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Global patterns reveal strong population structure in *Haemonchus contortus*, a nematode parasite of domesticated ruminants

Karin Troell, Annie Engström, David A. Morrison, Jens G. Mattsson, Johan Höglund *

Department of Parasitology (SWEPAR), National Veterinary Institute and Swedish University of Agricultural Sciences, 751 89 Uppsala, Sweden

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Abstract

We have examined the global population genetic structure of *Haemonchus contortus*. The genetic variability was studied using both amplified fragment length polymorphism (AFLP) and *nad4* sequences of the mitochondrial genome. To examine the performance and information content of the two different marker systems, comparative assessment of population genetic diversity was undertaken in 19 isolates of *H. contortus*, a parasitic nematode of small ruminants. A total of 150 individual adult worms representing 14 countries from all inhabited continents were analysed. Altogether 1,429 informative AFLP markers were generated using four different primer combinations. Also, the genetic variation was high, which agrees with results from previous AFLP studies of nematode parasites of livestock. The genetic structure was high, indicating limited gene flow between the different isolates and populations from each continent mostly formed monophyletic groups in the phylogenetic analysis. However, for isolates representing Australia, Greece and one laboratory strain that originated from South Africa (WRS), there was no clear genetic relationship between the isolates and the distance between their geographical origins. Basically the same pattern was observed for the mitochondrial marker, although the phylogenetic analysis was less resolved than for AFLP. In contrast with previous findings on the population genetic structure of *H. contortus*, the calculation of population structure gave high values (Nst = 0.59). The strong structure was present also for the four Swedish isolates (Nst = 0.16) representing a small geographical area.

Keywords: Amplified fragment length polymorphism; mtDNA; *nad4*; Nematoda; Population genetics; Sheep

1. Introduction

Environmental and geographical barriers, population size and life histories may all shape the genetic structure of populations. As the geographical distribution of a species is usually greater than an individual's dispersal capability, populations are often more or less genetically differentiated through isolation by distance. Host–parasite systems are usually more complex than single-species studies, as the life histories of the hosts and parasites directly affect each other as part of the co-evolutionary process (Poulin et al., 2000). Comparisons of genetic diversity and structure between different types of parasites can provide significant insight into the extent to which the life-history characteristics of the hosts influence the geographical scale of population differentiation of the parasites.

The population genetics of parasites has been a comparatively neglected field (Grant, 1994) and it is only in the last decade that it has received any serious attention (reviewed by Anderson et al., 1998; Gasser and Chilton, 2001; Hu et al., 2004). This means that some quite basic predictions about the structure of parasite populations remain largely untested. For parasitic nematodes of domesticated animals, it has been shown that gene flow is determined by the life histories of both the parasite and its host (Anderson et al., 1998; Poulin, 1998), although it is still unclear which factors predominate in creating genetic patterns within the parasite populations. One important suggestion is that low

* Corresponding author. Fax: +46 18 30 91 62.
E-mail address: Johan.Hoglund@bvf.slu.se (J. Höglund).

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genetic structure is a general feature of any nematode parasite of land vertebrates (Fisher and Viney, 1998). For example, for nematode parasites of domestic ruminant hosts most of the genetic variation is often observed to be within farms, rather than between farms both in North America (Blouin et al., 1995) and Europe (Leignel and Humbert, 2001), creating large effective population sizes. This pattern has been attributed to large-scale movements of the domesticated hosts (Blouin et al., 1995), although this situation does not always apply (Leignel and Humbert, 2001). Thus, host vagility may be a major determinant for parasites with limited dispersal capacity in their free-living stages (Blouin et al., 1995; Nadler, 1995). However, for parasites that have external life stages (i.e. outside the host) the relationship between the non-host environment and the population structure may be another pertinent factor (Höglund et al., 2004). For example, an unfavourable climate can result in extreme bottlenecks and thereby affect the genetic structure of these parasites. In addition, in a study of populations representing a large geographical area it should be expected that vicariance can have a strong impact on population structure. Very few hypotheses concerning the relative importance of these various effects on the population genetics of parasites have yet been tested, an issue that we address here.

In this work, both amplified fragment length polymorphism (AFLP) data and nucleotide sequences of the nicotinamide dehydrogenase subunit 4 gene (*nad4*) of the mitochondria were used to study the population genetic structure (PGS) of *Haemonchus contortus* (Nematoda: Trichostrongyloidea), a gastrointestinal parasite of small ruminants worldwide. This parasite is responsible for sudden outbreaks of disease (haemonchosis) that may be associated with sudden mortalities, particularly in young animals in the subtropics and tropics (Urquhart et al., 1996). However, haemonchosis is also a major threat to productivity and welfare of livestock in temperate climates (Lindqvist et al., 2001; Eysker et al., 2005a,b). Like other trichostrongyloids, *H. contortus* has a free-living period when the egg and larval stages are exposed to the environment outside the host. Thus, non-host environmental conditions have a major impact on the external larval stages and low temperatures are a major limiting factor for the completion of the parasite's lifecycle (Gordon, 1948; Silverman and Campbell, 1959; Rossanigo and Gruner, 1995; Troell et al., 2005).

Although there has been an increase in the number of studies on parasitic nematode population genetics, there is still limited information available. AFLP has previously been shown to be efficient for use on individual parasitic nematodes (Otsen et al., 2001; Höglund et al., 2004; Nejsum et al., 2005), although sequencing of various mitochondrial genes has been more widely used to study the population genetics of nematode parasites of livestock (reviewed by Hu et al., 2004). In particular, the *nad4* and *cox1* genes have been studied for several parasitic nematodes for this purpose (reviewed by Höglund et al., 2006). Only *nad4* has previously been used for *H. contortus*, so we used this gene in order to compare our data with previosuly published work.

The main question addressed in our study is to what extent geographical distance has had an effect on the PGS of *H. contortus*. We used AFLP data and *nad4* sequences sampled for the same individuals and compared their variation and utility to resolve the population structure of *H. contortus*. We included isolates of *H. contortus* from 14 countries representing all inhabited continents, with replicate samples from three locations. We compared within- and among-population genetic diversity and inferred the phylogenetic relationships between the studied isolates in relation to their geographical origin. We also included two intensively studied laboratory isolates to determine if these two isolates, from Sweden and Kenya, can be considered as representative of natural populations from two climatically different origins, i.e. cold temperate and tropical.

2. Materials and methods

2.1. Parasite material

Adult worms were collected from 19 isolates (Table 1). An isolate consists of worms from one or more hosts on a single farm (a field isolate) or from artificially infected hosts of an earlier-isolated parasite strain (a laboratory isolate). Each isolate thus represents the local parasite population as sampled by the grazing activities of the hosts in the season prior to collection. An attempt was made to obtain multiple isolates for each continental area (Table 1). The worms were collected and preserved in several laboratories and abattoirs and sent fresh-frozen or frozen in alcohol to Department of Parasitology (SWEPAR), National Veterinary Institute and Swedish University of Agricultural Sciences. Upon arrival, worms were stored at −70 °C until DNA extraction was performed. Randomly chosen adult worms of both sexes were used in all cases.

2.2. DNA extraction

DNA was prepared from individual worms using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's recommendation. RNase A (1.7 mg/ml) was used to obtain RNA-free samples. The purified DNA was stored at 4 °C until use.

2.3. AFLP procedure

In total 150 worms were used for AFLP analysis (Table 1). The AFLP procedure was performed basically as described by Applied Biosystems (ABI) in their Plant Mapping Protocol and as described in Höglund et al. (2004). All reagents were supplied in the AFLP™ Plant Mapping Kit except the restriction enzymes and the T4 DNA ligase, which were from New England BioLabs.

Table 1
Sources of 150 worms from 19 different isolates used for amplified fragment length polymorphism analysis and *nad4* sequencing

Code	Continent	Geographic origin	Animal host	Origin	Number of worms
BZ	Unknown	Unknown, BZ-resistant	Sheep	Lab	8
WRS	Africa	South Africa, WRS	Sheep	Lab	8
Ken	Africa	Kenya	Sheep	Lab	8
Eti_II	Africa	Ethiopia, Farm II	Sheep	Field	8
EtiIII	Africa	Ethiopia, Farm III	Sheep	Field	8
Aus	Australia	Australia	Sheep	Lab	8
Java	S-E Asia	Indonesia, Java	Sheep	Field	8
Kam	S-E Asia	Cambodia	Goat	Field	8
Grek	Europe	Greece, Thessaloniki	Sheep	Field	8
Ty	Europe	Germany, Hanover	Sheep	Field	8
Sve	Europe	Sweden	Sheep	Lab	8
Berg	Europe	Sweden, Bergshamra	Sheep	Field	8
Vis	Europe	Sweden, Visby	Sheep	Field	10
Ors	Europe	Sweden, Örsundsbro	Sheep	Field	7
Gua	C. America	Guadeloupe	Goat	Lab	8
Kan	N. America	Canada	Sheep	Field	8
Argl	S. America	Argentina	Sheep	Lab	8
Argf	S. America	Argentina, Buenos Aires	Sheep	Field	5
Bra	S. America	Brazil	Sheep	Field	8

For all worms, the maximum allowed volume, 5.5 μl, of extracted DNA was used. The DNA from each individual worm was digested with 1 unit of *Mse*I and 5 units of *Eco*RI, and *Mse*I adaptor and *Eco*RI adaptor were ligated in the same reaction as the digestion. After the restriction–ligation reaction, the mixture was diluted to a total volume of 200 μl with $TE_{0.1}$ (20 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). From the restriction–ligation reaction, 4 μl of the diluted DNA was mixed with 1 μl of the ABI preselective primer pairs (5 μM) and 15 μl of ABI's Core Mix for preselective amplification. The amplified products were diluted to a final volume of 200 μl.

Four primer combinations were used for selective amplification (*Eco*RI-ACA + *Mse*I-CTC; *Eco*RI-ACA + *Mse*I-CTA; *Eco*RI-ACT + *Mse*I-CAG and *Eco*RI-ACA + *Mse*I-CAA). In each case, the *Eco*RI selective primer was labelled with fluorescent dye FAM. From the selective amplification products, 1.0 μl was mixed with 9.6 μl deionised formamide and 0.4 μl GeneScan-500 size standard (ABI). The mixture was denaturated for 3 min at 95 °C before being loaded onto the Genetic Analyser. The collected data were analysed using GeneScan Analysis software (version 3.7.1) and the tabular data for all peaks stronger than 200 U were summarised.

All peak information was transferred to Excel (Microsoft). The peaks were judged on size and coded as present or absent. Genetic diversity (Hj, measuring variability among haplotypes) and population genetic structure (Fst, measuring the proportion of within-population variation to the total) for the AFLP dataset were computed using the Hickory program of Holsinger and Lewis (Holsinger, K.E., Lewis, P.O. (2003). Hickory v0.8: A Package for Analysis of Population Genetic Data. Department of Ecology and Evolutionary Biology, University of Connecticut, USA), with 25,000 samples taken after a burn-in of 5,000 generations. To obtain better acceptance values for the Markov chain, the denomTheta parameter was set to 100 and denomF to 20; all other settings were the defaults for the program.

2.4. Nad4 amplification and sequencing

The same 150 individual worms used for AFLP were sequenced for *nad4*. The 3′ end was amplified from genomic DNA by PCR, which generated fragments 607–806 bp long depending on the primers used. The amplification was performed in 50 μl reaction volumes containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 μM of each primer, 0.2 μM deoxyribonucleotide triphosphate and 1 U Ampli*Taq* DNA polymerase. Amplification was performed on a PTC-200 (MJ Research) using the following program: 5 min at 95 °C, 30× (30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C), 5 min at 72 °C. Different primers were used for different worms, as none of the primer pairs produced amplicons from all worms. As forward primer, OP439, 5′-GGATTTGGTCAGCA AATTGAA, or OP494, 5′-TCATTTGTGGTTACCTAA AGC were used, and as reverse primer OP440, 5′-GC CTGCAAATGAATTAACA or OP495, 5′-CCTGCA AATGAATTAACAATC. Aliquots of PCR products were analysed by electrophoresis on an agarose gel containing ethidium bromide (0.5 μg/ml). The remaining products were purified using Montage PCR_{96} Cleanup Kit (Millipore) and eluted in H_2O, prior to sequencing.

The amplicons were sequenced with Big Dye chemistry (ABI) according to the manufacturer's description. The same primers were used as for the PCR. All sequencing products were purified with Montage SEQ_{96} Cleanup Kit (Millipore). The sequences were determined with an ABI Prism 3100 automatic sequencer. The sequences were edited and analysed with Vector NTI suite 9, programs ContigExpress and AlignX (InforMax). The fragments were

trimmed to equal length for all individuals. After alignment and trimming, the final sequence length used was 411 bp. The sequences were compared for positions 618–1029, based on available database sequences of *H. contortus* (Accession No. AF070785) and *Cooperia oncophora* (Accession No. AY265417).

The calculations of nucleotide diversity (Pi, the nucleotide analogue of Hj) and population genetic structure (Nst, the nucleotide analogue of Fst) were computed using the DNAsp ver. 4.10 (Rozas et al., 2003). All standard settings in the program were used.

2.5. Phylogenetic analyses and structural hypotheses

Phylogenetic trees were constructed separately for the *nad4* and AFLP data via a range of techniques, using PAUP* ver. 4.0b10 (Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), version 4.0b10. Sinauer Associates, Sunderland, Massachussetts). For the AFLP data, pairwise genetic distances between the individual nematodes were calculated following Nei and Li (1979), while for the *nad4* data HKY + invariant + gamma distances were calculated based on the parameter estimates provided by the ModelTest ver. 3.6 program (Posada and Crandall, 1998). Phylogenetic trees were then constructed separately from these distances via both neighbour-joining (with random initial seed) and minimum-evolution (with neighbour-joining starting tree and TBR, tree bisection–reconnection, branch swapping). For both data sets, phylogenetic trees were also constructed via unweighted parsimony, with all of the program default values (e.g. stepwise addition starting tree and TBR branch swapping). The robustness of the branches on the minimum-evolution trees was assessed via the bootstrap method, with 1,000 iterations and TBR branch swapping for the AFLP data, and with 200 iterations and SPR, subtree pruning regrafting, branch swapping for the *nad4* data (as lack of phylogenetic resolution meant that for some pseudoreplicates there were literally hundreds of thousands of equally optimal trees).

Of the sequences obtained for *nad4*, 263 bp overlapped with the previously published sequences of *nad4* of 50 individual *H. contortus* from the USA (Blouin et al., 1995, 1998). All 200 overlapping sequences were thus aligned and a minimum-evolution tree was constructed as above.

The genetic differentiation among the continental areas was formally tested using analysis of molecular variance (AMOVA) for the *nad4* data (Excoffier et al., 1992) and Mantel tests for the AFLP data (Miller, 1999), based on the genetic distances described above. Three hierarchical levels were tested for the *nad4* data: between continents, between populations within continents and between individuals within populations, each with 10,000 permutations, using the Arlequin ver. 2.0 program. Two hierarchical levels were tested for the AFLP data, between populations and between individuals within continents, with 10,000 permutations, using the Mantel-Struct ver. 1.0 program. Four continental areas were recognised a priori: Europe, Africa, South-east Asia plus Australia and the Americas. The BZ and WRS isolates were excluded from the tests, as explained below.

In order to evaluate possible a posteriori grouping patterns of genetic structure among the isolates, we used the Structure ver. 2.1 program (Pritchard et al., 2000) on the AFLP data set. All 19 isolates were included and all potential group numbers from 1 to 10 were assessed, each with at least two replicate chains based on the NoAdmixture model and 20,000 samples taken after a burn-in of 10,000 generations; all other settings were the default for the program.

The BZ and WRS isolates have both been maintained in the laboratory for many years, and thus may no longer be representative samples. Therefore, each nematode in these two samples was tested with a maximum-likelihood allocation technique based on the AFLP data, using the AFLPOP ver 1.1 program (Duchesne and Bernatchez, 2002) with 2,000 simulations, in order to assess the probability that each of the other 17 isolates has acted as the source of these nematodes. Note that we do not imply by these tests that our other isolates have necessarily sampled the true origin of these laboratory isolates but we are merely using the tests as a heuristic guide to the possible continental area of origin. Indeed, all of the allocation probabilities were small.

3. Results

3.1. AFLP and nad4 sequences

A total of 1,429 informative AFLP markers were generated using four primer combinations (Table 2). Only two peaks were present in 150 worms. Between 294 and 723 peaks were amplified for each isolate, with an average of 105 peaks per primer combination. No notable differences in the number of amplified peaks were observed between the different primers (data not shown). For individual worms the mean number of peaks for all primer combinations was 175 and each worm had a unique haplotype. The number of unique (or private) peaks varied greatly between the different populations (from 1 to 92; Table 2). In most cases the unique peaks were present in only one to three worms per population.

Variable sites were detected at 103 (25%) of the 411 positions analysed for *nad4* sequences. All sequences had the same length, so no alignment gaps were needed. In total, 94 haplotypes were found among 150 worms, with an average of five haplotypes per population. As expected for a nematode mitochondrial gene, the base composition was very AT-rich (78.2%). Translation of the sequences to amino acids (aa) revealed 21 out of 137 aa were variable.

3.2. Phylogeny

The phylogenetic trees differed both between the two data sets (AFLP vs *nad4*) and between the three tree-con-

Table 2
Genetic diversity (Hj and Pi), and variability detected for amplified fragment length polymorphism (AFLP) and *nad4* data of the isolates studied

Isolate	AFLP	*Nad4*	Number of peaks in AFLP		
	Hj	Pi	Total	Mono.	Unique
BZ	0.09	0.027	465	31	39
WRS	0.08	0.017	335	62	4
Ken	0.11	0.015	723	21	92
Eti_II	0.10	0.018	538	17	66
EtiIII	0.10	0.017	555	33	43
Java	0.08	0.013	294	83	7
Kam	0.09	0.015	413	48	11
Aus	0.09	0.009	357	41	10
Grek	0.08	0.014	377	54	6
Ty	0.08	0.016	351	55	1
Sve	0.09	0.013	380	62	4
Berg	0.08	0.034	355	50	1
Vis	0.08	0.032	398	46	9
Ors	0.09	0.018	386	0	7
Kan	0.08	0.017	341	45	2
Argl	0.09	0.015	430	41	9
Argf	0.09	0.015	383	0	8
Bra	0.10	0.009	470	30	20
Gua	0.10	0.001	459	40	20
Mean	0.11	0.037	422	40	19

struction methods (neighbour-joining, minimum-evolution, parsimony). For convenience, the minimum-evolution trees have been chosen for presentation here (Figs. 1, 2), with the differences for the other methods highlighted separately (Table 4). The trees are unrooted since there are no outgroup sequences but they have been orientated in the figures to highlight those isolates that form potentially monophyletic groups. The basic difference between the two data sets is that the AFLP data yielded much stronger phylogenetic patterns, so these data will be discussed first.

The unrooted minimum-evolution tree based on the AFLP data (Fig. 1) showed strong geographical structuring at several spatial scales. Each worm formed a unique haplotype and the terminal branches of the tree were generally much longer than the internal branches. For the minimum-evolution analysis (Fig. 1), all individuals from the same isolate were clustered (i.e. they potentially form a monophyletic group, depending on where the root is placed), although there were occasional minor exceptions for the other two analyses (Table 4). This general pattern thus seems to be robust.

Many of the isolates from the same continent also formed clusters in the minimum-evolution analysis (Fig. 1), notably those from northern Europe, south-east Asia and Africa and these patterns also appeared in the other two analyses (Table 4). This pattern thus seems to be robust. However, the American isolates formed a cluster only in the parsimony analysis, with the Argentinian isolates being separated in the other two analyses, so that this pattern is not especially robust. Furthermore, in all of our analyses the Greek (i.e. southern Europe) and Australian isolates clustered together and the WRS isolate, which originated from South Africa, clustered with some subsets of isolates from northern Europe (Fig. 1). The BZ isolate, of undetermined origin, clustered either with the African isolates or as a basal relationship to the other isolates, depending on where the tree was rooted. In all cases, the estimates of the relationships between the continental areas were not robust, as they differed strongly between the three analyses (Table 4).

The mitochondrial marker showed less phylogenetic resolution than did the AFLP dataset (i.e. resolved the relationships among fewer groups), although some of the phylogenetic analyses revealed patterns that were consistent with those revealed by the AFLP data. Firstly, some of the haplotypes were shared by several worms, usually from the same isolate but, if not, then at least from the same continental area (Fig. 2). The only exception to this was the haplotype shared by Arg367 (Argentina) and Ors337 plus Ors338 (Sweden). Second, only a few of the isolates formed potentially monophyletic groups (e.g. Gua in the minimum-evolution analysis; Fig. 2). Third, the grouping of continental areas was less clear, with only the south-east Asian samples forming a cluster (Fig. 2), usually with low bootstrap support. The clustering of the African isolates was disrupted by some of the BZ worms; and the Americas plus most of the northern European samples formed a single cluster (Fig. 2). The relationship between the Greek and Australian samples was disrupted by some of the samples from Sweden plus a BZ worm.

The single tree constructed, based on the overlap of 263 bp between our nad4 sequences and those previously published in GenBank (Blouin et al., 1995, 1998), was consistent with the minimum-evolution tree of Fig. 2. Several of the database sequences had identical haplotypes to some of our worms. Of the 50 sequences representing *H. contortus* from the USA, 49 clustered with isolates from the Americas sequenced for this study (tree not shown), while the remaining sequence was identical to some of the Gua sequences.

3.3. Population genetic structure

The estimates of genetic diversity for the AFLP data were very consistent between the different isolates (Hj ranged from 0.08 to 0.11; Table 2) and also across primer combinations within each isolate (data not shown). On the other hand, the calculations for *nad4* showed very variable nucleotide diversity (Pi) among isolates, ranging from 0.001 to 0.034 (Table 2). There appeared to be no pattern to this latter variation, suggesting that this mitochondrial marker may be less suitable than the AFLP data for studying the population genetics of *H. contortus*.

As expected from the phylogenetic results, strong population structure was found for *H. contortus* in our samples when all isolates in the study where included (Table 3). The difference between the values for the two markers was confounded by expected differences in ploidy (assuming haploid for *nad4* and diploid for AFLP) and inheritance

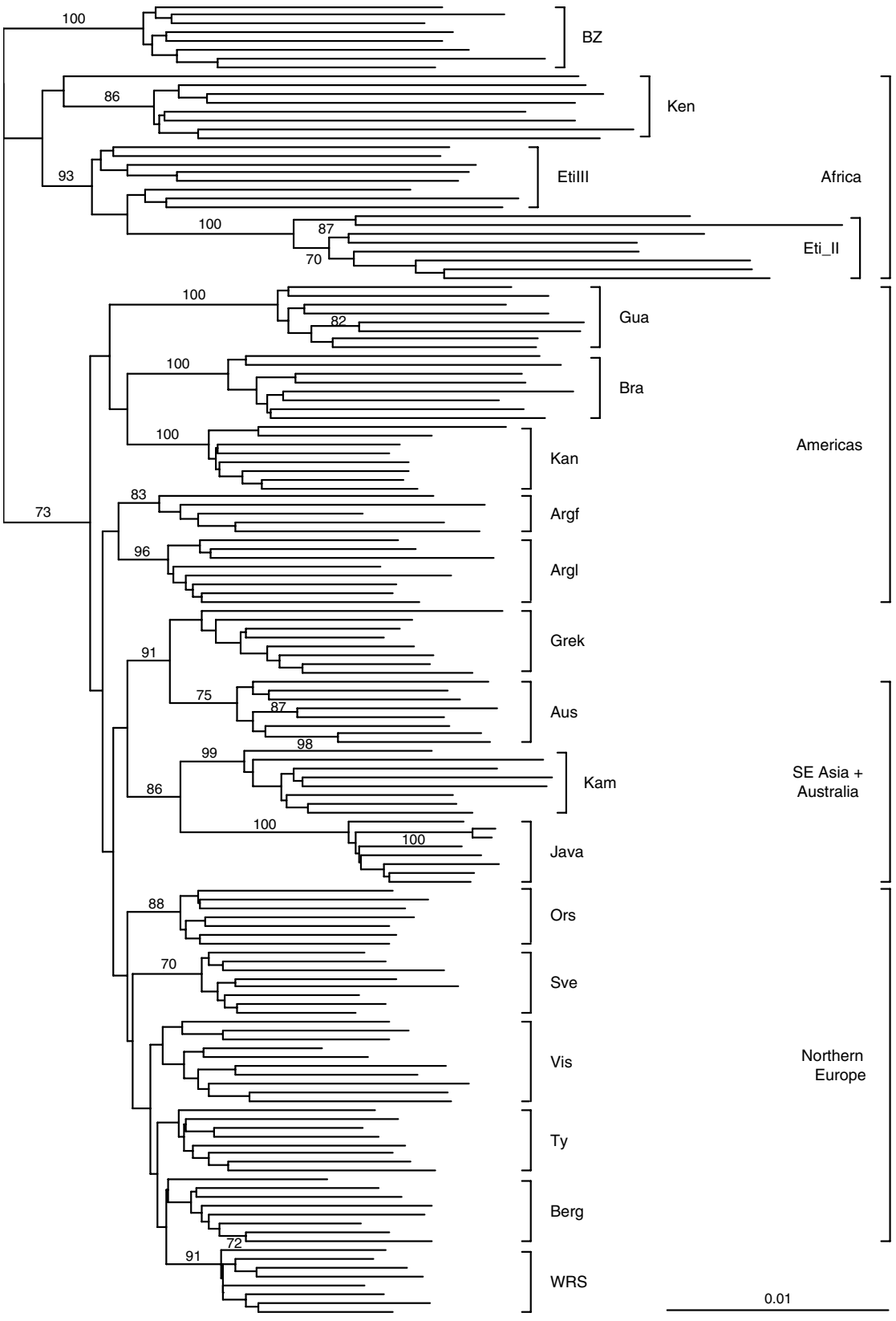

Fig. 1. Unrooted minimum-evolution tree of 150 *Haemonchus contortus* isolates based on amplified fragment length polymorphism presence/absence polymorphism. Each terminal branch represents one individual worm (unlabelled). Also shown are the isolate codes (see Table 1) and the geographical source of the isolates, along with those bootstrap values >70%. The scale is the inferred number of substitutions.

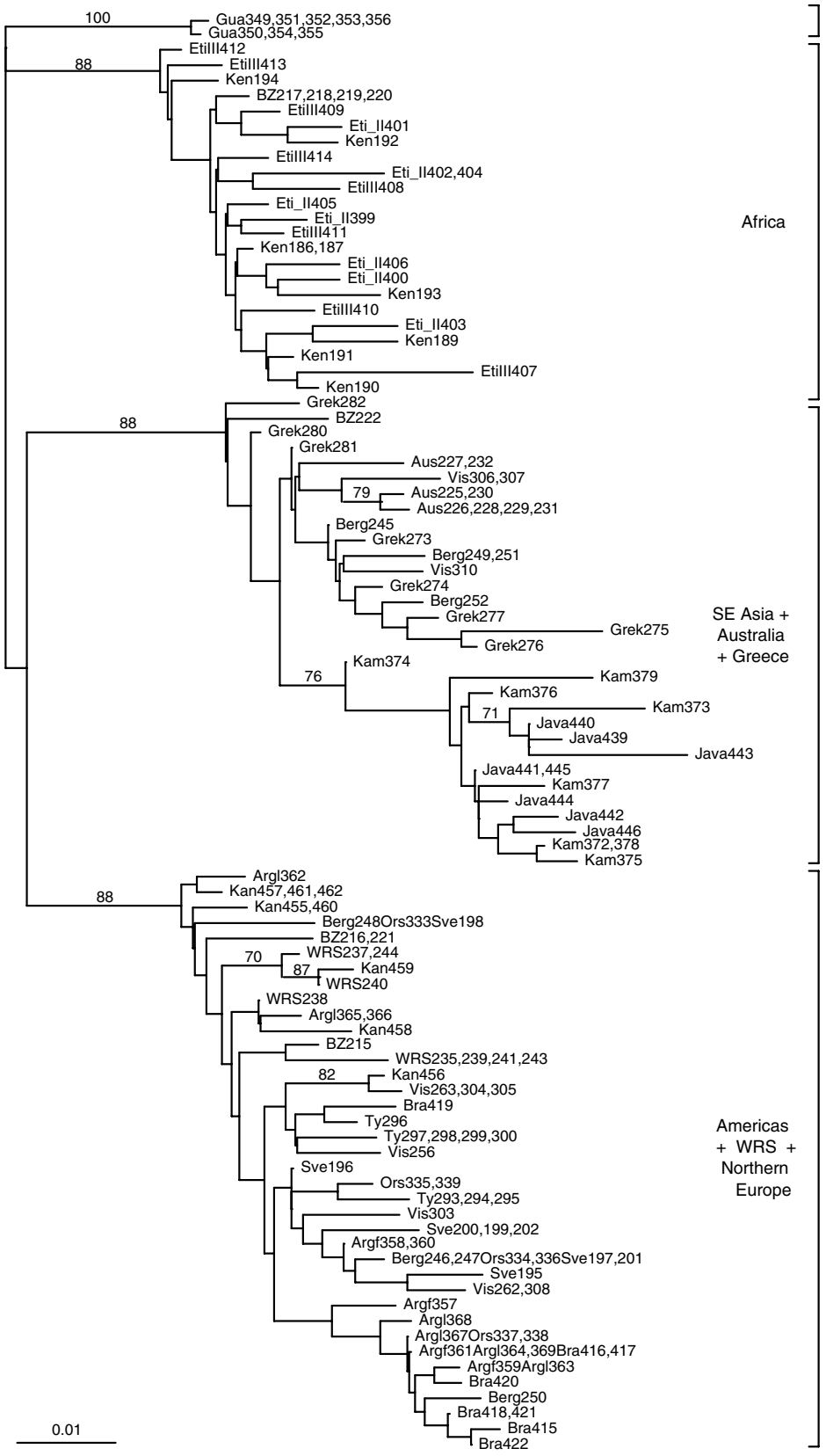

Fig. 2. Unrooted minimum-evolution tree of 150 *Haemonchus contortus* isolates based on *nad4* sequence polymorphism. Each terminal branch represents one haplotype, with the individual worms within each haplotype labelled according to the isolate codes in Table 1. Also shown is the geographical source of the isolates, along with those bootstrap values >70%. The scale is the inferred number of substitutions.

Table 3
Differences observed between the trees from the different phylogenetic analyses performed for the amplified fragment length polymorphism dataset

Isolates	Neighbor-joining	Minimum-evolution	Parsimony
Both Ethiopia	√	√	√
Kenya	√	√	All but Ken192
Ethiopia + Kenya	√	√	All but Ken192
Java	√	√	√
Cambodia	√	√	√
Java + Cambodia	√	√	√
Sister to	Americas	Greece and Australia	Basal
Australia	√	√	√
Greece	All but Grek275	√	√
Australia + Greece	√	√	√
Sister to	Sweden lab (Sve)	S-E Asia	Basal
Argentina lab	√	√	√
Argentina field	√	√	√
Both Argentina	√	√	—
Guadeloupe	√	√	√
Brazil	√	√	√
Canada	√	√	√
Gua + Bra + Can	√	√	√
All Americas	—	—	√
Sweden lab (Sve)	√	√	√
Sweden (Berg)	√	√	—
Sweden (Vis)	√	√	—
Sweden (Ors)	√	√	√
All Sweden	All but Sweden lab	—	—
Germany	√	√	√
Northern Europe	—	+WRS	–

A √ indicates that the isolate or group of isolates forms a potentially monophyletic group in that analysis.

Table 4
Genetic structure (Fst and Nst) for amplified fragment length polymorphism (AFLP) and *nad4* data of the isolates studied

Isolates	Number of isolates	AFLP Fst	*Nad4* Nst
All isolates	19	0.18	0.57
Field isolates	12	0.21	0.54
Lab isolates	7	0.24	0.63
Europe	6	0.14	0.35
S-E Asia + Australia	3	0.31	0.54
Africa (excl. WRS)	3	0.14	0.01
Americas	5	0.21	0.58
Sweden	4	0.13	0.16

(assuming maternal for *nad4* and biparental for AFLP) but in spite of this the AFLP data seemed to reflect stronger structuring, which is also consistent with the phylogenetic results.

The same patterns were observed when the laboratory and field isolates were analysed separately (Table 3), suggesting that the results from these two types of isolates are directly comparable (which is an important assumption of our sampling strategy). However, the phylogenetic analyses made it clear that the BZ and WRS isolates, both of which have been maintained in the laboratory for several decades, were distinct from the other laboratory isolates (which have been maintained for much shorter periods), and so they were excluded from the statistical tests. The maximum-likelihood allocation procedure for the AFLP data indicated that all eight of the WRS nematodes had most similarity to the northern European nematodes rather than to the African nematodes, as expected from the phylogenetic analysis. However, this procedure also indicated that four of the BZ nematodes had most similarity to the northern European nematodes while the other four had most similarity to the African nematodes, suggesting that this isolate may be of mixed stock. This result is consistent with the phylogenetic analysis of the *nad4* data set but not the AFLP data, so that the phylogenetic analysis of the AFLP data seems to have missed this particular pattern.

For the AFLP data, genetic differentiation between continental areas was significant at $P < 0.001$ for all pairwise comparisons, as expected from the phylogenetic results. For the *nad4* data, 38.0% of the genetic variation was attributed to differences between individuals within populations, 27.1% to differences between populations within continents and 34.8% to differences between continental areas. All three hierarchical levels were significant at $P < 0.001$, in spite of the poorer differentiation of the *nad4* data compared with the AFLP data. Thus, our results confirm that within continental areas there is less genetic differentiation between populations than there is within populations, so that the individuals form relatively large gene pools. However, there is also a great deal of genetic differentiation between continental areas, implying that at a global scale there are strong barriers to gene flow.

The a posteriori test of genetic structure consistently identified three main clusters of isolates: Africa (sometimes with BZ); the Americas; and Europe + south-east Asia + Australia + WRS. However, the optimal number of groups was four, with a different isolate forming the fourth group in different runs of the markov chain (e.g. BZ, Java). The Argf isolate was consistently given a high probability for both the second and third clusters, suggesting that it might be a mixed isolate.

Most the continental areas maintained the same levels of genetic structuring as did the overall data sets (Table 3). The main exceptions occurred for the African populations, which showed almost no genetic structuring within the *nad4* data and the south-east Asia + Australia group, which showed much stronger structuring for the AFLP data (due to differences between Australia and south-east Asia). Even geographically close populations within Sweden showed relatively strong population structure (Table 3), although the Fst and Nst values were considerably reduced compared with those for the full data sets.

4. Discussion

Little is known about the population genetic structure of parasites of domesticated ruminants, although the host is recognised to play an essential role in determining the genetic variability and gene flow between parasite populations (McCoy et al., 2003). A number of nematode species form large gene pools within continental areas and this has been attributed to widespread transportation of the hosts and thus mixing of the parasite gene pool. We tested this hypothesis for a domesticated host–parasite system by quantifying the structure of genetic variation among 19 isolates of *H. contortus*, representing populations from domesticated ruminants throughout the distributional range of the parasite, based on sampling of AFLP markers and *nad4* sequences from the same individuals. We found evidence of considerable population structure, at several spatial scales.

4.1. Comparison of the two genetic markers

The two markers produced patterns of genetic differentiation that were consistent with each other but the AFLP markers showed stronger patterns of genetic structure. A simplistic analysis suggests that the AFLP markers might show Fst values that are one-quarter those of the mitochondrial sequences (because of differences in ploidy and inheritance; Page and Holmes, 1998), but the AFLP values that we observed were generally one-third as large, suggesting that the AFLP loci detected substantially more genetic differentiation among the sampled populations than did the *nad4* locus. Since both data sets were from the same individuals, the differences in genetic structure are unlikely to reflect different population processes, and presumably reflect the better sampling of the genome by the AFLP technique.

This pattern is confirmed by the fact that the AFLP data detected a broader array of haplotypes than did the *nad4* data (150 and 94, respectively), had much more consistent estimates of genetic diversity among the isolates, and had much clearer patterns of phylogenetic relationship. A high degree of variability in AFLP data has previously been shown not only for *H. contortus* (Otsen et al., 2001) but also for the bovine lungworm *Dictyocaulus viviparus* (Höglund et al., 2004) and for the pig roundworm *Ascaris suum* (Nejsum et al., 2005). Although *nad4* sequences are commonly used for studies of nematode populations (reviewed by Höglund et al., 2006), and the high degree of diversity that we observed is in agreement with earlier published data on trichostrongyloid nematodes (Blouin et al., 1995; Fisher and Viney, 1998; Braisher et al., 2004), there are clearly limitations to the population patterns that this locus can detect and AFLP loci may be of more practical utility.

Adult whole worm preparations of both sexes were used as the source of DNA for this study. It is thus possible that there may have been contamination by sperm and/or egg DNA in fertilised eggs (Anderson et al., 2003). However, this will compromise neither the results nor our conclusions. For the mitochondrial marker (*nad4*), presence of male DNA will not have an effect, as the mitochondrial genetic makeup of the offspring is predominantly maternally inherited (Hu et al., 2004). In contrast, foreign DNA may be detected with AFLP. However, even in the most extreme case this would result in a more complex banding pattern, since more genotypes will be present in individual samples, so that more genotypes will be detected than if we were dealing with pure DNA from a single individual. If so, this would have resulted in an underestimation of the level of inbreeding, which in itself would have generated an even stronger genetic structure. In this case, our estimates of genetic structure will be underestimates.

4.2. Assessment of laboratory isolates

We sampled both field and laboratory isolates, including in the latter five recently derived strains and two long-standing strains (BZ and WRS). Our analyses indicate that the relatively recent isolates can be considered to be representative of natural populations, as they showed no consistent differences from their closely related field isolates in any of our analyses. This indicates that the overall genetic variation is still representative of natural populations and that it has not dramatically changed following short-term laboratory maintenance.

Furthermore, we have confirmed that two intensively studied laboratory isolates, from Sweden (Sve) and Kenya (Ken), appear to be characteristic of their different climatic origins. It has been shown that *Haemonchus* of both sheep and goats in Sweden are the same genetic species as *H. contortus* in the tropics (Troell et al., 2003). However, no major differences in survival were found between these isolates when larvae were subjected to long-term cold-stress or in a range of other phenotypic characters studied (Troell et al., 2005, 2006). We therefore considered whether these laboratory strains are still representative of the diversity encountered in natural populations, after having adapted to a stable environment in the laboratory, since routine passages of parasites in the laboratory are likely to decrease the genetic polymorphism in an isolate (Gasnier and Cabaret, 1998). However, the genetic diversity does not seem to have decreased in any of the laboratory isolates that we included. This is consistent with the results of both Otsen et al. (2001) and Höglund et al. (2004).

On the other hand, the two longest-standing isolates in our study, BZ and WRS, showed unusual genetic patterns. The WRS strain was originally isolated from South Africa (van Wyk et al., 1987) and yet both our AFLP and *nad4* data clearly showed that its genetic relationships are with the northern European isolates rather than the tropical African isolates. This could be due either to accidental sampling of recently arrived nematode immigrants when the strain was first isolated or fortuitous genetic changes during the years of passages through the laboratory hosts. These alternative hypotheses could be tested by comparing

the many different samples of this strain that are maintained in laboratories around the world. The BZ strain is of undetermined origin; and our *nad4* data and the allocation test of the AFLP data both indicate that this strain is of mixed stock. This hypothesis has important consequences for the use of this strain in laboratory experiments and it thus needs to be investigated further.

4.3. Within-continent patterns of field isolates

The remaining 17 isolates showed strong genetic structuring at several spatial scales. Notably, much of the genetic variation (cf. 35% for the *nad4* data) is due to differentiation among individuals within the local population, while less variation (cf. 25%) is due to differentiation among populations within continental areas. This means that individuals can form large gene pools, as has been reported at this spatial scale for this and other nematode species.

There are few previous AFLP data with which ours can be compared but Höglund et al. (2006) provide a summary of the available data from *nad4* sequences at this spatial scale, with Nst values recalculated from the original data in a consistent manner. Three scenarios are evident from these data: (i) nematodes of domesticated hosts have small Nst values (0.001–0.14, indicating very little differentiation between populations and presumably strong gene flow); (ii) nematodes of non-domesticated hosts but with expanding populations (cf. Morrison and Höglund, 2005) also have small Nst values (−0.01 to 0.05); and (iii) nematodes of other non-domesticated hosts have larger Nst values (0.32–0.85, indicating large differentiation between populations and presumably restricted gene flow). These patterns seem to be independent of the host species and are also consistent with available data from other mitochondrial genes (*atp6*, *cox3*, *nad5*), so that they provide a benchmark for comparison.

The previous *nad4* data for *H. contortus*, from five populations in the USA, yielded Nst = 0.14 (Höglund et al., 2006), which compares with our value of Nst = 0.16 for the four Swedish isolates. However, our values for most other geographical areas are much larger than this (Table 3), indicating that even at the within-continent spatial scale we have detected more genetic structure than has previously been reported for most nematodes of domesticated hosts. This suggests that many of the current assumptions about the nature of population structure in such parasites, especially those involving large gene pools, may be overly simplistic. A more realistic hypothesis seems to be that there may be few barriers to gene flow at local scales but at larger scales such barriers may be important.

4.4. Between-continent patterns of field isolates

Our most important result is that for this nematode species there is considerable genetic differentiation between continental areas, implying that at a global scale there are strong barriers to gene flow. This geographic pattern was revealed in both the *nad4* polymorphism (c. 40% of the genetic differentiation) and in both a priori and a posteriori testing of the AFLP polymorphism. An obvious explanation for this genetic subdivision is the poor dispersal ability of the parasite across strong geographic barriers such as different continents, along with greatly restricted opportunities for host movement between these geographical locations. This suggestion agrees with the data of Leignel and Humbert (2001), who found genetic subdivision between *Teladorsagia* (Nematoda:Trichostrongyloidea) populations in France and Morocco, where there is a strong oceanic barrier to host movement.

This question of large-scale geographical variation has not been directly addressed for animal-parasitic nematodes before. In particular, earlier comparative studies of parasites of domestic livestock have been restricted to isolates collected from relatively small spatial scales, within countries (Blouin et al., 1995, 1998; Braisher et al., 2004), from different hosts (Brant and Orti, 2003) or following selection with anthelmintics (Otsen et al., 2001). Global patterns of genetic relationships have been explored for several human parasites but even then most of the comparative population data relate to a limited number of geographical areas (usually areas of endemic disease).

The continental areas sampled were consistently clustered into four groups: Africa; the Americas; south-east Asia plus Australia (and Greece); and Northern Europe. Unfortunately, there is no clear pattern of genetic relationship between these continental areas, for either the AFLP or *nad4* data, although there is some indication that the latter two groups may be the most closely related. A deeper investigation into patterns is likely to reveal the patterns of introduction of the nematodes into the different continents along with their domesticated hosts.

Of particular interest is the relative lack of apparent genetic differentiation between North, Central and South America in the available samples (ours as well as the database *nad4* sequences), especially compared with areas of equivalent size such as Europe and Africa. Perhaps the available samples do not reflect the true amount of diversity, as there may be relatively isolated pockets of genetically distinct *H. contortus*. It seems likely, however, that climatic patterns do not have an important effect on gene flow in *H. contortus* in the Americas, given the broad latitudinal spread from which samples have already been taken.

The most unexpected pattern was the close genetic relationship between the isolate from Greece and the single isolate that we were able to obtain from the Australian continent. At first sight it might be hypothesised that this represents previous transport of nematodes from Europe to Australia, presumably in an infected host (or hosts) when sheep were first introduced by Europeans. However, this suggestion is contradicted by the facts that (i) the Australian isolate also shows (weaker) genetic ties to the south-east Asian isolates and (ii) the Greek isolate shows little

genetic similarity to any of the other European samples. The simplest scenario then becomes that the nematodes were introduced into Greece from Australia, an intriguing suggestion that bears further investigation.

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