymorphism analysis of the parasitic nematode *Dictyocaulus viviparus* the lungworm of cattle

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## Abstract

We have examined the population genetic structure in a collection of nine isolates of the parasitic lungworm *Dictyocaulus viviparus*. Eight of the isolates were sampled from cattle in geographically separated farms throughout south-central Sweden, and one isolate was a laboratory strain that has been maintained in experimentally infected calves for almost four decades. A total of 72 worms were examined, with eight individual worms from the same individual host representing each isolate. The genetic variation as revealed by amplified fragment length polymorphism analysis using four selective primer combinations was high. Depending on the primer combination a total of 66–79 restriction fragments were amplified, with 26–44 peaks of similar complexity from each of the isolates. The heterozygosity within populations was relatively small, as were the population mutation and immigration rates, which seemed to be in neutral equilibrium. The genetic diversity was therefore reasonably well structured in the field; and the laboratory isolate was quite distinct from the field samples. There was no relationship between the patterns of genetic diversity and the geographical proximity of the farms. The estimates of heterozygosity were much larger and more consistent than those previously estimated for this nematode species using mitochondrial sequencing, and the genetic structuring was thus much less pronounced and the gene flow greater. We attribute these differences in estimation to the broader sampling of loci available using amplified fragment length polymorphism markers, which may therefore constitute a superior technique for the study of patterns of lungworm diversity. Furthermore, the data estimating gene flow for *D. viviparus* was less than previously reported for closely related species in North America. This might be related to different rates of movements of infected hosts. It seems likely that lungworm infections are rather persistent on different farms, and the sudden outbreaks of disease that can be observed with host movements are most likely to be related to the introduction of susceptible stock.

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## 1. Introduction

Throughout the world, grazing livestock are infected by a range of nematode parasites. Most species in domestic ruminants belong to the superfamily Trichostrongyloidea, which often are a cause of substantial economic loss. The trichostrongyloids are dioecious, with members exhibiting direct one-host life cycles (Anderson, 1992). Following sexual reproduction inside the host these parasites are transmitted as third stage infective larvae that develop in the faeces and are picked up on pasture. Inside the host the adult parasites, depending on

the species, reside either in the gastrointestinal tract or in the lungs. In cattle of temperate regions of the world the most important nematodes are the gastrointestinal species *Ostertagia ostertagi* and *Cooperia oncophora* as well as the lungworm *Dictyocaulus viviparus*.

Compared to many other groups of organisms, there have been relatively few studies of the population biology of nematodes (Silva and Russo, 2000). However, for some years there has been a steadily increasing interest in the population genetic structure (PGS) of parasitic nematodes of livestock. For example, the PGS reveals specific information about the genetic similarities of worms, which can then be used to understand the patterns of spread of parasites (Anderson, 1998; Anderson et al.,

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1998; Paterson and Viney, 2000). Because the long-term survival of pasture-borne parasites of livestock depends on their external larval stages, PGS can help in understanding whether there is locally adapted sub-structured populations, or if the parasite consists of a single panmictic population (Blouin et al., 1995). This is of great relevance for the understanding of the dynamics of infection, at local, regional and global scales, which may be used to predict the potential risk for spread of anthelmintic resistance.

Although molecular based information about the PGS of livestock parasites is still very limited, a low genetic diversity has been found among different isolates of *O. ostertagi* in cattle in the USA, as revealed by variation in the cytochrome oxidase subunit 1 and NADH dehydrogenase regions of the mtDNA (Dame et al., 1993). This was explained as being due to recent host movements within the country that contributed to the spread of this parasite. However, in contrast to this, we have found evidence of structured subpopulations of *D. viviparus* in south-central Sweden based on studies of mtDNA, despite similar kinds of host movements (Hu et al., 2002).

In order to further assess the genetic diversity of various lungworm isolates, a genetic comparison was undertaken based on Amplified fragment length polymorphism (AFLP) analysis. This is a polymerase chain reaction (PCR)-based molecular fingerprinting technique that requires no prior sequence information. In contrast to studies based on sequence information in the mtDNA, AFLP is a whole-genome approach that can be applied to any organism in order to estimate population genetic diversity and genetic structure parameters below the species level (Vos et al., 1995). Since AFLP markers occur throughout the genome, this makes them potentially more valuable than other genetic markers because many unlinked nuclear loci are needed to estimate population parameters reliably (Pluzhnikov and Donnelly, 1996; Wakeley and Hey, 1997; Campbell et al., 2003).

This study was carried out to assess the applicability of the AFLP technology as a method for DNA fingerprinting in the trichostrongyloid nematode *D. viviparus*. The aim

was to further determine whether different natural geographic subpopulations of *D. viviparus* exist based on information revealed by AFLP. An isolate that has been maintained through passages in live animals for several decades, was also included as a distantly related reference strain.

## 2. Materials and methods

### 2.1. Populations and extraction of genomic DNA

Field isolates of adult *D. viviparus* were collected from eight farms at different locations in Sweden (for details see Table 1). One isolate from a laboratory strain that has been maintained for several decades by biannual passages in experimentally inoculated calves was also included. The worms were fresh frozen and then stored at  $-70^{\circ}\text{C}$  before use in this study.

DNA from all isolates, except N29/00, was prepared from individual worms using QIAamp DNA Mini Kit (Qiagen). The procedure was performed according to the tissue protocol provided by the manufacturer. To obtain RNA-free DNA the tissue lysis step was followed by incubation with 1.7 mg/ml RNase A, for 2 min at room temperature. The DNA was eluted in the supplied elution buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0). The eluted DNA was stored at  $+4^{\circ}\text{C}$  until further use.

DNA from worms of the isolate N29/00 was isolated using Genovision's GenoPrep™ Tissue Kit which is a magnetic bead-based system used with a robotic workstation, Genom™48/BioRobot M48. The samples were disintegrated in lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 100 mM EDTA, 1% SDS) complemented with Proteinase K to give a final concentration of 1 mg/ml, and incubated on a shaking platform at  $37^{\circ}\text{C}$  over night. The tissue lysis step was followed by incubation with RNase A, 0.9 mg/ml for 5 min at room temperature. The solutions were then loaded onto the robot and the DNA was eluted with distilled  $\text{H}_2\text{O}$  and stored at  $4^{\circ}\text{C}$  until further use.

The DNA concentration was determined by measuring the absorbance at 260 nm. The integrity of the genomic

Table 1  
*Dictyocaulus viviparus* isolates used in this study

Code	Geographic origin	Country	Position
L10: 1, 2, 3, 4, 5, 6, 8, 9	Lab strain, Intervet®	Netherlands	
F29: 1, 2, 4, 5, 7, 9, 10, 11	Kopparberg, Västmanland	Sweden	59°52'N14°59'E
F34: L, M, P, Q, S, T, U, V	Linköping, Östergötland	Sweden	58°15'N15°37'E
F36: M, N, O, P, R, T, U, X	Åtorp, Närke	Sweden	59°12'N15°10'E
F38: 3, 4, 5, 6, 7, 8, 9, 10	Gamleby, Småland	Sweden	57°54'N16°24'E
F49: 18, 19, 20, 21, 24, 25, 26, 27	Ljungbyhed Skåne	Sweden	56°04'N13°12'E
F65: 1, 2, 3, 4, 5, 7, 8, 10	Mantorp, Östergötland	Sweden	58°19'N15°08'E
F68: 2, 4, 5, 6, 7, 8, 10, 11	Linghem, Östergötland	Sweden	58°25'N15°37'E
F76: 1, 2, 5, 6, 7, 8, 9, 10	Kävlinge, Skåne	Sweden	55°48'N13°06'E

DNA was determined through gel electrophoresis on 0.8% agarose gel in 1 × TAE buffer (40 mM Tris–acetate, 1 mM EDTA) stained with ethidium bromide (~0.8 µg/ml).

## 2.2. AFLP procedure

The AFLP procedure was basically performed as described by Applied Biosystems (ABI, Foster City, CA) in their Plant Mapping Protocol. All reagents were supplied in the AFLP™ Plant Mapping kit except the restriction enzymes and T4 DNA ligase (New England BioLabs, Beverly, MA).

### 2.2.1. DNA restriction and ligation of adapters

Approximately 50 ng of DNA from each individual worm was digested with 1 unit of *Mse* I and 5 units of *Eco* RI (*Mse* I 10 U/µl, *Eco* RI 100 U/µl). *Mse* I adaptor and *Eco* RI adaptor were ligated in the same reaction as the digestion, using 20 pmol of *Mse* I adaptor and 2 pmol of *Eco* RI adaptor, 1 Weiss Unit of T4 DNA Ligase in 1.1 µl T4 DNA ligase buffer (50 mM Tris–HCl; pH 7.8, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA), 1.1 µl 0.5 M NaCl, and 0.55 µg BSA to a total volume of 11 µl. The restriction-ligation reaction was performed for 2 h at 37 °C. The mixture was then diluted to a total volume of 200 µl with TE<sub>0.1</sub> (20 mM Tris–HCl, 0.1 mM EDTA, pH 8.0).

### 2.2.2. Preselective amplification

From the restriction-ligation reaction 4 µl of the diluted DNA was mixed with 1 µl of ABI's preselective primer pairs (5 µM) and 15 µl of ABI's Core Mix. The core mix contains buffer, dNTPs, MgCl<sub>2</sub> and enzyme. Amplification was performed in a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA) using the following program: 2 min at 72 °C, 20 × [20 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C], 30 min at 60 °C. Ramp was set to 0.9 °C/s. To verify the production of the preselective amplification products, 10 µl was run on a 1.5% agarose gel in 1 × TAE buffer stained with ethidium bromide. The remaining amplification products were diluted with TE<sub>0.1</sub> in a final volume of 200 µl.

### 2.2.3. Selective amplification

Four primer combinations were used to produce AFLP fingerprints from each of eight adult worms of each of the nine *D. viviparus* isolates (for details see Table 2). Two classes of primers were used. The primers of the first class were complementary to the *Mse* I adaptor and have three additional selective nucleotides at their 3' end. The primers of the other class were complementary to the *Eco* RI adaptor and also have three additional selective nucleotides at their 3' end. For the detection of the fragments on the Genetic Analyzer (ABI 3100), the *Eco* RI primers contain fluorescent dye labels on their 5' end. From the diluted

Table 2

AFLP-primers used, numbers of peaks scored and number of peaks observed in each of the isolates included in this study, *n* = 8 individual adult *Dictyocaulus viviparus* worms from nine isolates

Isolate	L10	F29	F34	F36	F38	F49	F65	F68	F76	Mean
Combination A, <i>Eco</i> RI– <i>Mse</i> I, (5'–3') ACA (E35)/CTC (M60)										
Amplified peaks	38	32	33	26	26	34	31	28	41	32
Monomorphic peaks	19	27	16	17	18	17	19	17	17	19
Polymorphic peaks	14	4	11	7	7	12	9	11	21	11
Unique peaks	5	1	6	2	1	5	3	0	3	3
Combination B, <i>Eco</i> RI– <i>Mse</i> I, (5'–3') ACA (E35)/CTA (M59)										
Amplified peaks	43	42	36	28	31	37	36	33	43	37
Monomorphic peaks	23	25	15	18	23	16	15	24	17	20
Polymorphic peaks	18	8	16	8	7	14	16	6	21	13
Unique peaks	2	9	5	2	1	7	5	3	5	4
Combination C, <i>Eco</i> RI– <i>Mse</i> I, (5'–3') ACT (E38)/CAG (M49)										
Amplified peaks	38	33	42	32	33	42	43	40	39	38
Monomorphic peaks	23	26	24	23	26	25	26	25	23	25
Polymorphic peaks	12	3	11	5	5	14	16	10	12	10
Unique peaks	3	4	7	4	2	3	1	5	4	4
Combination D, <i>Eco</i> RI– <i>Mse</i> I, ACA (E35)/CAA (M47)										
Amplified peaks	44	34	35	28	35	35	34	33	41	35
Monomorphic peaks	26	24	15	19	22	19	22	21	17	21
Polymorphic peaks	13	10	18	9	11	14	8	7	21	12
Unique peaks	5	0	2	0	2	2	4	5	3	3
Combination A–D, all primer combinations combined										
Amplified peaks	163	141	146	114	125	148	144	134	164	142
Monomorphic peaks	91	102	70	77	89	77	82	87	74	83
Polymorphic peaks	57	25	56	29	30	54	49	34	75	45
Unique peaks	15	14	20	8	6	17	13	13	15	13

Within brackets are the primer names according to the KeyGene abbreviations for primer nomenclature.

preamplification products 3.0  $\mu$ l was mixed with 1.0  $\mu$ l of *Mse*I primer (5  $\mu$ M), 1.0  $\mu$ l of *Eco*RI primer (1  $\mu$ M) and 15  $\mu$ l Core Mix. Amplification was performed in a PTC-200 Peltier Thermal Cycler (MJ Research) using the following program: 2 min at 94 °C, 10  $\times$  [20 s at 94 °C, 30 s at 66 °C, 2 min at 72 °C] where the annealing temperature was decreased by 1.0 °C per cycle. The first 10 cycles were followed by 20  $\times$  [20 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C], 30 min at 60 °C. Ramp was set to 0.9 °C/s.

#### 2.2.4. Electrophoresis

From the selective amplification products 1  $\mu$ l was mixed with 9.6  $\mu$ l deionised formamide and 0.4  $\mu$ l GeneScan-500 size standard (ABI). The mixture was denaturated for 3 min at 95 °C before being loaded onto the Genetic Analyzer (ABI 3100, POP4, 36 cm array). The collected data were analysed using GeneScan Analysis software (version 3.7 and 3.7.1), and the tabular data for all peaks higher than 300 U was summarised. The values were then converted to binary characters (0, for absence, 1, presence of peaks) for further analysis. The digital peaks of the AFLP patterns were normalised by alignment to the size standard included. The peak patterns could thus be combined by assigning this reference as a standard and alignment of all other tracks versus this standard.

#### 2.3. Data analysis

The estimates of allele frequencies for each isolate used the Bayesian method with informative priors of Zhivotovskiy (1999). The calculation of genetic diversity ( $H_j$ ) and population structure ( $F_{st}$ ) from these estimates followed the procedures of Lynch and Milligan (1994), with 2000 randomisations used for the statistical tests. These statistics were all computed using the AFLPSurvey program of Vekemans (2002). However, since these methods assume Hardy-Weinberg genotypic proportions (i.e. no inbreeding,  $F_{is}$ ), for comparison  $F_{st}$  and  $F_{is}$  were calculated using the Markov Chain Monte Carlo (MCMC) Bayesian method described by Holsinger et al. (2002), using the Hickory program of Holsinger and Lewis (2003), with 25 000 samples taken after a burn-in of 5000 generations.

For the eight field samples, the population mutation rate ( $4N\mu$ ) was estimated using the iterative procedure described by Chakraborty and Weiss (1991) based on  $H_j$ . Gene flow between the field isolates ( $N_m$ ) was estimated using the  $F_{st}$  method described by Slatkin and Barton (1989). The Ewens–Watterson test for selective neutrality followed the method of Manly (1985), with 2000 randomisations, computed using the POPGENE program of Yeh et al. (1999).

Pairwise genetic distances between the field isolates were calculated following Reynolds et al. (1983), using AFLP-Survey and based on the absence or presence of bands. These distances were compared to the geographical distances between the farms via Mantel tests, with 2000

randomisations, using the ZT program of Bonnet and van der Peer (2002).

Pairwise genetic distances between the individual nematodes were calculated following Nei and Li (1979) as modified by Felsenstein (2002), using the PHYLIP computer package (Felsenstein, 2002). Phylogenetic trees were then constructed from these distances via neighbor-joining, using PAUP\* (Swofford, 1998), with minimum evolution and random initial seed. The robustness of the branches on the final tree was assessed via the bootstrap method with 1000 iterations; and the final tree was drawn with TreeView version 1.6.6 (Page, 1996). The similarity of each nematode to the other members of the same population was also tested using the maximum-likelihood jackknife re-allocation technique described by Campbell et al. (2003), using the AFLPOP program of Duchesne and Bernatchez (2002).

Where appropriate, all calculations were performed separately for each primer pair as well as for the combined data, and also for the farm/field isolates alone (i.e. without the laboratory isolate). In all cases, default values were used for all of the computer programs except as individually specified above.

For comparison, the data of Hu et al. (2002) were also reanalysed. The population mutation rate ( $4N\mu$ ) and the gene flow between populations ( $N_m$ ) were estimated simultaneously via the MCMC maximum-likelihood coalescent approach of Beerli and Felsenstein (2001), using the MIGRATE program of Beerli (2003), based on three long chains of 100 000 samples each after 20 short chains of 10 000 samples each. Appropriate adjustments were made for the haploid genome, and the number of males was assumed to equal the number of females.

### 3. Results

With the given reaction conditions and a cut-off value of 300 U, the size of the DNA restriction fragments varied between 36 and 486 bp and, depending on the primer combination used, between 26 and 44 fragments were amplified (Table 2). On average 32, 37, 38 and 35 peaks were obtained using primer combination A, B, C and D, respectively. Within each isolate three kinds of peaks were identified: (1) monomorphic peaks that were present in all individuals, (2) polymorphic peaks that were at least shared by a minimum of two worms, and (3) unique peaks that were only observed in single individual worms. As seen in Table 2 the number of monomorphic peaks, polymorphic peaks and unique peaks ranged, depending on the primer combination, from 15 to 27, 3 to 21 and 0 to 9, respectively.

The AFLP analysis thus provided 281 markers using the four primer pairs, 247 (87.9%) of which were variable. The primers differed in their ability to detect polymorphism within populations. The average over all populations of polymorphic bands amplified per primer pair varied from

Table 3  
Average values of the measures of genetic diversity ( $H_j$ ) and genetic population structure ( $F_{st}$ ), along with the results of the statistical tests

Genetic measure	Isolate	Primer combination				
		A	B	C	D	All
Hj	L10	0.16	0.15	0.13	0.15	0.15
	F29	0.05	0.10	0.05	0.10	0.08
	F34	0.12	0.14	0.14	0.18	0.14
	F36	0.08	0.08	0.07	0.09	0.08
	F38	0.07	0.06	0.06	0.12	0.08
	F49	0.13	0.13	0.16	0.15	0.14
	F65	0.10	0.14	0.15	0.09	0.12
	F68	0.11	0.06	0.12	0.09	0.09
	F76	0.21	0.18	0.13	0.22	0.19
Farm av.	0.11	0.11	0.11	0.13	0.12	
Fst <sup>a</sup>	All	0.41	0.52	0.41	0.44	0.45
<i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001
Fst <sup>b</sup>	All	0.51	0.58	0.52	0.50	0.53
Fst <sup>a</sup>	Farm	0.37	0.45	0.39	0.35	0.39
<i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001
Fst <sup>b</sup>	Farm	0.47	0.53	0.49	0.48	0.49
4 N $\mu$	Farm	0.09	0.10	0.09	0.12	0.10
Nm	Farm	0.36	0.23	0.37	0.32	0.31
Mantel <i>r</i>	Farm	0.01	−0.28	−0.18	−0.28	−0.21
<i>P</i>		0.483	0.160	0.249	0.141	0.228
Re-allocation success (%)		94	99	97	93	100

Values on the population mutation rate (4 N $\mu$ ), gene flow between the field isolates (Nm), and comparisons of the genetic and the geographical distances between farms (Mantel *r*), are also presented.

<sup>a</sup> Assuming  $F_{is} = 0$ .

<sup>b</sup>  $F_{is}$  allowed to vary.

54 (pair C) to 74 (pair B), and from 5 (pair A in Farm 29) to 43 (pair B in Farm 76 and pair C in Farm 65) for individual populations. The laboratory strain had a consistently high number of polymorphic bands for all primer pairs (38–44 bands).

Estimates of genetic diversity ( $H_j$ ; Table 3) were fairly consistent across primer pairs, with Farm 76 varying most from 0.13 to 0.22. Based on all loci, Farms 29, 36, 38 and 68 had similarly low diversity values, while Farm 76 had a much higher value. The laboratory strain had similar genetic diversity to Farms 34, 49 and 65. Thus, the overall variation in  $H_j$  between different primer combinations was low. This indicates that the results were consistent irrespective of which of the primer combinations that were used, although given the relatively low  $H_j$  values they are expected to provide a better answer when combined.

Genetic differentiation between isolates was statistically significant, with approximately half of the total variation being between populations (Table 3), the estimates varying from 45 to 53% depending on whether variation in the inbreeding coefficient ( $F_{is}$ ) was taken into account or not. The estimated values of  $F_{is}$  themselves were unreasonably high (>0.9) and have thus been ignored—this is a known

potential problem with the Hickory program when a large number of loci are used (Holsinger and Lewis, 2003). The genetic differentiation among the farm populations alone (i.e. excluding the laboratory isolate) was also statistically significant, although for this subset only 40–50% of the total variation was between populations (Table 3). The Mantel tests (Table 3) indicated that there was no relationship between the patterns of genetic similarity among the field populations and their geographical proximity to each other.

The population mutation rate for the farm nematodes (4 N $\mu$ ; Table 3) was relatively low, estimated as 0.1. On the other hand, gene flow between the field populations (Nm; Table 3) was slightly higher, estimated as 0.3 effective migrants per generation. Nevertheless, the tests of neutral evolution for the 247 polymorphic loci were rejected in only 27 cases (i.e. 89% were accepted), indicating that mutation and migration were in approximate equilibrium in these populations.

Given the strong structuring of the genetic variation between and within populations, this can most appropriately be displayed as a phylogenetic tree rather than as a network. The unrooted neighbour-joining tree (Fig. 1)

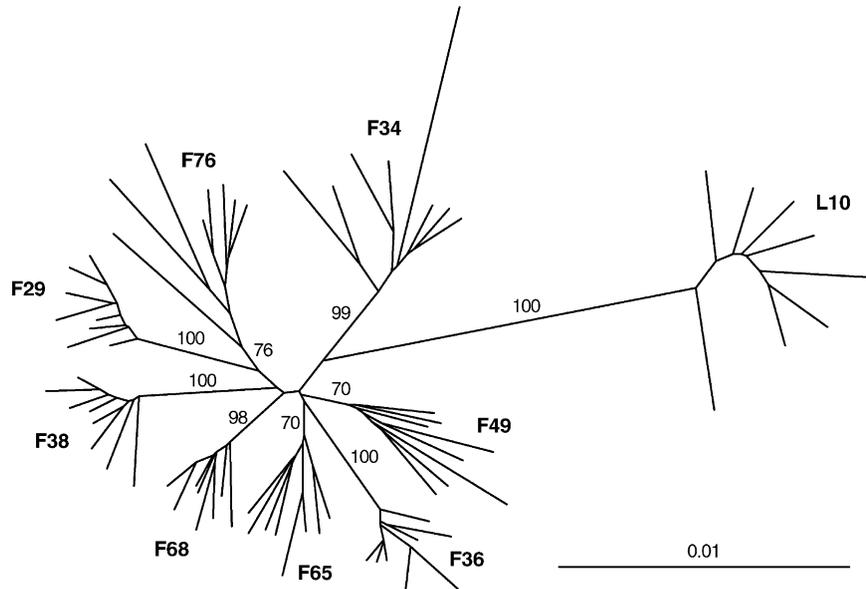


Fig. 1. Unrooted neighbour-joining tree of nine *Dictyocaulus viviparus* isolates based on amplified fragment length polymorphism (AFLP) presence/absence polymorphism. Each terminal branch represents one individual worm. The population labelled with L came from the laboratory maintained isolate, whereas those labelled with F were from the field. Also shown are the bootstrap values as the percentage of 1000 iterations and the 0.01 scale generated by PHYLIP which indicates the probability of differences between taxa.

shows the clear genetic isolation of the laboratory strain from the eight field samples, as well as the considerable within-population variation of this strain. There is much less inter-population variation among the farms. Furthermore, there is more variation among individuals within Farms 34, 49, 65 and 76 compared to the other four farms, and Farms 34 and 76 clearly have some rather aberrant individuals. Slightly different trees were obtained when the data obtained with the four primer combinations were analysed independently. In the majority of the analyses individual worms from the field isolates grouped together based on their origin. Exceptions from this were found within isolates F49 and F65, which in two out of the four data sets had one individual worm each that was separated from the majority of individuals in the group. Further, isolates F68 and F76 occasionally had up to three worms that were separated from the rest of the groups.

However, when the four data sets were combined all worms of the same origin grouped together (Fig. 1). We found statistical support for this pattern with bootstrap values that varied between 70 and 100%. Furthermore, the re-allocation analysis assigned each individual to its correct subpopulation almost all of the time (Table 3), confirming that they are genetically distinct. The unsuccessful re-allocations involved the same individuals and populations identified by the phylogenetic analysis.

For the data of Hu et al. (2002), the population mutation rate ( $4N\mu$ ) was estimated to be  $<0.01$ , and gene flow between the field populations ( $Nm$ ) was estimated to be 0.36 migrants per generation.

#### 4. Discussion

In this study we have described the PGS within and between nine isolates of *D. viviparus*, a parasitic nematode in the lungs of cattle. The number of loci detected was typical of reported AFLP studies, with an average of 70 bands per primer pair. The proportion of polymorphic loci is also typical of AFLP studies, with 90% of the bands being variable. However, the proportions of polymorphic loci were low for some of the populations, with the extreme being 7.5% for primer pair A in Farm 29.

The heterozygosity within populations was small, as were the population mutation and immigration rates. The genetic diversity was therefore well structured in the field. The laboratory isolate was also genetically distinct from the field isolates. Several different types of data analyses were run for most of the parameter estimates, in an effort to assess the robustness of the values obtained to different assumptions about the data. The results from comparable analyses were congruent, leading us to conclude that our estimates are reliable.

The partitioning of the genetic variation indicated that overall approximately 50% of the variation occurred between and 50% within populations. However, much of the between-isolate variation was due to the genetic distinction of the laboratory strain from the field samples, rather than from differentiation among the farms. When the laboratory strain was excluded from the calculations, ~40% of the genetic variation was between farms and 60% within farms. We detected only low levels of mutation within farms

and modest levels of migration among farms. This indicates relatively restricted gene flow. There was no evidence of deviation from selective neutrality.

The results we obtained from the genetic variation based on AFLP data thus indicate that this parasite forms separate subpopulations in Sweden, since genetic structure between worms isolated from different farms was found. This result differs from those of some closely related trichostrongyloid nematodes of ruminants in the USA (Blouin et al., 1992, 1995) and some other hosts elsewhere (Anderson et al., 1998), which were characterised by high genetic diversities within populations and with a high gene flow among populations, so that the  $F_{st}$  values were less than one-third of those that we observed. This alternative pattern was attributed to the extent of host movement; and the relatively few high-observed  $F_{st}$  values have usually been attributed to some form of subdivision within the species (Anderson et al., 1998). However, our conclusions are in accordance with a similar study of *D. viviparus* in Sweden based on the variation as revealed by single-strand conformation polymorphism (SSCP) analysis of the *cox1* gene of mtDNA (Hu et al., 2002). Thus, host movement may be less in Sweden than in North America, or lungworms may show different demographic behaviour compared to other trichostrongyloids (see below). Interestingly,  $F_{st}$  values comparable to ours have been reported for plant parasitic nematodes that by definition infect hosts that are sessile (Anderson et al., 1998).

If lungworms of cattle form separate subpopulations in Sweden and there is a limited gene flow between populations on different farms, then this has important practical implications, as it indicates the way in which host movements are associated with disease outbreaks. Based on the present finding it seems likely that the sudden and often unpredictable outbreaks of disease that sometimes are observed in Sweden, are probably due to local mass propagation of the parasite as a result of the introduction of susceptible animals into the herd, rather than to a recent introduction of the parasite on afflicted farms. The results also suggest that lungworms on different farms might be adapted to local conditions such as the microclimate. It may also be hypothesised that, with the observed PGS, in case of the development of anthelmintic resistance towards this parasite then the spread between farms will be delayed.

Although we found structured genetic variation between populations at different locations, there was no apparent correlation between genetic similarity as determined by the AFLP data and geographical distance, i.e. isolates from geographically close farms were not more similar to each other than were isolates from more distantly located farms. This result is in agreement with previous findings of the PGS of *D. viviparus* (Hu et al., 2002). Interestingly, this observation somewhat corresponds with the variability in genetic structure that has been determined for the ascarid nematode *Ascaris suum* of pigs (Nadler et al., 1995; Anderson and Jaenike, 1997). For the pig roundworm

*A. suum* it has been suggested that genetic structuring of various isolates into subpopulations was combined with high rates of host migration (Anderson and Jaenike, 1997). In the case of *D. viviparus*, it has been suggested that host movement does not play a major role in the spread of lungworms between farms due to few opportunities for genetic variants of this parasite to move across among farms (Hu et al., 2002). Although cattle are transported between farms in Sweden, lungworms are probably prevented from using this mode of dispersal as the prevalence in infected herds is normally low (~10%), and especially in older cattle that mount an effective protective immune response against this parasite the prevalence is in general low. Furthermore, as most cattle in Sweden are moved between farms mainly as calves and before they have been out on pasture, the likelihood of transferring this parasite between farms is small.

Taken together it seems likely that both *D. viviparus* and *A. suum* entertain high levels of inbreeding within populations, which in turn will result in a relatively low genetic diversity within subpopulations on different farms. Although both species are rather common parasites of livestock, there are at the same time major biological differences between the two. For example, survival of the larval stages inside the eggs on pasture over winter is a common phenomenon amongst pig roundworm (Larsen and Roepstorff, 1999), whereas this seems to occur more sporadically when it comes to the lungworm (Borgsteede et al., 1994). One character both parasites have in common, and which is in sharp contrast to most trichostrongyloids of ruminants, is the low effective population size. Gastrointestinal nematodes can be found virtually in every individual that has been out on pasture, and intensities in the range of 10 000–400 000 worms per individual host are not uncommon (Eysker and Kooyman, 1993). Lungworms, on the other hand, are normally only found in a smaller proportion of the animals in infected herds, and the intensity of these infections are also considerably lower than for the gastrointestinal parasites (Eysker and Kooyman, 1993). In a Dutch tracer test that lasted for about a week, ~60 *D. viviparus* were found at slaughter, whereas the numbers of *O. ostertagi* and *C. oncophora* were 40 000 and 180 000, respectively (Eysker et al., 1993). To what extent this contributes to the differences in the PGSs of these parasites definitely requires more attention.

One of the most interesting results is the large within-isolate variation shown by the laboratory sample. Differentiation of this strain from the field samples is to be expected, given that these farms are not where the isolate was first collected. However, the fact that the laboratory strain shows more within-population variation than do most of the farms is unexpected. This has been rarely reported for other nematodes (e.g. Grant and Whittington, 1994). It indicates that the effective population size in the field is approximately the same as in the laboratory, and that a relatively small population size is sufficient for maintaining the degree of genetic diversity observed in the field.

The mechanisms that generate the high level of polymorphism and how this is maintained are largely unknown. The variation observed between the various subpopulations is probably a result of genetic drift, and it indicates a limited gene flow between farms that probably has been ongoing for many generations. However, more detailed genetic work needs to be carried out, as we still know little about the evolutionary forces shaping these populations, which is relevant to disease control.

Although a range of DNA fingerprinting techniques have been used to describe the PGS of parasitic nematodes, papers based on the AFLP technique are scarce (Masiga et al., 2000). Most of these studies are focussed on nematodes of crops (Folkertsma et al., 1996; Semblat et al., 1998, 2000; Marche et al., 2001), whereas those of livestock nematodes are almost absent (Roos et al., 1998; Otsen et al., 2001). A major biological difference between nematode parasites of plants and livestock is that at least in some plant parasitic genera males are absent or rare and reproduction is by means of self-fertilisation. In addition, they exhibit poor dispersal mechanisms and they are parasites of non-mobile hosts, which complicates the comparison. Nevertheless, when (Folkertsma et al., 1996; Semblat et al., 1998) described the PGS of potato cyst and root-knot nematode populations, they found a great deal of genetic diversity as determined with the AFLP technology, that was of the same magnitude as in the present study.

Comparison of AFLP data with data from mitochondria is not necessarily straightforward, because it is confounded by issues related to ploidy (diploid + haploid versus haploid genomes) and gender (paternal + maternal inheritance versus maternal inheritance). Thus, these systems may differ considerably in their underlying rates and mechanisms of genetic change (Buonaccorsi et al., 2001), even in the absence of selection, with the mitochondrial genome potentially evolving faster on average. Nevertheless, such comparisons can be fruitful, and so we compared our data directly to those of Hu et al. (2002).

For the mitochondrial data of Hu et al. (2002), the heterozygosity (H) was reported to be in the range 0–0.5 for their sampled populations, which is much more variable and in general much larger than we found for the AFLP markers (0.05–0.22). Consequently, the reported  $F_{st}$  value (0.77) was much higher than ours (0.35–0.53), indicating much stronger population differentiation. Furthermore, the population mutation rate ( $4N\mu$ ,  $<0.01$ ) was estimated to be much less than ours (0.10) although the estimates of gene flow between the field populations ( $Nm$ ) were quite similar (0.36 versus 0.31).

These differences between the estimates from the mitochondrial and AFLP data are not unexpected (Silva and Russo, 2000), as genetic theory suggests that the small effective population size and limited dispersal of mitochondrial markers should enhance apparent differentiation among populations relative to nuclear markers. For example, (Salvato et al., 2002) also found that AFLP-derived diversity

was about two-thirds that of mitochondrial (*cox1* and *cox2* genes) nucleotide diversity in their study of European moths. Nevertheless, it is interesting to note that in spite of the differences in most of the population parameters the two estimates of lungworm migration were very similar. This accords with the results of Buonaccorsi et al. (2001) for blue marlin, who found that differences in  $F_{st}$  values estimated from four different types of genetic marker also disappeared when the values were converted to  $Nm$ . They attributed the divergent  $F_{st}$  values to the differential effects of genetic drift, mutation and migration on the various marker classes.

This result highlights the necessity of interpreting population genetic parameters within the context of the marker system used. Our estimates of the population genetic parameters are likely to be more reliable than those of Hu et al. (2002), because estimation error is reduced when information from multiple unlinked loci are combined (Pluzhnikov and Donnelly, 1996; Wakeley and Hey, 1997). Hu et al. (2002) found 15 variable nucleotide positions at what is effectively one locus (since there is little recombination in the mitochondrial genome, all of the genes are linked), while we had potentially 281 variable loci (although presumably some of these are not independent, because linkage is expected to increase with the number of bands). Importantly, AFLP data can capture a genome-wide picture of genetic variation, whereas sequencing of a single mitochondrial gene provides a much more restricted picture. Furthermore, parameters that measure population differentiation, such as  $F_{st}$ , can be very sensitive to the level of within-population variation, and so failure to detect such variation must lead to biased estimates. AFLP data should lead to unbiased sampling among loci, and thus avoid this problem.

In spite of these potential advantages, analysis of AFLP data can be more complicated than for other types of molecular markers. Since the AFLP peaks were scored as presence/absence, the analyses need to assume that these are dominant markers, with each peak representing a locus with two phenotypes. Therefore, it is not possible to identify heterozygotes and thus the departure from Hardy-Weinberg equilibrium, which complicates the quantification of allele frequency, which is the basis of most of the data analysis techniques. This does not necessarily lead to biased estimates (Krauss, 2000), but assessment of the level of inbreeding is not possible. It is now technically feasible to make quantitative measurements of peak intensity, which then allows co-dominant scoring, and this approach is to be preferred. Other potential problems, such as band homology for scoring the markers and the low allelic diversity (i.e. diallelic), only further complicate the general issues such as the underlying models on which the analyses are based (e.g. equilibrium populations of equal size, equal numbers and migration probabilities of males and females, infinite sites/alleles/islands).

This is the first time that the use of AFLP for determining of the genetic variability of individual worms from various

isolates of *D. viviparus* has been demonstrated. Our results showed that this technique is an effective method that could be used to reveal the intra- and inter-specific genetic variation among geographical isolates of this parasite. We found strong genetic structure, and the results obtained were also highly reproducible. The AFLP technique is somewhat related to the random amplification of polymorphic DNA (RAPD)-PCR techniques that have been quite extensively used to describe genetic variation in nematode parasites of livestock including lungworms (Gasser, 1999; Gasser and Newton, 2000). Although AFLP and RAPD-PCR techniques share several features (Yan et al., 1999), it seems likely that AFLP provides more consistent results due to much more stringent reaction conditions. Like the RAPD-PCR techniques, AFLP is based on minute amounts of DNA, and prior sequence knowledge is not required.

It is usually considered that one primer pair may be sufficient for 95% successful population assignment (Campbell et al., 2003), and this was certainly the case for the lungworms that we have studied. Overall, the most successful primer pairs in all of the analyses were B and C and the least successful were D and A, either of the former having sufficient information for most of the analyses. They can thus be recommended for future studies of lungworm PGS.

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