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Nuclear and Mitochondrial DNA Sequence Diversity in the Antarctic Nematode *Scottnema lindsayae*[1]

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Abstract: A nematode, *Scottnema lindsayae,* is the dominant metazoan found in soils of the McMurdo Dry Valleys, Antarctica. The distribution of *S. lindsayae* is patchy within and between these dry valleys; nevertheless, it is unclear to what extent these populations are genetically isolated. We investigated genetic diversity in this nematode using nuclear and mitochondrial gene sequences that encode ribosomal RNA. In 169 nematodes surveyed, only one variable site was found in each of two different expansion segments of nuclear rRNA. While most nematodes have only one sequence type, some nematodes were found to contain a mixture of both sequences. No fixed differences in nuclear sequences were observed between populations. This pattern of nuclear variation is most consistent with a single species of nematode defined morphologically as *S. lindsayae.* For mitochondrial DNA sequences, we found 10 variable positions defining 12 haplotypes among 188 nematodes surveyed. While all observed haplotypes are closely related, significant differences in haplotype frequencies were observed between geographically defined populations. The nuclear and mitochondrial variation suggests populations of *S. lindsayae* represent a single polymorphic species with some restriction of gene flow between geographic populations.

Key words: Antarctic, ecology, extreme environment, genetic diversity, genetics, mitochondrial DNA, morphology, nematode, ribosomal RNA, *Scottnema lindsayae,* soil biodiversity, systematics, taxonomy.

The Antarctic McMurdo Dry Valleys (77°100′S, 169°152′E) comprise nearly 4,000 km^2 of ice-free land (Fig. 1). This system is composed of approximately 15 named and distinct valleys, each with a different geological history, soil, climate, geographic orientation, and distance from the ocean. These valleys are considered to have among the most extreme environments on earth due to the high winds and the cold, arid environment, with mean annual temperatures of −19 °C (Schwerdtfeger, 1984) and mean annual precipitation less than 100 mm water equivalent (Bromley, 1985). Studies of microbial distributions in the dry valleys by Cameron et al. (1970) and Vishniac (1993) have led to the belief that these soil ecosystems are largely devoid of life.

Recent studies suggest that most of the Antarctic Dry Valleys' biological diversity is contained within the soil (Walton, 1987). Nematodes are important components of all soil ecosystems. In dry Antarctic soils, nematodes typically represent the top of the food chain (Freckman and Virginia, 1991, 1997). Compared to temperate desert soils and other ecosystems, Antarctic nematode species richness is very low (Freckman and Virginia, 1991; Powers et al., 1995). Antarctic ecosystems are under intense scrutiny because of their potential susceptibility to environmental disturbance (Freckman and Virginia, 1997; Weiler and Penhale, 1994). Consequently, a detailed understanding of biological systems in this fragile environment is greatly needed.

Scottnema lindsayae Timm 1971 Rhabditida, a free-living microbivore with separate sexes (Overhoff et al., 1993), dominates Dry Valley soil communities (Freckman and Virginia, 1991) and is the only genus endemic to continental Antarctica (Maslen, 1979). While we are rapidly developing a more detailed understanding of the biology of this nematode, it is not known whether *S. lindsayae* disperses easily between valleys, thus ensuring genetic exchange, or whether

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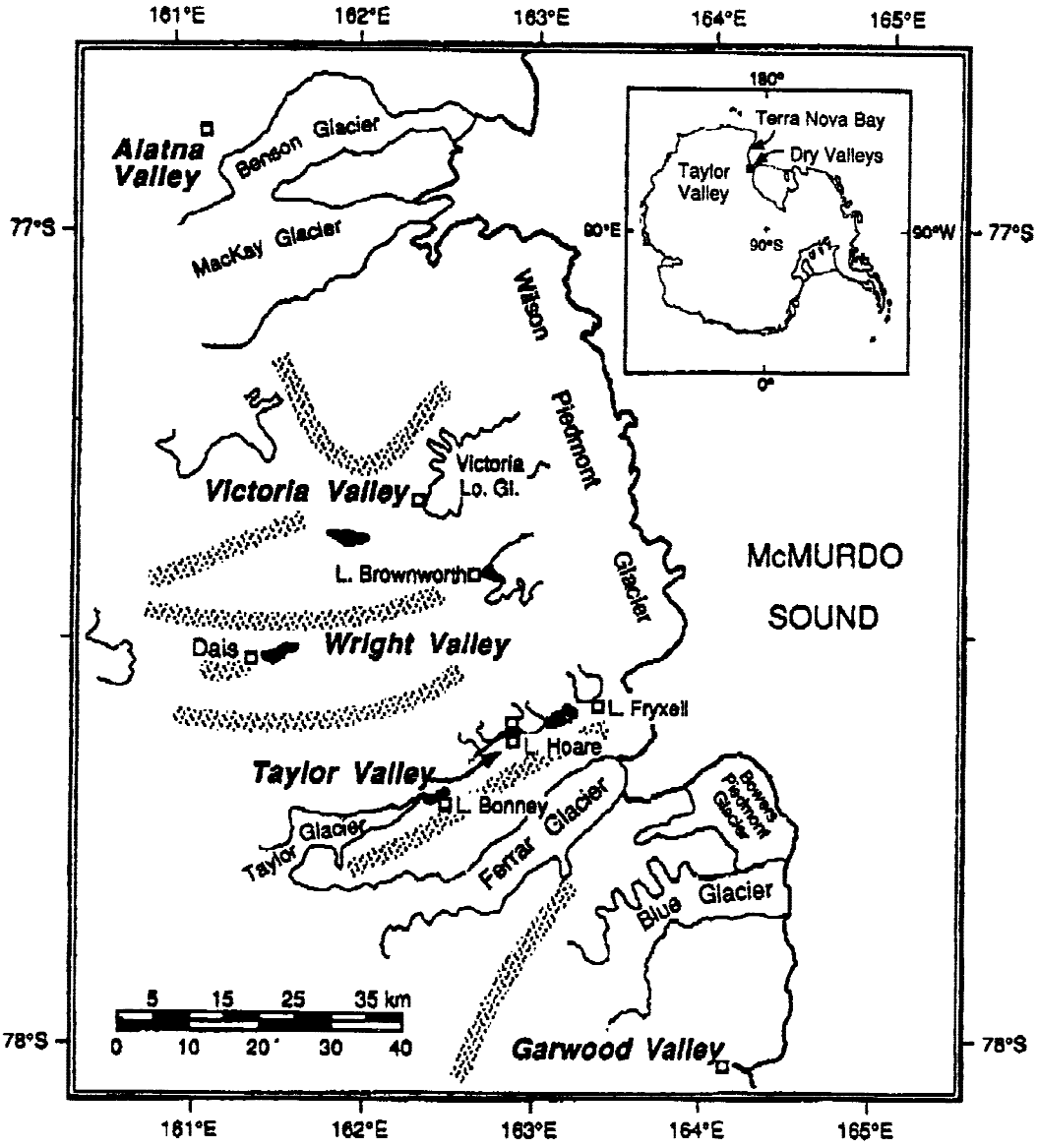

FIG. 1. Map of sites (boxes) sampled in Taylor, Victoria, Wright, Garwood, and Alatna Valleys. Taylor Valley: Lake Bonney, Lake Hoare (north), Lake Hoare (south), Lake Fryxell; Victoria Valley: Victoria Lower Glacier; Wright Valley: Lake Brownworth, Dais. Speckled areas indicate mountain ranges. Insert depicts location of Dry Valleys and Terra Nova Bay on the Antarctic continent.

populations are geographically isolated and show genetic drift and adaptation to local environmental conditions.

Distinguishing among nematode species can be difficult due to morphological similarities, variation in morphological characters with habitat and food source (Anderson, 1968; Boström and Gydemo, 1983; Schiemer, 1982), and a generally underdeveloped taxonomy. The existence of cryptic nematode species that are morphologically indistinguishable yet genetically divergent (Butler et al., 1981; Emmons et al., 1979; Thomas and Wilson, 1991) suggests that morphologically defined species such as *S. lindsayae* may represent multiple, reproductively isolated and evolutionarily unique entities.

An important fundamental question concerns the potential genetic isolation of *S. lindsayae* populations. *Scottnema lindsayae* populations occur across a wide geographic range, including sites effectively isolated by mountainous barriers (Freckman and Virginia, 1991, 1997, 1998; Wharton and Brown, 1989). Wind is a major mode of colonization for biota in Antarctica (Ellis-Evans and Walton, 1990; Linskens et al., 1993; Marshall, 1996; Walton, 1984; Wynn-Williams, 1992). Nematodes, as anhydrobiotes, could be dispersed by wind (Carroll and Viglierchio, 1981; Orr and Newton, 1971), particularly in the Dry Valleys, where wind speed often exceeds 30 m/second (Mullan and Sinclair, 1990) and even small pebbles may be transported through the air (Campbell and Claridge, 1987). If nematodes undergo frequent, large-scale dispersal by wind or other mechanisms, populations may not be genetically isolated and the genetic diversity of *S. lindsayae* may lack geographic structure. While dispersal may be widespread, establishment and population persistence may be limited to only a few favorable habitats or microsites (Freckman and Virginia, 1998). Severe restrictions in the effective population size for these nematodes may result in limited genetic diversity (Walton, 1987).

This research was designed to address two fundamental questions concerning *S. lindsayae*'s genetic diversity: (i) Does the morphology defined as *S. lindsayae* represent a single species or does this morphology encompass multiple, potentially ecologically unique groups? (ii) How genetically diverse and structured are *S. lindsayae* populations?

Materials and Methods

Sample collection: Individuals of *S. lindsayae* were isolated from soil samples taken from Taylor, Victoria, Wright, Garwood and Alatna Valleys, and Terra Nova Bay (Fig. 1) during the 1993–1994 austral summer and during the 1995 summer field season (Table 1). Pre-sterilized plastic sampling scoops (Nasco, Modesto, CA) and sterile Whirl-pak bags (Nasco, Modesto, CA) were used to collect approximately 1,500 g of soil to a 10-cm depth at each location (Freckman and Virginia, 1993). Soil samples taken in 1993–1994 and from Terra Nova Bay (1995) were packed in insulated boxes and transported at – 20 °C to Colorado State University. Shipping took approximately 3 months, after which the samples were stored in a freezer at –10 °C for 1 year. Remaining soil samples taken in 1995 were transported to Crary Laboratory at McMurdo Station (United States Antarctic Research Program), where they were refrigerated at 1 °C (Overhoff et al., 1993) and processed within 48 hours.

Nematodes were extracted from subsamples of soil with a sugar flotation-centrifugation technique (Freckman and Virginia, 1993). For samples collected during 1993–1994 and at Terra Nova Bay in

Table 1. Coordinates and numbers of *Scottnema lindsayae* at six Antarctic locations.

Location	Coordinates	Nematodes collected 1993–1994 and 1995
Taylor Valley		
Lake Bonney	77°42.92′S, 162°27.65′E	4/0
Lake Hoare, N.	77°37.49′S, 162°54.31′E	11/0
Lake Hoare, S.	77°38.03′S, 162°52.75′E	21/18
Lake Fryxell	77°55.94′S, 163°22.68′E	20/0
Victoria Valley		
Victoria Lower Glacier	77°21.81′S, 162°19.11′E	19/19
Wright Valley		
Lake Brownworth	77°26.13′S, 162°42.61′E	9/0
Dais		0/18
Garwood Valley	78°02′S, 164°10′E	22/0
Alatna Valley	76°52′S, 161°05′E	0/20
Terra Nova Bay	74°20′S, 165°08′E	0/18

1995, individual nematodes, usually adult, were picked and transferred into a 0.5-ml microcentrifuge tube containing 25 µl of water within 24 hours following extraction. Nematodes collected during 1995 were extracted within 24 to 48 hours of collection. Within 24 hours following extraction, individual nematodes were picked and transferred into 0.5-ml microcentrifuge tubes containing 10 µl of water.

DNA extraction: The extraction of DNA from individual nematodes was modified from Williams et al. (1992). Nematodes were placed into 15 µl of lysis buffer (60 µg/ml proteinase K in 10 mM Tris pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% TWEEN 20, and 0.05% gelatin), frozen at −70 °C for at least 15 minutes, incubated at 60 °C for 1 hour, and incubated at 95 °C for 15 minutes to inactivate the proteinase K.

Amplifications and sequencing: For the 1993–1994 nematodes and some 1995 nematodes, the D2 and D3 expansion segments of the large nuclear rRNA subunit, and a section of mitochondrial large rRNA (16S) subunit, were amplified by the polymerase chain reaction (PCR) and sequenced. The primer pairs used were D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D2B (5′-AATCCGTGTTTCAAGACGGG-3′), D3A(5′-GACCCGTCTTGAAACACGGA-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (Nunn, 1992), and mtA (5′-GGCGGATCCTACATCGATGTTGTAT-3′) and mtB (5′-GGCGGATCCWKTTCCTCTCGTACT-3′). These primers selectively amplify metazoan ribosomal DNAs (rDNA) and do not amplify common contaminants such as bacteria, fungi, or plant material.

Double-strand amplifications were performed in 25 µl of a solution containing 67 mM Tris pH 8.8, 6.7 mM $MgSO_4$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 1 mM of each deoxynucleoside triphosphate (dNTP), 1 mM of each primer, 2.5 µl of DNA from the extraction described above, and 2.5 units of *Thermus aquaticus* DNA polymerase (Perkin Elmer, Foster City, CA). An extract control consisting of lysis buffer only and a negative PCR control containing only the amplification mixture were included. Amplification consisted of denaturation at 94 °C for 40 seconds, annealing at 50 °C for 1 minute, and extension at 72 °C for 1 minute for 30 cycles. The products were separated by electrophoresis in a 2% NuSieve agarose gel (FMC Bioproducts, Rockland, ME). The resulting band was excised from the gel and diluted with 100 µl of water.

Single-strand DNA for sequencing was generated by the unbalanced priming method (Gyllensten and Erlich, 1988). Single-strand amplifications were performed with 1.5 µl of the diluted isolated gel product under the same conditions as the double-strand amplifications except one primer was diluted 100-fold relative to the other. Following amplification, salts and nucleotides were removed by filtration in Millipore Ultrafree 30,000 NMWL filter units (Millipore, Bedford, MA), and amplification products were collected in 10 to 15 µl of water. From these collected amplification products, 3.5 µl were used in dideoxy sequencing reactions with ^{35}S-dATP and Sequenase enzyme (United States Biochemical Co., Cleveland, OH) according to standard protocols. In addition to the amplification primers, the internal primer ID3B (5′-TAGWTCRCCATCTTTCGGGT-3′) was used for sequencing the D3 expansion segment. The products of the sequencing reactions were separated in 6% or 8% polyacrylamide 7 M urea gels.

Mismatch PCR: Mismatch PCR was performed on nematodes collected in 1995 to assay variable sites in the D2 and D3 expansion segments. The variable sites were identified in direct-sequence comparisons of the 1993–1994 nematodes.

Double-strand amplifications of DNA were performed in 25-µl reactions as described above. For mismatch PCR of the D2 expansion segment, the D2A primer and a mismatch primer (D2 Sal I) (5′-CAGATCCCATACGGGAGATCCAAGCATGCCGCACGAAGTC-3′) were used. The mismatch primer differs from the target sequence by a single base (underlined) that generates a Sal I restriction site when a cy-

tosine (C) is present at the variable site just 3′ of this primer (Fig. 2A). Thirty cycles of amplification consisted of denaturation at 94 °C for 40 seconds, annealing at 53 °C for 1 minute, and extension at 72 °C for 2 minutes. Digests containing 7.5 µl of the amplification product and 2.5 units of Sal I (Promega, Madison, WI) were incubated at 37 °C for 1 hour.

For mismatch PCR of the D3 expansion segment, the D3B primer and the mismatch primer (D3 Hpa II) (5′-GAAGCGATCTTCCGATGCTGATATGCGATCCGTAGTGCTC-3′) were used. The mismatch primer differs from the target sequence by a single base (underlined) that generates an Hpa II restriction site when a C is present at the variable site just 3′ of the primer (Fig. 2B). Thirty cycles of amplification consisted of denaturation at 94 °C for 40 seconds, annealing at 47 °C for 1 minute, and extension at 72 °C for 2 minutes. Digests containing 7.5 µl of the amplification product and 2 units of Hpa II (Amersham Pharmacia, Piscataway, NJ) were incubated at 37 °C for 1 hour. All digestion products were separated by electrophoresis in 3% NuSieve (FMC Bioproducts, Rockland, ME) agarose gels. The mismatch approaches above were designed such that the majority of samples would be digested.

Results

Ribosomal DNA: For 97 nematodes from 1993–1994 samples, three different D2 genotypes were identified (Table 2). The majority of nematodes examined (88) had a C at position 86 (type 1-D2). Nematodes with this common genotype were found at all locations sampled. Only three nematodes from soil samples collected at Victoria Lower Glacier had a genotype with an adenine (A) at position 86 (type 3-D2). In addition, six individuals containing both an A and a C at position 86 in approximately equal ratios were identified (type 2-D2). Five of these nematodes were from Victoria Lower Glacier, and one was from Lake Bonney.

Similarly, three genotypes were also found for the D3 expansion segment based on 98 nematodes from samples collected in 1993–1994 (Table 2). A common genotype (type

A. D2 sequence

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GTACTTTGAAGAGAGAGTTCAAGAGGACGTGAAACCGGTAGATGGAAACGGATAGAGCCA   60
ACGAACAAGCCCGTATTCAGCTAGTCGGCTTCGTGCGGCATGCTTGGATCTCCCGTATGG  120
GATCTGAGTGTGTGCGGCATGCGGCTGGCTAGTGCATTTGCGGGTGATGTGCGCTGAGGC  180
GTGTGGTGGAGTGCTGCAAACTGCGTAGAGAGGCCTCGTCTTCGGACTTGGAACCTATGC  240
GTGGGGAGTAGTGTTTCTGCTGCATGTAGATATGCGGTTAGGTGCAATCGCACTGCTGCG  300
TTGTCGGGTCGGTTCGTTGGGCGNGCATGCGACTTGCTCTTTGGGCTGGCTTTGGTGGNG  360
TAGTAGTGCCCTAATCGATCCTCCGCTTTTACC                              393
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B. D3 sequence

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CCAAGGAGTCTAGCGTATGCGCGAGTCATTGGGTGGAAAACCCATAGGCGAAATGAAAGT   60
GAAGGCATCTTCCGATGCTGATATGCGATCCGTAGTGCTTCGGCGCTGCGGAGCAGCATA  120
GCCCCGTCTCTACCGCTTGCGGTGGGGCGGAGGTAGAGCGTATTCGCTGGTACCCGAAAG  180
ATGGTGAACTATGCCTGAGCAGGATGAAGCCAGAGGAAACTCTGGTGGAGGTCCGAAGCG  240
CTTCTGACGTGCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATC        296
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C. mtDNA sequence

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CTCAATTGTACAGCGGCTTAGAGTCTAGTCAGTACATTCAGACTGTTCCGCTGTTAATTA   60
AGTAATACTCCAGAACGTGATATTAGTTTAATTCGACGCAAGTCAGAGTGGTTTATCTGG  120
ATAAGTGTTTTTATTTGTTAGACGGCT                                    146
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FIG. 2. Representative sequences for the three *Scottnema lindsayae* rDNA genes studied. In each case the sequence is the most common type found among all individuals. The D2 region (A) corresponds to nucleotides 3019–3305, and the D3 region (B) corresponds to nucleotides 3326–3628 of the published nuclear rDNA sequence (Ellis et al., 1986). The mitochondrial fragment sequence (C) corresponds to 11170–11302 of the published *C. elegans* mitochondrial sequence (Okimoto et al., 1992). Variable sites in each sequence are underlined. *Scottnema lindsayae* sequences are numbered beginning with the first nucleotide after the primer on the 5′ end (D2A, D3A, or mtA).

TABLE 2. Geographic distribution of D2, D3, and mtDNA types of *Scottnema lindsayae* collected in Taylor, Victoria, Wright, Garwood, and Alatna Valleys, and Terra Nova Bay, Antarctica[a]

Location	DNA sequence type (no. of nematodes)		
	D2 Genotype (*n*)[a]	D3 Genotype (*n*)[b]	MtDNA Genotype (*n*)
Taylor Valley			
Lake Bonney	1 (3)	1 (2)	5 (1)
	2 (1)		
Lake Hoare North	1 (10)	1 (10)	2 (9)
		3 (1)	
Lake Hoare South	1 (18)	1 (13) (1993–1994)	1 (8) (1993–1994)
		1 (13) (1995)	1 (16) (1995)
			2 (7)
		2 (3) (1993–1994)	3 (6) (1993–1994)
		2 (3) (1995)	3 (1) (1995)
		3 (0) (1993–1994)	
		3 (2) (1995)	
Lake Fryxell	1 (16)	1 (19)	1 (4)
		3 (1)	2 (6)
			3 (9)
Victoria Valley			
Victoria Lower Glacier	1 (10) (1993–1994)	1 (19)	1 (7) (1993–1994)
	1 (13), (1995)		1 (9) (1995)
			3 (2) (1993–1994)
			3 (2) (1995)
	2 (5) 1993–1994)		4 (0) (1993–1994)
	2(5) (1995)		4 (2) (1995)
			5 (6)
	3 (3) (1993–1994)		
	3 (1) (1995)		
			6 (0) (1993–1994)
			6 (1) (1995)
			9 (1) (1993–1994)
			9 (1) (1995)
			11 (0) (1993–1994)
			11 (1) (1995)
			12 (0) (1993–1994)
			12 (3) (1995)
Wright Valley			
Lake Brownworth	1 (9)	1 (6)	2 (8)
		2 (2)	
Dais	1 (17)	1 (15)	1 (1)
			3 (2)
			4 (7)
			6 (5)
			8 (3)
Garwood Valley	1 (22)	1 (18)	1 (13)
		2 (4)	2 (3)
			3 (5)
			10 (1)
Alatna Valley	1 (20)	1 (18)	1 (20)
		2 (2)	
Terra Nova Bay	1 (17)	1 (15)	1 (10)
		2 (1)	3 (5)
			7 (3)

[a] Genotype columns refer to the genotype (1, 2, or 3) for D2 and D3 sequences or the mitochondrial haplotypes (1–12).
[b] Number of nematodes (*n*) in each genotype given for each collecting period (1993–1994 and 1995).

1-D3) with a C at position 101 was found in 87 nematodes from all locations sampled (Table 3). A genotype with a thymine (T) at position 101 was found in two nematodes (type 3-D3)—one from Lake Fryxell and one from the north shore of Lake Hoare.

TABLE 3. Variable sites defining 12 haplotypes of *S. lindsayae* mtDNA. *S. lindsayae* were from samples collected in Taylor, Wright, Victoria, Garwood, and Alatna Valleys, and Terra Nova Bay, Antarctica.

Haplotype	Number of nematodes	21	25	31	37	39	99	125	130	132	136
1	88	G	C	A	T	C	C	G	T	A	G
2	33	G	C	A	T	C	C	G	T	T	G
3	32	G	T	A	T	C	C	G	T	A	G
4	9	G	C	A	T	T	C	G	C	A	G
5	7	G	T	A	T	C	T	G	T	A	G
6	6	G	C	A	T	T	C	G	T	A	G
7	3	A	T	A	T	C	C	G	T	A	G
8	3	G	C	A	T	T	C	A	T	A	G
9	3	G	C	G	T	C	C	G	T	A	G
10	2	G	C	A	T	T	C	G	C	A	A
11	1	G	C	A	T	C	C	A	T	A	G
12	1	G	C	A	A	C	C	G	T	A	G

Polymorphic positions are numbered starting with the first nucleotide after the mtA primer.

Nine individuals containing both a C and T at position 101 in approximately equal ratios (type 2-D3) were identified from several locations.

Mismatch PCR: Using a mismatch PCR approach, we examined the variable positions in the D2 and D3 expansion segments identified above in 72 nematodes collected during 1995. In addition to resampling Victoria Lower Glacier and Lake Hoare (south), these nematodes represented three new localities: the Dais in Wright Valley, Alatna Valley, and Terra Nova Bay. Amplifications using either the mismatch primer D3 Hpa II or the mismatch primer D2 Sal I produced a single product of the expected size. Digestion of the PCR product with the corresponding enzyme gave three overlapping genotypic classes corresponding to type 1, type 2, and type 3(Fig. 3). Type 1 is completely uncut (lane 3, Fig. 3) type 2 contains both cut and uncut fragment (Lanes 2, 4, 5, 7, and 8, Fig. 3), and type 3 is completely cut with no detectable undigested fragment (lanes 1, 6, 9, and 10, Fig. 3).

Mitochondrial DNA variation: The mitochondrial primers amplified a single 146-base-pair segment (Fig. 2C) from all nematodes assayed. Mitochondrial DNA sequenced for this region varies at 10 positions defining 12 haplotypes (Table 3). Halotypes 1 and (or) 2 occurred at every sampled site except Lake Bonney (Table 2). Haplotypes 3–6 were found at multiple localities, while haplotypes 7–12 were found only at single localities (Table 2). To test the significance of haplotype frequency differences between populations, we compared the haplotype frequencies using the method of Roff and Bentzen (1989). This analysis demonstrated that significant differences in haplotype frequencies do exist between the populations when grouped geographically (Table 1).

DISCUSSION

Genetic continuity of S. lindsayae rDNA sequences: The genotypes represented in Figure 3 were generally consistent with the results from direct sequencing. However, the detection of rare genotypes appears to be more sensitive using mismatch PCR. For example, the nematodes D2 expansion segment analyzed by mismatch PCR in lane 8 of

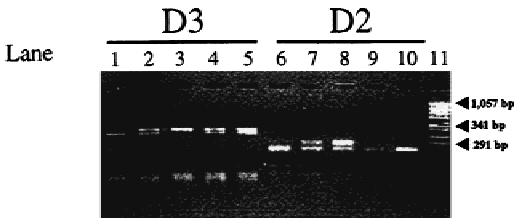

FIG. 3. Agarose gel of mismatch PCR products showing a range of genotypes for the D2 and D3 expansion segments. Lanes 1–5 show five nematodes amplified with the primers D3B and D3Hpa II and digested with the enzyme Hpa II. Lanes 6–10 show five nematodes amplified with the primers D2A and D2Sal I and digested with the enzyme Sal I. Lane 11 is the size standard Hinc II-digested $\phi\times174$.

Figure 3 is predominately uncut by the enzyme Sal I and was originally scored as a type 3 sequence (not Sal I site). Nevertheless, some fraction of the molecules in this PCR product are digested by Sal I, and are presumed to represent copies of the common genotype. In several cases, samples were analyzed by both sequencing and mismatch approaches. In each case, nematodes originally scored with sequencing as type 1 or type 3 were found to have low but detectable levels of the alternative sequence when examined with mismatch PCR (Fig. 3). Nematodes clearly containing both sequences in approximately equal amounts by direct sequencing were also found to have both genotypes in approximately equal ratios when scored by mismatch PCR. When a nematode rDNA expansion segment was amplified in multiple, separate experiments, the observed ratio of the two products was approximately the same in all cases. These results were consistent with the presence of two different rDNA sequences within a single nematode genome and a non-allelic inheritance of these sequences, as would be expected for a multicopy array. No attempt has been made to quantify the number of copies representing each sequence in each nematode.

Of particular interest is the observation of nematodes that appear to have two different nuclear rDNA sequences. Mixed genotypes could represent naturally occurring variation in the rDNA arrays. Alternatively, these mixed genotypes could reflect hybridization during the transfer and storage of soil from Antarctica (approximately 15 months at −20 °C to −10 °C). Although there is no evidence that nematodes are active at these storage temperatures, with this long storage period and a generation time estimated to be 218 days at 10 °C (Overhoff et al., 1993), it is conceivable that some nematodes isolated may be the result of matings that occurred during the long cold-storage period. To test the possibility that mixed genotypes are not found in nature, two localities with the highest frequency of mixed genotypes were resampled in 1995 (Table 2), with nematodes isolated from soil samples less than 48 hours after collection.

Of the 19 nematodes collected at Lower Victoria Glacier assayed by mismatch PCR for variation in the D2 region, five nematodes were of the mixed genotype (Table 2). Similarly, three of the 18 nematodes collected in 1995 from south of Lake Hoare showed a mixed genotype for the D3 region (Table 2). In both populations, the frequencies of the three nuclear rDNA genotypes observed were similar to the 1993–1994 samples (Table 2). The observation of mixed genotypes in the rapidly processed 1995 samples supported the hypothesis that these mixed genotypes occur in natural populations. In most animals the rRNA genes occur in multicopy arrays and show a concerted pattern of evolution, such that within a species a single sequence is found in the vast majority of gene copies. To be an effective marker of species differences, sufficient time must have passed to allow reproductively isolated groups to accumulate and fix nucleotide substitutions in all rDNA copies. Logically, breeding between nematodes representing two distinct genotypes would result in offspring of mixed genotype. These would not simply be heterozygous individuals because the processes of unequal crossing-over and gene conversion within the potentially large arrays complicate the inheritance of this locus. Therefore, the observation of fixed rDNA sequence differences between nematodes would be consistent with the existence of multiple noninterbreeding groups.

Scottnema lindsayae with apparently fixed differences in rDNA sequence were observed; however, the pattern of genotypes does not support the existence of reproductively isolated groups. For both expansion segments (D2 and D3), most nematodes contain a single sequence (type 1) for all copies within the array. A few nematode sequences contain a fixed nucleotide substitution (type 3) for all copies within the array. The rare rDNA sequence types are found together in soil samples with common sequence types. In addition, close scrutiny of genotypes revealed many nematodes with both sequence variants. While the inheritance of this locus is fundamentally complicated, the observed variation in the two

rDNA sequences does not support the existence of multiple, reproductively isolated groups among the specimens morphologically defined as *S. lindsayae.*

Mitochondrial DNA variation: We observed 12 mitochondrial haplotypes defined by variation at 10 positions among 188 nematodes. These 12 haplotypes can be linked together into a minimal evolutionary framework by single mutational steps (Fig. 4). There are a minimum of 11 nucleotide substitutions, of which nine (82%) are transitions, a bias typical of mitochondrial evolution (Thomas and Wilson, 1991).

The mitochondrial haplotype frequencies show a pattern common for invertebrates where there are a few relatively common haplotypes (1, 2, 3) and numerous rare variants. The evolutionary framework is also interesting in that rare haplotypes appear at the tips of branches having only a single connection to other types. By contrast, the most common haplotype has a central position and is inferred to have given rise to numerous other haplotypes (Fig. 4). Examination of the relationships among haplotypes suggests no ''genetic breaks'' or phylogenetically distinct groups of haplotypes. Thus, the phylogeographic pattern of mitochondrial haplotypes does not support the existence of long-term zoogeographic barriers among *S. lindsayae* populations. This observation is consistent with the pattern of variation of nuclear rDNA loci as discussed above.

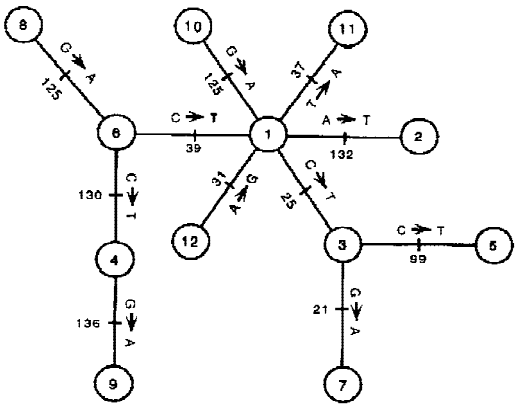

FIG. 4. A minimal evolutionary framework for mtDNA haplotypes. Haplotypes are linked with single mutational changes. Position and kind of mutation are indicated at the hashmark.

Frequency differences of mitochondrial haplotypes: To test the null hypothesis that populations of *S. lindsayae* are genetically homogeneous, multiple analyses of geographic variation of mitochondrial haplotype frequencies were performed. Because many of the cells in an χ^2 contingency test have expected values <1 and few have expected values >5, we used the method of Roff and Bentzen (1989) to evaluate significance. In a comparison of all six localities, we found that significant heterogeneity of haplotype frequency exists ($P < 0.001$) with no χ^2 values from the 1,000 randomized data sets exceeding the observed values. We also tested the heterogeneity for all 56 possible combinations of localities and haplotypes. In nine of these, χ^2 values from randomized data were found to exceed the observed values. In four pairwise comparisons of populations (Taylor Valley and Garwood Valley, Victoria Valley and Garwood Valley, Victoria Valley and Terra Nova Bay, and Garwood Valley and Terra Nova Bay, Table 4), we were unable to reject the hypothesis of homogeneity ($P > 0.005$).

For two valleys (Taylor and Wright), multiple sites within each valley were sampled. If each site within a valley is treated as a separate population and the same tests of heterogeneity as above are performed, none of the χ^2 values from randomized data exceed the observed values and in both cases we can reject the hypothesis of homogeneity within these valleys (Table 4).

The observations above suggest that significant differences in haplotype frequency exist between valleys and even between smaller-scale geographic sites. These genetic differences may reflect geographic isolation over time; however, little is known about the temporal stability of these nematode populations. Genetic differences may reflect the establishment of local populations by a small number of founding individuals. At two localities (south shore of Lake Hoare and Lower Victoria Valley), samples were collected from the same site for two consecutive years. When tested as above for haplotype frequency differences, it was possible to reject the hypothesis of homogeneity between temporal samples from the Lake

TABLE 4. Haplotype frequency analysis for mitochondrial DNA.

Populations	χ^2	Number[a]	P[b]
Interregion haplotype frequency analysis			
Taylor Valley, Garwood Valley, Terra Nova Bay	16.2	1	0.001
Victoria Valley, Alatna Valley, Terra Nova Bay	17.59	5	0.005
Garwood Valley, Alatna Valley, Terra Nova Bay	11.66	1	0.001
Taylor Valley, Garwood Valley	2.62	154	0.154
Victoria Valley, Garwood Valley	6.67	26	0.026
Victoria Valley, Alatna Valley	8.91	2	0.002
Victoria Valley, Alatna Valley	4.96	74	0.074
Garwood Valley, Alatna Valley	5.73	1	0.001
Garwood Valley, Terra Nova Bay	2.75	151	0.151
Intraregion haplotype frequency analysis			
Taylor Valley	41.14		
Wright Valley	18.05		
Temporal haplotype frequency analysis			
Victoria Lower Glacier	6.47	16	0.016
Lake Hoare, S.	9.36	2	0.002

Analyses were performed on 1,000 randomized data sets (Roff and Bentzen, 1989).
[a] Number represents the number of χ^2 values from randomized data that exceeded the observed χ^2 value.
[b] P is the proportion of χ^2 values from randomized data that the observed χ^2 value. For interregion analyses, only those population combinations for which randomized data sets exceeded the observed χ^2 values are presented.

Hoare population (Table 4). This temporal comparison is confounded by our incomplete understanding of detailed geographic population structure. Nevertheless, these observations suggest the need for temporal studies of genetic continuity and small-scale geographic variation.

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