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Genetic Variation in *Nacobbus aberrans*: An Approach toward Taxonomic Resolution¹

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Abstract: Biochemical and molecular analyses of genetic variation were evaluated to address the taxonomic status of *Nacobbus aberrans*. Isolates from Mexico, Peru, and Argentina, cultured on tomato in the greenhouse, were analyzed with respect to isozyme and DNA marker variation. Although acid phosphatase and malate dehydrogenase revealed distinct profiles for each isolate, non-specific esterases revealed possible affinities between the Peruvian isolates and between the isolates from Mexico and Peru. Two of 10 RAPD primers revealed affinities suggested by esterase profiles. RFLP analysis of the rDNA repeating unit with six restriction enzymes revealed identical cleavage patterns between the Peru isolates and a distinct profile shared by isolates from Mexico and Argentina. Nucleotide sequence analysis of the 5.8S rRNA coding region revealed differences among the four isolates at eight of 157 positions; sequences of the Peruvian isolates differed from each other at only one position, whereas the Mexican and Argentine isolates were identical and could be distinguished from the Peruvian isolates. A distance matrix from unweighted pairwise comparisons of the 5.8S rDNA revealed apparent elevated intraspecific divergence in *N. aberrans* comparable to intergeneric divergence between *Heterodera* and *Globodera*. Analysis of additional *N. aberrans* isolates from throughout the distribution range should help determine the full extent of intraspecific genetic variation that underlies the phenotypic and morphologic diversity of the genus.

Key words: esterase, false root-knot nematode, molecular biology, *Nacobbus aberrans*, nematode, RAPD, rDNA, RFLP, 5.8S rRNA, taxonomy.

Physiological variation of nematode species described on the basis of morphology alone often confounds development of reliable taxonomic systems and management strategies. *Nacobbus aberrans* Thorne, 1935 (Thorne & Allen, 1944), one of the most compelling examples, is comprised of geographical variants with great heterogeneity in host range and pathogenicity. The species is distributed widely throughout tropical and temperate regions of the Americas with variants that collectively parasitize more than 40 host genera (Baldwin and Cap,

1992; Canto, 1992; Doucet and Gardenal, 1992; Inserra et al., 1985), sometimes causing extensive agricultural losses (Baldwin and Cap, 1992; Inserra et al., 1985). These losses can be minimized most effectively by crop rotation or development of resistant cultivars. The implementation of these management regimes depends upon the taxonomic clarification of *N. aberrans*.

Phenotypic variation within *N. aberrans* has resulted in taxonomic controversy. Thorne and Allen (1944) first described the genus *Nacobbus* the false root-knot nematode. They reported two morphologically defined nominal species, *N. aberrans* and *N. dorsalis* Thorne and Allen, 1944. Morphological divergence was much more ambiguous for subsequently described species and subspecies, including *N. batatiformis* Thorne and Schuster, 1956 (Thorne and Schuster, 1956), *N. serendipiticus* Franklin, 1959 (Franklin, 1959), and *N. serendipiticus bolivi- anus* Lordello, Zamith & Brock, 1961 (Lordello et al., 1961). Later, Sher (1971) revised the genus and considered that *N. ab-*

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errans and *N. dorsalis* were the only two species, synonymizing all others into a physiologically heterogeneous *N. aberrans*. Under the extant taxonomy, intraspecific physiologic diversity is particularly great among isolates in Mexico, Peru, and Argentina (Baldwin and Cap, 1992; Cap, Canto, and Cid del Prado [pers. comm.]). Consequently, there have been some recommendations to recognize distinct physiological races, pathotypes, or even additional new species within *N. aberrans* (Boluarte and Jatala, 1992; Canto-Saenz et al., 1997; Inserra et al., 1985; Manzanilla-Lopez et al., 1997; Toledo et al., 1993).

The taxonomic limits of *N. aberrans* have been morphology-based, and conclusions have been unsatisfying and inconsistent. The original limits defining *N. aberrans* became more restricted as additional species were described on the basis of morphology. Then recognition of overlap of morphological characters among putative species led to synonymies and a new, more inclusive morphological definition of *N. aberrans*. This current definition remains controversial, in part because of the high degree of intraspecific morphological variation of *N. aberrans*, and the apparent lack of congruence or predictive value with respect to pathogenesis or geography. Morphological studies beyond light microscopy, including scanning electron microscopy, have not resolved questions of the taxonomic limits of *N. aberrans*, suggesting the need to explore other tools that might provide a suitable basis to define this variable species (Baldwin and Cap, 1992).

Biochemistry and molecular biology offer promising approaches for addressing the taxonomic questions raised by intraspecific variation of *N. aberrans*. These approaches have been particularly productive for addressing similar taxonomic concerns recently reviewed for a wide variety of organisms (Ferraris and Palumbi, 1996), including phytonematodes (Caswell-Chen et al., 1993; Hyman and Powers, 1991; Hyman and Whipple, 1996). Although few such studies have been made on *N. aberrans*, they generally confirm genetic variability but do not

provide information in a biogeographic or phylogenetic context (Doucet and Di Rienzo, 1991; Doucet and Gardenal, 1992; Mayorga and Jatala, 1990). In the present study we explored the most appropriate biochemical or molecular approaches for assessing diversity by analyzing four isolates of *N. aberrans* from sites in Mexico and South America. Once general approaches have been established using these four isolates, the investigation can be expanded to a substantial number of isolates and outgroups to search for more general patterns linked to biogeography and phylogeny, and as a basis for taxonomic resolution.

MATERIALS AND METHODS

Nematode populations: Four isolates of *N. aberrans* were kindly provided by M. Canto (Peru), I. Cid del Prado (Mexico), and G. Cap (Argentina) and are hereafter designated F1, F2, F4, and F5. The Peruvian isolates (F1, F2) were obtained from table beet and potato, respectively; the Mexican and Argentine populations (F4 and F5, respectively) were both isolated from tomato. The four isolates were maintained on tomato (*Lycopersicon esculentum* cv. Tropic) at 25 °C to 27 °C in a University of California-Riverside glasshouse with a 16-hour day length under 45% average relative humidity. Females were dissected from galled roots and placed in an iced cavity glass block. Single females or mixtures of up to 10 pooled females were transferred to microcentrifuge tubes containing sterile distilled water and frozen at -80 °C.

Isozyme extract preparation and gel electrophoresis: Frozen females were thawed on ice and resuspended in 20 µl extraction buffer (2% Triton X-100, 20% glycerol, 0.01% bromophenol blue). Nematodes were homogenized on ice with a small plastic pestle and the lysate centrifuged at 14,000g for 10 minutes at 4 °C. The 20-µl clarified supernatant was used immediately for electrophoresis.

For non-specific esterase detection, samples were fractionated with an LKB Multiphor focusing gel system with an ampholine pH range of 3 to 10. Cathode and anode electrode strips were soaked in 1.0 M

NaOH and 1.0 M H_3PO_4 , respectively. The gel was pre-focused for 30 minutes with 800V at 4 °C, followed by electrophoresis for 3 hours under the same conditions. For acid phosphatase (AP) and malate dehydrogenase resolution, polyacrylamide gel electrophoresis was carried out as previously described (Esbenshade and Triantaphyllou, 1987). Non-specific esterases were detected with isoelectric focusing using standard methods (Ibrahim and Rowe, 1995). Gel electrophoresis experiments were conducted a minimum of five times to ensure reproducibility of the banding patterns.

DNA extraction: DNA samples were obtained from both pooled and individual females. For bulked samples, 10 females were washed in sterile distilled water and suspended in 20 μ l lysis buffer (10 mM Tris (pH 8.0), 1 mM EDTA, 1% nonidet P-40, 100 μ g/ml proteinase K) in a 1.5-ml microcentrifuge tube. After homogenization with a pellet pestle (Biomedix, UK), the extract was incubated at 94 °C for 3 minutes. DNA was obtained from individual females by resuspension in 5 μ l distilled water and treated as above. These crude DNA extracts were suitable for polymerase chain reaction (PCR) amplification without further treatment.

rDNA and RAPD amplification: A portion of *N. aberrans* rDNA containing the 3' end of the 18S rRNA coding sequence, intact internal transcribed spacer (ITS)1, the complete 5.8S rRNA gene, intact ITS 2, and the 5' end of the 28S rRNA coding sequence was amplified with PCR primers developed by Vrain et al. (1992). Duplicate 25- μ l reaction mixtures contained 10 mM Tris (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μ M dNTPs, 30 pM of each primer, 1 μ l cellular DNA extract (1 to 50 ng DNA template), and 2.5 units of Tfl thermostable DNA polymerase (Epicenter Technologies, Madison, WI). Thirty amplification cycles were conducted at 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 2 minutes. Reactions containing the Operon "M" Kit RAPD primers (Operon Technologies, Alameda, CA) were conducted as described above for 35 cycles at 94 °C for 1 minute, annealing at 35 °C for

2 minutes, and a 72 °C extension for 2 minutes.

Restriction enzyme digestion and gel electrophoresis: Restriction enzymes were purchased from commercial sources and used according to manufacturers' specifications. All DNA preparations were electrophoretically fractionated on Tris-Borate-EDTA (TBE)-buffered 0.7% to 1.2% (w/v) agarose gels depending upon the application (Maniatis et al., 1982), and repeated a minimum of five times. DNA was visualized by ethidium bromide staining (1.0 μ g/ml) and illumination with a Fotodyne 3-3000 UV transilluminator.

Molecular cloning, DNA sequencing, and DNA sequence analysis: PCR-generated rDNA was ligated into the pCRII cloning vector (InVitrogen, San Diego, CA). The ligation mixture was introduced into *Escherichia coli* INV α F' (supplied by InVitrogen), rendered competent for DNA-mediated transformation (Hanahan, 1983). DNA sequences were determined using the "dideoxy" chain termination method (Sanger et al., 1977).

Multiple sequence alignments were generated with CLUSTAL W (v. 1.4; Higgins et al., 1991). Endpoints delimiting the complete 5.8S rRNA gene were deduced by alignment of the *N. aberrans* sequence with available 5.8S gene sequences from other phytonematodes (Ferris et al., 1994; Ferris et al., 1995; Vahidi and Honda, unpubl. GenBank submission) and from *Caenorhabditis elegans* (Ellis et al., 1986). Distance measurements and phenogram construction were conducted with PHYLIP (v. 3.57). Estimates of confidence in dendrogram branching patterns were obtained by performing a bootstrap test (500 replications).

RESULTS

Isozyme analysis: Isozyme analysis was used for initial evaluation of genetic variation among the four *N. aberrans* isolates. Both acid phosphatase and malate dehydrogenase revealed distinct patterns for all four isolates tested (data not shown). Esterase banding patterns also differed among these same four isolates (Fig. 1) but provided

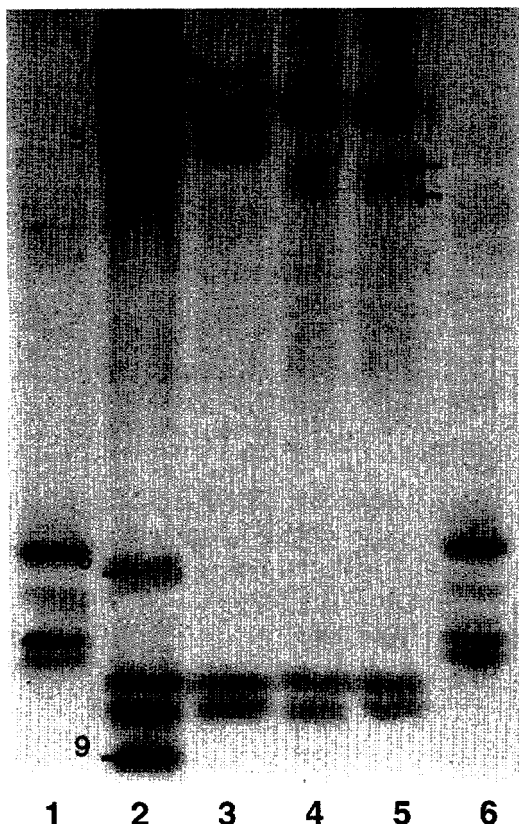


FIG. 1. Isoelectric focusing of non-specific esterases. Lanes 1 and 6, *Meloidogyne javanica*. Lanes 2–5, *Nacobbus aberrans* populations F1, F2, F4, and F5, respectively. Numbers correspond to band numbers used in the text; arrows indicate migration of weakly staining non-specific esterases shared by populations F4 and F5.

some indication of genetic affinity between F1 and F2 and between F4 and F5. To facilitate description of the *N. aberrans* esterases, we divided them into two electromorph groups. Within the cluster of slowly migrating esterases, bands 2 through 5 were shared among isolates F1 and F2; isolates F4 and F5 shared a single high molecular weight form and two diffuse, weakly staining bands (Fig. 1 arrows) not found in F1 and F2. Among the esterases of high electrophoretic mobility (bands 6–9), bands 6 and 9 were found only in isolate F1.

RAPD analysis: To survey a larger portion of the *N. aberrans* genome for genetic variation among these four isolates, we evaluated a panel of 10 RAPD primers with PCR amplification. When total cellular DNA prepared from an individual female representing each isolate was used as template for

RAPD amplification in triplicate reactions, most individual primers typically generated reproducible differences in the numbers and sizes of PCR products. One such example was primer OPM-10 (Fig. 2A). However, a few primers generated amplified fragments that were shared among two of the four isolates. When OPM-04 was used (Fig. 2B), RAPD products identical in size and number were shared between isolates F4 and F5. Four of five PCR products were shared between isolates F1 and F2 when primer OPM-09 was used (Fig. 2C). While RAPD analysis typically revealed genetic variation among each of the four isolates, results with primers M4 and M9 provided support for affinities between F1 and F2 and between F4 and F5, as first suggested by the esterase studies.

rDNA analysis: Results of isozyme and RAPD studies demonstrated genetic variation among these four isolates and identified potential genetic affinities among the populations. To further test these possibilities, we examined divergence within the *N. aberrans* rDNA repeating unit. With use of PCR primers that target the 18S and 28S rRNA coding regions, a 850-bp fragment that spanned the ITS1-5.8S-ITS2 region was amplified from all four *N. aberrans* isolates. Subsequently, these PCR products were digested with 13 different restriction enzymes, six of which revealed restriction site polymorphism (Alu I, Ava II, BstX I, Hae III, Hinf I, and Taq I). With all six enzymes, restriction fragment length polymorphism (RFLP) analysis revealed identical cleavage patterns for isolates F1 and F2; isolates F4 and F5 also shared identically sized restriction products that were readily distinguished from the RFLP characteristic of F1 and F2. Examples of cleavage products generated with Taq I and Hinf I are displayed in Fig. 3. These results were obtained from template DNAs isolated from mixtures of 10 females or individual nematodes representing each isolate.

Within the 850-bp *N. aberrans* rDNA amplification product, the 5.8S rRNA coding region was anticipated to exhibit the greatest sequence similarity relative to flanking ITS sequences. Alignment of the complete

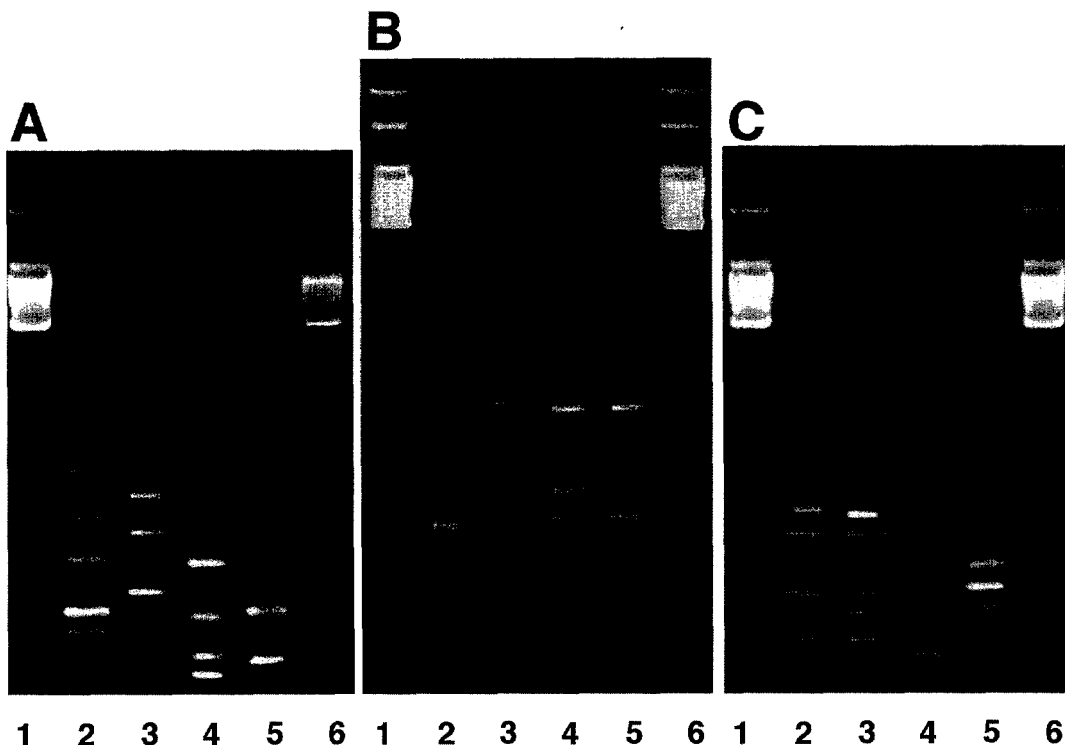


FIG. 2. *Nacobbus aberrans* RAPD amplification product profiles. Cellular DNA obtained from individual females and used as template in PCR reactions with A) primer OPM-10 (5'-TCTGGCGCAC-3'); B) OPM-04 (5'-GGCGGTGTC-3'); C) OPM-09 (5'-GTCTTGCGGA-3'). After fractionation with agarose gel electrophoresis, RAPD-generated bands were visualized by ethidium bromide staining. In each panel, lanes 1 and 6 contain 123 bp DNA size-standard ladder. Lanes 2-5: *N. aberrans* populations F1, F2, F4, and F5, respectively.

5.8S rRNA gene sequence among the four *N. aberrans* isolates likely would provide a conservative estimate of intraspecific genetic variation, and provide a more rigorous test of the affinities among the four isolates as suggested by isozyme, RAPD, and rDNA-RFLP analysis.

Nucleotide differences among the four *N. aberrans* isolates occurred at 8 of 157 positions (Fig. 4) with a striking substitution pattern. Excluding the single G \leftrightarrow A transition at position 4, F1 and F2 5.8S rDNA sequences were identical to each other, as were F4 and F5; substitution only generated dissimilarity between the F1-F2 and F4-F5 pairs. This result was entirely consistent with affinities among the four isolates first observed in the isozyme, RAPD, and RFLP experiments.

Pairwise comparisons of the complete *N. aberrans* 5.8S rRNA genes, along with published intact 5.8S rDNAs for other phytonematodes and *C. elegans*, were displayed in

an uncorrected distance matrix (Table 1) and illustrated in a phenogram compiled from the same distance data (Fig. 5). Nucleotide dissimilarity ranged from 0% for the F1-F2 pair to 5.1% when F1-F2 was compared with F4. Identical results were obtained with methods that incorporate assumptions involving individual nucleotide frequencies, unequal nucleotide substitution rates, transition-transversion ratios, and among-site heterogeneity. These additional analyses included the Jukes-Cantor (1969) one-parameter, Kimura (1980) two-parameter, and LogDet-paralinear (Lockhart et al., 1994) algorithms.

Inclusion of available 5.8S rDNA sequence data for other phytonematodes into this data set permitted comparison of dissimilarity values at several phylogenetic levels. Interspecific comparisons of 5.8S rDNA sequences from the cereal and sugarbeet cyst nematodes, *Heterodera avenae* and *H. schachtii* (Ferris et al., 1994, 1995), gener-

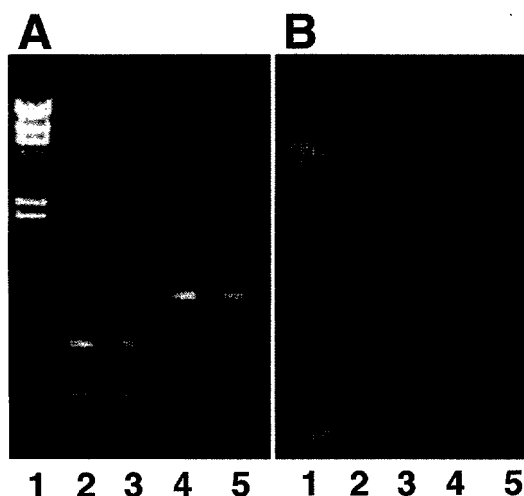


FIG. 3. Restriction fragment length polymorphism analysis of the *Nacobbus aberrans* ITS1-5.8S-ITS2 rDNA region. The 850-bp rDNA fragment amplified from DNA prepared from four *N. aberrans* populations was cleaved with restriction enzymes and the resultant restriction fragments resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. A) Taq I digestion; B) Hinf I digestion. In each panel, lane 1 contains a λ -Hind III (A) or 123 bp ladder (B) size standard, and lanes 2–5 contain rDNA restriction fragments representing populations F1, F2, F4, and F5, respectively.

ated a dissimilarity value approaching 1.0%, compared to 1% to 5% dissimilarity within *N. aberrans* (Table 1). Intergeneric pairwise comparisons with 5.8S sequences from the *Heterodera* spp. and the potato cyst nematode, *Globodera rostochiensis*, were 2.0% to 2.6% dissimilar. These relative diversities were illustrated with branch-length comparisons (Fig. 5), where distances among the *N. aberrans* lineages were equal to or greater than those exhibited by the interspecific comparisons among *Heterodera* spp. or the intergeneric distances among *G. rostochiensis* and representative species of *Heterodera*.

DISCUSSION

The objective of this investigation was to evaluate meaningful approaches for addressing the taxonomic controversy and the inconsistent and unsatisfying species concept applied to *N. aberrans* (Baldwin and Cap, 1992). Four independent tests applied in this investigation consistently supported two groups of isolates within the first four *N.*

aberrans isolates examined by biochemical and molecular methods. For our initial molecular analysis, we examined rDNA, a genomic locus that has become increasingly popular as markers for population and evolutionary studies (Hillis and Dixon, 1991). Affinities among helminths (Qu et al. 1986), including nematodes (Nadler, 1992; Ferris et al., 1994, 1995), are among taxa that have been studied with rDNA analysis.

The two *N. aberrans* groups are divergent with respect to complete 5.8S rRNA gene sequences at a depth that might be expected of species or genera, when compared to other taxa (*Heterodera* and *Globodera*) previously examined in Tylenchida. The 5% dissimilarity observed in comparisons involving *N. aberrans* isolates is four- to eight-fold greater than that observed within *H. avenae*, and is five times that found when interspecific comparisons involving *H. avenae* and *H. schachtii* are considered (Ferris et al., 1994). The *N. aberrans* 5.8S rDNA distances are most equivalent to the 2% to 3% dissimilarity values exhibited by intergeneric comparisons involving *Heterodera* and *Globodera*. In a preliminary study, three unpublished *M. arenaria* 5.8S rRNA gene sequences deposited in the GenBank sequence database also exhibit elevated within-species genetic distances (9% dissimilarity). However, a recent expansive survey of root-knot nematode 5.8S rRNA coding sequences incorporating several different *Meloidogyne* spp. indicates that variation at this locus within *M. incognita* (0.6%) and between *M. incognita* and *M. javanica* (0.6% to 1.2%) is as low as that observed within *H. avenae* (B. J. Adams and T. O. Powers, pers. comm.). Given the diversity of morphological and physiological characters traditionally used to describe *N. aberrans* (Baldwin and Cap, 1992), it was anticipated that isolates of *N. aberrans* would display substantial genetic variation. The present data support this expectation.

Our *N. aberrans* data were not developed specifically to address phylogenetic questions, but some interesting sequence comparisons can be made with other genera where the relatively short, complete 5.8S rRNA gene sequence is known. A distance

1					
F1	CTAGTCTTAT	CGGTGGATCA	CTCGGCTCGT	AGTTCGATGA	AGAACGCAGC
F2	CTAGTCTTAT	CGGTGGATCA	CTCGGCTCGT	AGTTCGATGA	AGAACGCAGC
F4	CTA A TCTTAT	CGGTGGATCA	CTCGGCTCGT	AGTTCGATGA	AGAACGCAGC
F5	CTAGTCTTAT	CGGTGGATCA	CTCGGCTCGT	AGTTCGATGA	AGAACGCAGC
▲					
51					
F1	TAACTGCGAT	AA T TAGTGTG	AACTGCAGAA	GCATTGAGCA	C A GAATCTT T
F2	TAACTGCGAT	AA T TAGTGTG	AACTGCAGAA	GCATTGAGCA	C A GAATCTT T
F4	TAACTGCGAT	AA C TAGTGTG	AACTGCAGAA	GCATTGAGCA	C T GAATCTT C
F5	TAACTGCGAT	AA C TAGTGTG	AACTGCAGAA	GCATTGAGCA	C T GAATCTT C
101					
F1	GAATGCACAT	TGCGCCATTG	GATT T CG G TC	C A TTGGCACG	TTTGGTTCAG
F2	GAATGCACAT	TGCGCCATTG	GATT T CG G TC	C A TTGGCACG	TTTGGTTCAG
F4	GAATGCACAT	TGCGCCATTG	GATT C CG A TC	C T TTGGCACG	TTTGGTTCAG
F5	GAATGCACAT	TGCGCCATTG	GATT C CG A TC	C T TTGGCACG	TTTGGTTCAG
151					
F1	GGTCGT A				
F2	GGTCGT A				
F4	GGTCGT T				
F5	GGTCGT T				

FIG. 4. Alignment of 5.8S rRNA gene coding sequences among four *Nacobbus aberrans* populations. Alignments were conducted with the multiple-sequence alignment program Clustal W (v. 1.4). Nucleotides in bold and enlarged type identify eight substituted positions; seven reveal affinities between populations F1 and F2, and between F4 and F5. The arrowhead (▲) underneath position 4 identifies the eighth remaining substitution among the aligned *N. aberrans* 5.8S rRNA genes. These sequences are available in GenBank, accession numbers U71375 and U71376.

TABLE 1. Uncorrected pairwise distance matrix of nematode^a 5.8S rRNA gene sequences.

	F1	F2	F4	F5	MaA	MaB	MaC	Hsc	Hav	Gro
F2	0.00	—								
F4	0.05	0.05	—							
F5	0.04	0.04	0.01	—						
MaA	0.23	0.23	0.23	0.23	—					
MaB	0.26	0.26	0.25	0.25	0.09	—				
MaC	0.23	0.23	0.22	0.22	0.00	0.09	—			
Hsc	0.11	0.11	0.12	0.12	0.21	0.21	0.21	—		
Hav	0.11	0.11	0.12	0.12	0.20	0.20	0.20	0.01	—	
Gro	0.10	0.10	0.12	0.12	0.23	0.22	0.22	0.03	0.02	—
Cel	0.33	0.33	0.32	0.31	0.30	0.28	0.30	0.32	0.31	0.32

^a F1, F2, *Nacobbus aberrans* isolates from Peru; F4, *N. aberrans* from Mexico; F5, *N. aberrans* from Argentina; MaA-C, *Meloidogyne arenaria* (GenBank accession nos. x57216, x57217, x57220); Hsc, *Heterodera schachtii* (Ferris et al., 1994); Hav, *H. avenae* (U12389); Gro, *Globodera rostochiensis* (Ferris et al., 1995); Cel, *Caenorhabditis elegans* (x03680).

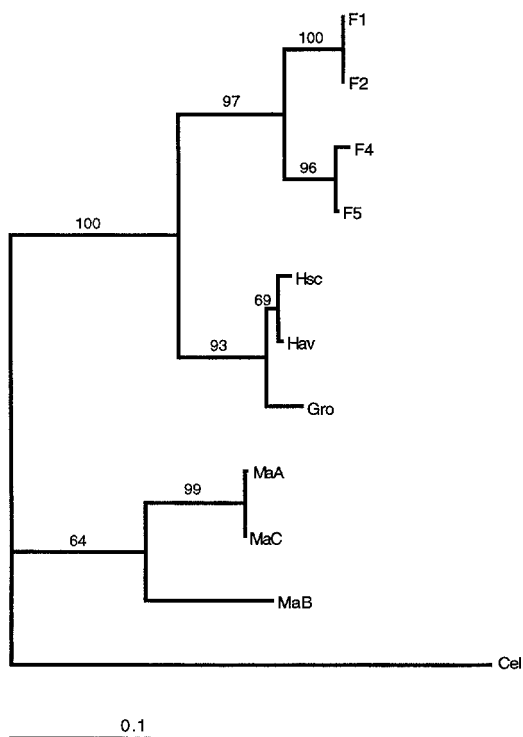


FIG. 5. Neighbor-joining phenogram of genetic distances among nematode 5.8S rRNA gene sequences. Values of bootstrap confidence levels are provided on each interior branch. Scale bar represents 10% dissimilarity.

phenogram compiled from unweighted pairwise sequence comparisons of this region does not contradict the hypothesis that *Nacobbus* and *Heterodera* are contained within a larger monophyletic group that excludes *Meloidogyne*. The argument that cyst nematodes (*Heterodera* and *Globodera*) are not monophyletic with *Meloidogyne* is supported by a wide range of morphological data (Baldwin, 1992). These relationships are further testable by investigations with sequences specifically selected to test phylogenetic relationships. Distance matrices also are consistent with the obvious consideration of *C. elegans* as a member of the outgroup of Tylenchida.

Although only four isolates of *N. aberrans* were evaluated, the broader integrity and monophyly of these two tentative subspecific categories is testable by extending 5.8S rRNA gene sequences to representative isolates of the full range of geographic and physiologic variability of *N. aberrans*. Once

consistent subspecific categories are recognized by sequence alignment, they can then be tested for congruence with phenotypic variables of morphology, phenotype, physiology, and host range, and also with geography. Currently, many such variables (characters) do not provide a basis for meaningful subspecific groupings with taxonomic status because evolution of these characters is likely to be convergent. For example, a particular host range may have evolved independently, and isolates sharing that phenotype would lack a consistent genealogy; congruence of host range and other characters would not be a meaningful marker of taxonomic status in this circumstance. Host range and virulence, as with other phenotypic characters, might simply represent alleles that vary in their frequencies within particular populations (Bakker et al., 1993; Roberts, 1995; Triantaphyllou, 1987). However, subspecific groupings firmly based on molecular sequences provide a framework upon which congruent phenotypic, geographic, or physiologic characters can be placed to extract those affinities that have minimal convergence and maximum consistency with the genotype. This approach will facilitate recognizing integral *N. aberrans* subspecific categories, which then might be considered for meaningful taxonomic status.

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