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Host Movement and the Genetic Structure of Populations of Parasitic Nematodes

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ABSTRACT

Mitochondrial DNA (mtDNA) sequence data were used to compare the population genetic structures of five species of parasitic nematodes from three different hosts: *Ostertagia ostertagi* and *Haemonchus placei* from cattle, *H. contortus* and *Teladorsagia circumcincta* from sheep, and *Mazamastrongylus odocoilei* from white-tailed deer. The parasites of sheep and cattle showed a pattern consistent with high gene flow among populations. The parasite of deer showed a pattern of substantial population subdivision and isolation by distance. It appears that host movement is an important determinant of population genetic structure in these nematodes. High gene flow in the parasites of livestock also indicates great opportunity for the spread of rare alleles that confer resistance to anthelmintic drugs. All species, including the parasite of deer, had unusually high within-population diversities (averages of 0.019–0.027 substitutions per site between pairs of individuals from the same population). Large effective population sizes (Ne), perhaps in combination with rapid mtDNA evolution, appear to be the most likely explanation for these high within-population diversities.

THE population genetic structure (PGS) of a parasite species has important implications for evolutionary processes such as host-race formation, adaptation to host defenses, and the evolution of drug resistance. Yet we still know surprisingly little about the PGS of most species of parasitic helminths, much less how PGS differs between helminths having different hosts or life cycles (Nadler 1990, 1995a; Thompson and Lymbery 1990; Blouin *et al.* 1992). The PGS of a set of populations (here defined as the total genetic diversity in a set of populations and its distribution into components within and among populations) is determined primarily by the effective sizes of the populations and the rates of gene flow among them. For parasitic helminths, effective sizes and rates of gene flow will be determined by aspects of the life history of both the parasite and the host. Which life history traits are most important for determining population genetic structure in different helminth species is still unknown.

Only recently have researchers begun using molecular techniques to partition genetic diversity among individuals of helminth species into components within and among populations and to estimate rates of gene flow among populations (Lydeard *et al.* 1989; Mulvey *et al.* 1991; Lymbery *et al.* 1990; Blouin *et al.* 1992; Anderson *et al.* 1993, 1995; Nascetti *et al.* 1993; Hugall *et al.* 1994; Nadler 1995b). Blouin *et al.* (1992) used mtDNA restriction site data to describe the population genetic structure of the trichostrongylid nematode *Ostertagia ostertagi*, a parasite of cattle in the United States. We found two unexpected results. First, 98% of the total genetic diversity in this species is partitioned within populations, a result consistent with very high gene flow among populations. Second, the average number of nucleotide substitutions per site among individuals from the same population is five to 10 times higher than typical estimates reported for species in other taxa. Trichostrongylid nematodes are parasites of ruminants worldwide. All have simple, one-host life cycles in which eggs pass in feces, hatch on pasture, and develop directly into infective larvae that are swallowed by grazing hosts. Individuals in the free-living stage have virtually no dispersal abilities, so gene flow in these species should be determined primarily by host movement. We hypothesized that the low differentiation among populations of *O. ostertagi* is caused by a tremendous rate of gene flow that results from the high rate at which livestock are shipped around the United States. We also discussed three hypotheses to explain the unusually high within-population diversity.

Here we compare the PGS of *O. ostertagi* with the PGSs of four other trichostrongylid parasites: one that infects cattle, two that infect domestic sheep, and a fifth that infects white-tailed deer (*Odocoileus virginianus*). The first goal of this project is to test the effect of host mobility on the distribution of genetic variance within and among populations by comparing the PGS of species that infect domestic hosts (cattle and sheep) with that of a species that infects a wild host (deer). Deer have not been moved nearly as much as domestic ruminants (Ellsworth *et al.* 1994), so the *a priori* prediction is that the parasite of deer will show more population

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TABLE 1

Sampling locales

Host	Parasite species	Sampling Locales		Contact person
		Abbreviation	Site	
Sheep	*Haemonchus contortus*	OH	Columbus, OH	Rupert Herd
		TN	Knoxville, TN	Craig Reinemeyer
		VA	Montgomery Co., VA	Allen Dahl
		WY	Pine Bluff, WY	Jeff Laursen
	Teladorsagi circumcincta	WA	Pullman, WA	Bill Foreyt
		TN	Knoxville, TN	Craig Reinemeyer
		VA	Montgomery Co., VA	Allen Dahl
		WY	Pine Bluff, WY	Jeff Laursen
Cattle	*Haemonchus placei*	FL	Alachua Co., FL	Charles Courtney
		GA	Experiment Station, GA	Rick Ciordia
		LA	Baton Rouge, LA	Jimmy Williams
		NC	Johnston Co., NC	David Edmiston
Deer	*Mazamastrongylus odocoilei*	SC1	Carolina Sandhills Wildlife Management Area, McBee, SC	Dale Guthrie
		SC2	Savanah River Ecology Laboratory, Aiken, SC	Margaret Mulvey
		FL1	Tide Swamp Wildlife Management Area, Perry, FL	Sammy Patrick
		FL2	Deseret Ranch, Melbourne, FL	Ray Kaplan
		AR	Holla Bend Wildlife Management Area, Russellville, AR	Martin Perry

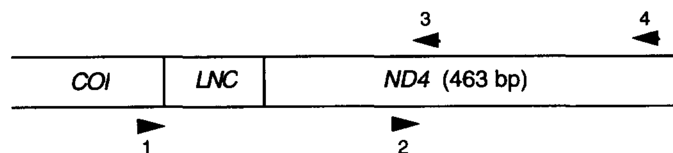
Abomasa from sheep and cattle were from one herd in each locale.

differentiation than the parasites of cattle and sheep. The second goal of this project is to determine whether the unusually high within-population diversity seen in *O. ostertagi* is also seen in all other trichostrongylids, is seen in just those that parasitize domestic hosts, or is unique to *O. ostertagi.*

Finally, there is an important practical reason for asking whether the other trichostrongylids that infect domestic ruminants have population genetic structure's similar to that of *O. ostertagi*. Trichostrongylids are serious economic pests of cattle, sheep and goats, and the rapid and widespread evolution of resistance to anthelmintic drugs in these parasites has become a major problem (Craig 1993; Shoop 1993; Waller 1993; Bjorn 1994). The population genetic structure we inferred for *O. ostertagi* will enhance the evolution of anthelmintic resistance (see discussion), so it is important for those planning strategies to control the evolution of resistance to know whether the other economically important trichostrongylids have a PGS similar to that of *O. ostertagi.*

MATERIALS AND METHODS

Sampling and sequencing overview: We sampled nine to 11 worms from each of four or five populations per species (Table 1). We collected worms from abomasa of deer killed



Species	Primer 1	Primer 2	Primer 3	Primer 4
H. contortus	CGACAAACCACCTTGATACTTTATAT	ATTCAATATTATACTGTTAGTG	TAATTTTTAGTATTRTGTTTGC	GCTTATTCTTCAGTTACACATATAAGA
H. placei	CGACAAACCACCTTGAT	TTTTCCTCAACATTATTCTG	same as *H. contortus*	CAAAGTGATTCCAAGTCATTGGC
T. circumcincta	CGACAAACCACCTTGATATTT	GAATAGTAAAATGCCACTAAA	CAAACAGCGGTGTGCCCCC	same as *H. placei*
M. odocoileus	same as *H. placei*	same as *T. circumcincta*	CTAATAGTGGTGTTCCACC	same as *H. placei*

Figure 1.—Orientation of primers used to amplify and sequence the *ND4* region from individuals of each species. *COI* refers to 5′ end of the cytochrome oxidase gene. *LNC* refers to a long, noncoding region that separates the *COI* and *ND4* genes. Entire piece was first amplified using primers 1 and 4. The two smaller pieces were then amplified for sequencing using primers 1 with 3 or 2 with 4.

TABLE 2

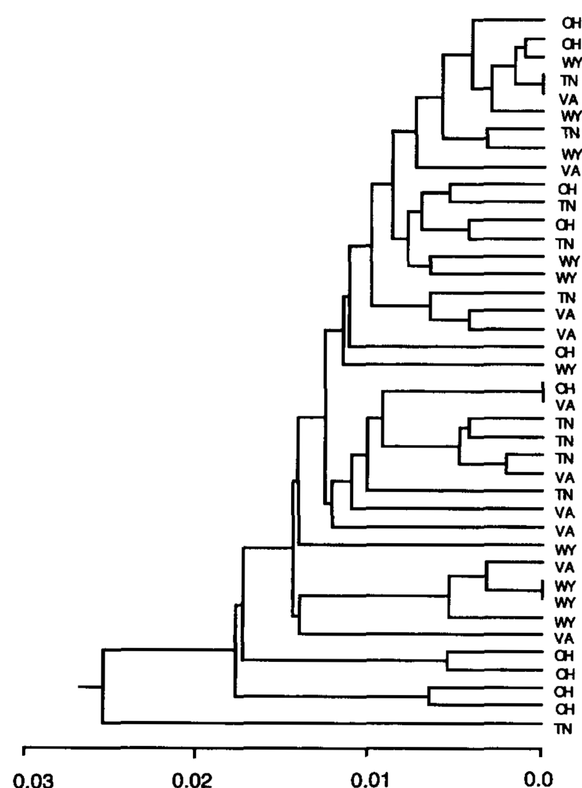
Average net diversity (substitutions per site) between populations, average within-population diversity, and fraction of total diversity distributed among populations for each species

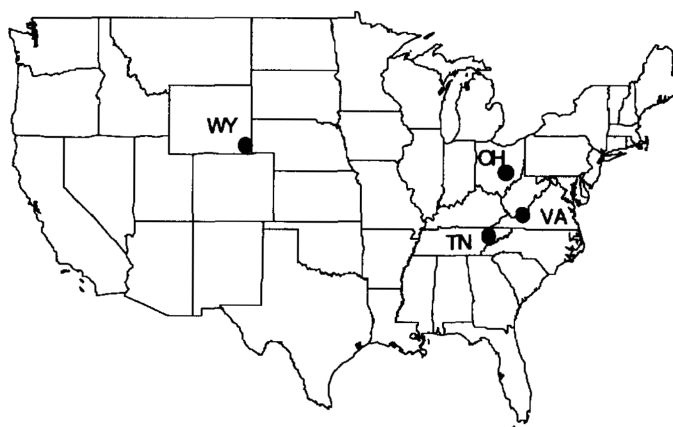
Species	V_b	V_w	N_{st}	Significance
H. contortus	0.0004 ± 0.0020	0.026 ± 0.002	0.01 ± 0.072	NS
T. circumcincta	0.0005 ± 0.0019	0.024 ± 0.002	0.02 ± 0.073	NS
H. placei	0.0008 ± 0.0027	0.019 ± 0.003	0.04 ± 0.128	NS
M. odocoileus				
Entire data set	0.0122 ± 0.0061	0.028 ± 0.003	0.31 ± 0.096	$P < 0.01$
Group A worms only (SC1, SC2, FL1, FL2 populations)	0.0035 ± 0.0024	0.026 ± 0.004	0.12 ± 0.058	$P < 0.05$

Estimates and standard errors calculated following LYNCH and CREASE (1990). N_{st} is analogous to WRIGHT's (1978) F_{st}, and is calculated as $N_{st} = V_b/(V_w + V_b)$. NS, not significant; V_b, average net diversity; V_w average within-population diversity; N_{st}, fraction of total diversity among populations.

by hunters in wildlife management areas, and from abomasa of cattle and sheep from individual farms (Table 1). From each worm, we PCR-amplified ~600 bp of an mtDNA fragment that contains 463 bp of the 3′ end of the *ND4* gene plus the variable-length long-noncoding (LNC) region that occurs between the *ND4* and *cytochrome oxidase I* genes (Figure 1; see also OKIMOTO *et al.* 1992). To sequence this product, we first reamplified, in two separate reactions, the 5′ and 3′ halves of the product using one original primer with one of two internal primers (Figure 1). The 5′ ends of these four primers contained extra bases corresponding to M13 forward or reverse sequencing primers (not shown in Figure 1). Incorporation of the M13 primer sequences into the second-round PCR products allowed us to efficiently sequence the second-round products using fluorescein-labeled M13 primers on an automated sequencer (dye primer sequencing method; Applied Biosystem, Inc.).

Detailed molecular methods: Individual adult male worms were identified by microscopic examination and then were extracted by grinding in 100 μl of buffer (10 mM Tris-HCL, pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCL, 0.1 mg/ml gelatin) with nonionic detergents (0.45% NP40 and 0.45% Tween 20) and 60 mg/ml proteinase K. Before PCR amplification, extracts were heated to 95° for 10 min to inactivate the proteinase K. A 5 μl aliquot of the extract was added to a 50 μl reaction mixture (10 mM Tris-HCL, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCL, 0.1 mg/ml gelatin, 0.2 mM dNTPs, 1 μM primer 1, 1 μM primer 4, and 1.25 units Taq polymerase). Thermocycling was performed under oil as follows (conditions in brackets indicate a repeated profile): 93° for 3 min, 35 cycles of [93° for 1 min,





Haemonchus contortus
(host = sheep)

FIGURE 2.—Geographic position of sampling locales for *H. contortus*, and UPGMA phenogram of relationships among the individual haplotypes. Letters at branch tips identify the origin of each haplotype. Scale shows the number of substitutions per site between the two branches connected by each node.

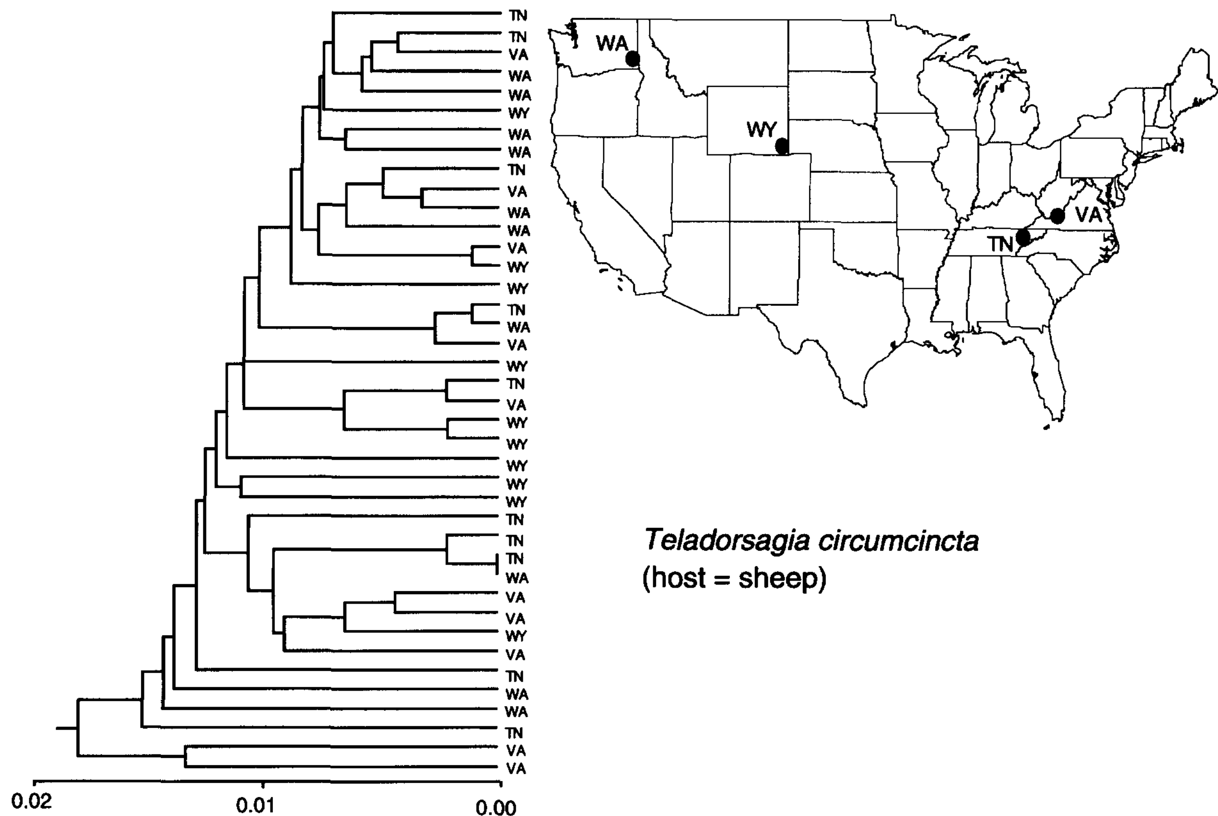


FIGURE 3.—Geographic position of sampling locales for *T. circumcincta*, and UPGMA phenogram of relationships among the individual haplotypes. Letters at branch tips identify the origin of each haplotype. Scale shows the number of substitutions per site between the two branches connected by each node.

36° for 1 min, and 72° for 1.5 min], followed by extension at 72° for 3 min. Conditions for amplification of the 5′ and 3′ subfragments were as above except that primers 1 with 3 or 2 with 4 were used, and thermocycling conditions were: 93° for 3 min, 10 cycles of [93° for 1 min, 36° for 1.5 min, and 72° for 3 min], 25 cycles of [93° for 1 min, 55° for 1.5 min, and 72° for 3 min], followed by extension at 72° for 3 min.

Data analysis: LNC sequence evolution is characterized by many rearrangements, insertions and deletions (unpublished data), so the analysis presented here is based only on the 463 bp of *ND4* gene sequence. The number of substitutions per site between each pair of haplotypes was estimated, and the total diversity was partitioned into components within and among populations, using the methods of LYNCH and CREASE (1990). UPGMA trees showing the relationships among individual haplotypes within each species were constructed using PHYLIP (FELSENSTEIN, 1991).

In our original study on *O. ostertagi*, we restriction mapped the entire mtDNA of each worm to obtain estimates of pairwise sequence divergence, which averaged 0.022 substitutions per site. Comparing estimates of within-population genetic diversity from that study with estimates based on *ND4* gene sequence from this study would be misleading if the *ND4* region evolves at a different rate than the mtDNA as a whole. Therefore, we sequenced 361 bp of the *ND4* region from 28 of the original 50 *O. ostertagi* individuals we used in the 1992 study (28 were all we had left). Using these 28 individuals, we estimated an average within-population diversity of 0.027 (SE = 0.003) using *ND4* sequence, *vs.* an average of 0.018 (SE = 0.005) by restriction mapping.

RESULTS

The population genetic structures of the parasites of cattle and sheep studied here were all very similar to that of *O. ostertagi*. Estimates of net nucleotide diversity between populations were very small, averaging 0.0004, 0.0008, and 0.0005 substitutions per site for *H. contortus*, *H. placei*, and *T. circumcincta*, respectively (Table 2). Estimates of within-population nucleotide diversity for these species averaged 0.026, 0.019, and 0.024, respectively (Table 2). These values are similar to the 0.027 estimate obtained for *O. ostertagi*. Almost all (96–99%) of the total genetic diversity was distributed within, rather than among, populations (Table 2). This result is reflected in the UPGMA dendrograms from each species, which show little correlation between the geographic origin of a haplotype and its position on the tree (Figures 2–4).

The trees for *H. contortus* and *T. circumcincta* (Figures 2 and 3), like that for *O. ostertagi*, showed more or less continuous variation between haplotypes, with no large discontinuities (distinct clusters of haplotypes, with haplotypes closely related within clusters, but distantly related between clusters). The tree for *H. placei* shows a discontinuity, with approximately equal numbers of individuals falling onto each of two main branches (Figure 4). Note, however, that the apparent strength of this bifurcation is exaggerated by the way the tree was drawn. The main branch is fairly shallow (at <0.015 substitutions/site), and some individuals within each branch are almost as distinct as individuals between the two branches. Regardless, in none of these trees do we

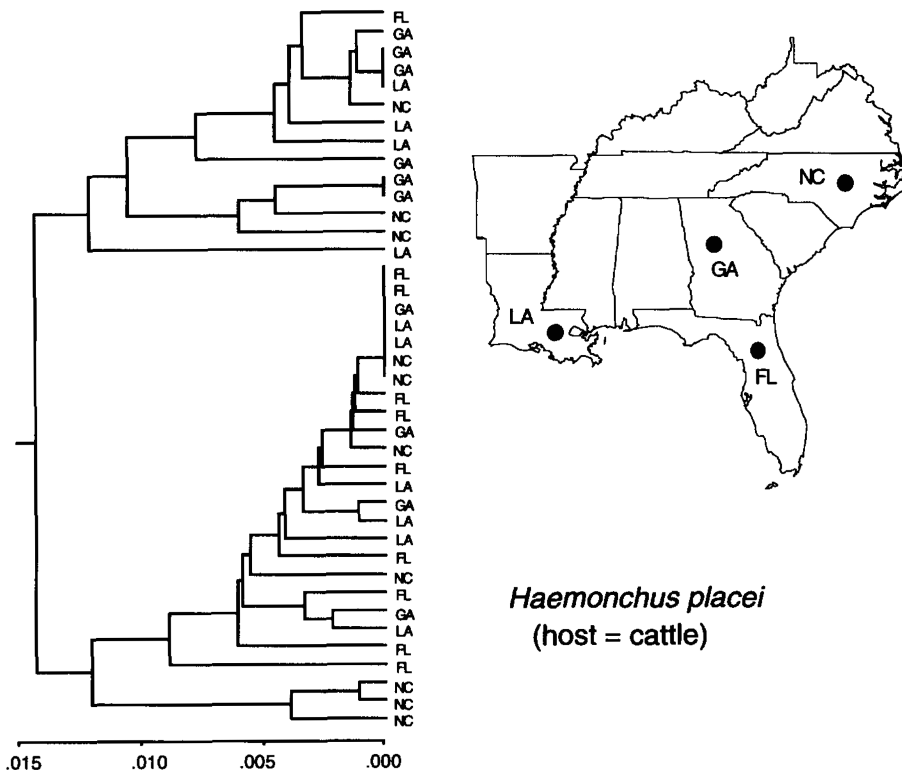


FIGURE 4.—Geographic position of sampling locales for *H. placei*, and UPGMA phenogram of relationships among the individual haplotypes. Letters at branch tips identify the origin of each haplotype. Scale shows the number of substitutions per site between the two branches connected by each node.

see the sort of large discontinuity that would clearly indicate a mixture of individuals from highly differentiated populations (*e.g.*, see AVISE *et al.* 1984; 1987).

The parasite of deer, *M. odocoilei*, showed much more population subdivision than the domestic parasites. In particular, all the AR worms, and one worm from each of the FL1 and FL2 populations, clustered in one very distinct branch of the tree (Figure 5). We will hereafter refer to these worms as group B and to the worms in the other branch of the tree as group A. Thirty percent of the total diversity is distributed among populations in this data set (Table 2), but this large Nst obviously results because all but two of the group B individuals are found in Arkansas. If we exclude the group B individuals from the analysis, and describe the PGS of only the group A worms (populations SC1, SC2, FL1 and FL2) we still find a statistically significant Nst of 12% (Table 2). We also see a general pattern of isolation by distance among these four populations, as one would expect if gene flow is a function of host movement (Figure 6). Although *M. odocoilei* has a much more subdivided population structure than the domestic species, it does not have less diverse populations (Tables 2 and 3). Indeed, the highest within-population diversity observed in this study was in the *M. odocoilei* FL2 population (0.037; Table 3).

DISCUSSION

Population subdivision and Gene flow in trichstrongylids: The cattle parasite (*H. placei*) and the two sheep parasites (*H. contortus* and *T. circumcincta*) all had PGSs remarkably similar to that of *O. ostertagi* with almost all of the total diversity distributed within populations. This PGS is consistent with very high gene flow among populations. In contrast, the deer parasite (*M. odocoilei*) showed significant population subdivision, and a pattern of isolation by distance. This pattern held even if we analyze worms from the A and B groups separately. Interestingly, the geographic location of the break between the A and B groups of *M. odocoilei* corresponds to a biogeographic break seen in the mtDNA of their host (ELLSWORTH *et al.* 1994) and in several other unrelated vertebrate taxa (AVISE *et al.* 1979; BERMINGHAM and AVISE 1986; HAYES and HARRISON 1992). These patterns support the prediction that for a parasite with no intermediate hosts and no dispersal abilities in the free-living stage, population subdivision is determined primarily by the movement of the host. Humans have just made cattle and sheep into supermobile hosts.

Explanations for high within-population diversity: All five species had within-population diversities that are much higher than typically observed in other taxa (*e.g.*, compare Table 2 of LYNCH and CREASE 1990). This result holds even after accounting for the faster rate of evolution in the *ND4* gene than in the mtDNA molecule as a whole. Surprisingly, the deer parasite had some of the most diverse populations of any species (Table 3). This result suggests that high within-population diversities are a general feature of the biology of trichostrongylids, and not some consequence of the domestication of livestock by humans.

BLOUIN *et al.* (1992) discussed three explanations for the high within-population diversity when it was first observed in Ostertagia. The first explanation was that

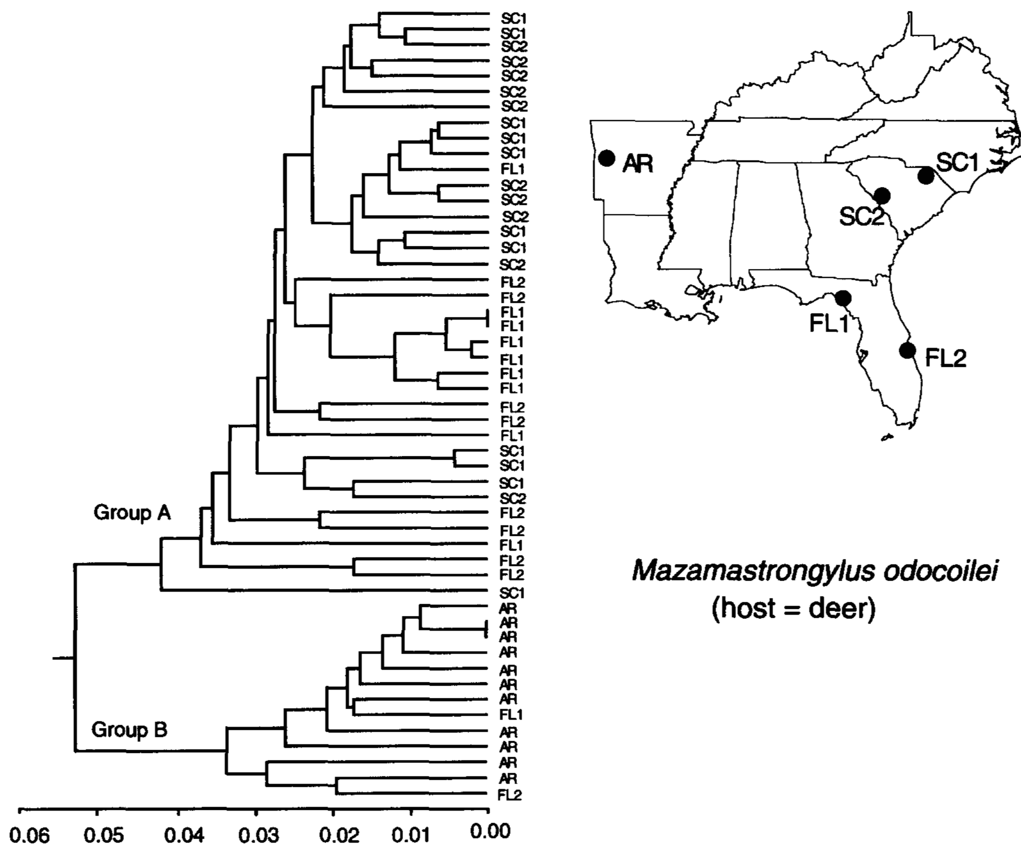


FIGURE 5.—Geographic position of sampling locales for *M. odocoilei,* and UPGMA phenogram of relationships among the individual haplotypes. Letters at branch tips identify the origin of each haplotype. Scale shows the number of substitutions per site between the two branches connected by each node.

sampled populations are a mixture of individuals from previously differentiated populations. This explanation seemed the most plausible at first because cattle have been shipped worldwide for decades. However, in a mixed population, one would expect large discontinuities in the haplotype phenograms. Phenograms from *O. ostertagi, T. circumcincta,* and *H. contortus* show more or less ladder-like topologies with continuous variation in genetic distance between individuals. The phenogram from *H. placei* suggests a discontinuity, although not a very distinct one. The phenogram from *M. odocoilei* shows a strong discontinuity, but this discontinuity corresponds with geographic locale, and so does not explain the high within-population diversities. Moreover, *M. odocoilei* has had much less opportunity for mixing than the domestic parasites, yet has some of the highest within-population diversities. In conclusion, the mixed-population hypothesis might explain some of the diversity seen in *H. placei* populations, but it fails as an explanation for high within-population diversities in all five species.

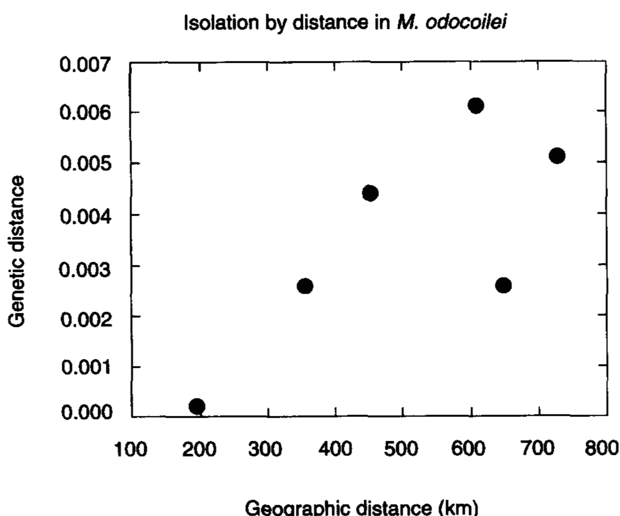


FIGURE 6.—Isolation by distance among the four eastern populations of *M. odocoilei* (SC1, SC2, FL1 and FL2; see map in Figure 5) excluding group B individuals. Genetic distance = net number of nucleotide substitutions per site between each pair of populations.

The second explanation for high within-population diversities was that mtDNA evolves faster in nematodes than in other taxa, and the third explanation was that sampled populations have had very large long-term effective sizes. Assuming that the vertebrate rate of mtDNA evolution also applies to nematodes, we estimated that long-term Ne within each population of *O. ostertagi* would have to be in the millions of individuals per generation to maintain the observed diversities (BLOUIN *et al.* 1992). We wondered whether individual farms can sustain populations of *O. ostertagi* this large,

TABLE 3

Substitutions per site between pairs of individuals within each population (within-population diversity)

Species	Population	Within-population Diversity
H. contortus	OH	0.030 ± 0.004 (10)
	TN	0.027 ± 0.006 (10)
	VA	0.025 ± 0.004 (10)
	WY	0.024 ± 0.004 (10)
T. circumcincta	TN	0.023 ± 0.004 (10)
	VA	0.025 ± 0.004 (10)
	WA	0.021 ± 0.003 (10)
	WY	0.025 ± 0.004 (10)
H. placei	FL	0.015 ± 0.005 (10)
	GA	0.020 ± 0.004 (10)
	LA	0.019 ± 0.004 (10)
	NC	0.021 ± 0.004 (10)
M. odocoileus	SC1	0.025 ± 0.004 (11)
	SC2	0.024 ± 0.004 (10)
	FL1[a]	0.019 ± 0.005 (9)
	FL2[a]	0.037 ± 0.005 (8)
	AR	0.024 ± 0.005 (11)

Values are means ± SE with number of individuals that diversity measurement is based on in parentheses.

[a] Calculated without the single group B worm found in each these populations. Including the group B worms increases the FL1 and FL2 diversities to 0.026 and 0.040, respectively.

so we suggested that massive gene flow might be making all the *O. ostertagi* populations into one huge population (under this condition the overall Ne would certainly be high enough to maintain the diversity observed within each farm). This explanation obviously does not work for the deer parasite, which is not characterized by high gene flow. Therefore, either effective sizes within each population really are large enough to maintain the observed diversities or else mtDNA evolution is accelerated (which lowers the Ne required to maintain the observed diversity). Interestingly, BEECH *et al.* (1994) found very high sequence diversity at two nuclear genes within a population of *H. contortus* (average substitutions per site of 0.094 for *β-tubulin-*1 and 0.091 for *β-tubulin-*2). Therefore, one must invoke accelerated evolution in both nuclear and mitochondrial DNA, or else accept that large Ne alone is responsible for the high diversities. We currently lack data to test directly the hypothesis that nucleotide substitution rates are faster in nematodes than in other taxa. However, when OKIMOTO *et al.* (1994) used mitochondrial ribosomal RNA genes to construct a phylogeny of the metazoan phyla, branch lengths from the common metazoan branch point to nematodes (*Ascaris suum* and *Caenorhabditis elegans*) were twice as long as branch lengths to other phyla. In a phylogeny of the metazoa constructed using nuclear 18s rRNA sequences, the longest branches were again to nematodes (Haemonchus, Strongyloides, and Caenorhabditis; PHILIPPE *et al.* 1994). These results suggest that nematode DNA does indeed evolve faster than DNA in other taxa. On the other hand, fast DNA evolution can't be the only explanation because not all parasitic nematodes have highly diverse populations. Ascaris populations that infect pigs tend to have much lower genetic diversity, and to be more subdivided, than trichostrongylid populations (ANDERSON *et al.* 1993, 1995; NADLER 1995b). Substitution rates are presumably the same in ascarids and trichostrongylids, and in both cases the parasites infect livestock. This leaves Ne as the main difference, which makes sense given one usually finds only a few to a few dozen Ascaris per host *vs.* thousands of trichostrongylids (NADLER 1995b). Therefore, as a working hypothesis, we propose that the high within-population diversities observed in trichostrongylid nematodes result mainly from large Ne, perhaps in combination with an accelerated rate of nucleotide substitution.

PGS and the evolution of drug resistance in trichostrongylids: Gene flow among populations can accelerate or hinder response to selection for pesticide resistance. It is well established that frequent immigration of susceptible individuals into a population under selection retards resistance evolution in the selected population (ROUSH and DALY 1990; TABASHNIK 1990). This situation may often occur with herbivorous insects, in which populations on native plants constantly send migrants into populations on crops. However, when all populations are under selection, migration will hurt by spreading resistance alleles from resistant populations to other populations in which those alleles have not yet appeared by mutation (UYENOYAMA 1986; CAPRIO and TABASHNIK 1992). Populations of trichostrongylids in the United States probably conform to this latter model. Most farms use anthelmintics, and there is no large reservoir of unselected individuals cycling through native wildlife. Our data suggest that the level of gene flow among populations of trichostrongylids parasitizing cattle and sheep in the U.S. is extremely high, so there is great opportunity for the spread of rare resistance alleles. The consequences of this PGS are not trivial. Organophosphate resistance in the mosquito *Culex pipiens* on three continents is controlled by the same amplification allele that evolved once and then spread worldwide (RAYMOND *et al.* 1991). BJORN (1994), VARADY *et al.* (1993), and VAN WYK (1990) describe suspected cases of anthelmintic resistance spread by the movement of sheep in Europe. Our data suggest the same could easily occur in the United States.

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