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Genotypic Analysis of *Heterorhabditis* Isolates from North Carolina

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Different species and strains of pathogens and predators differ in their effectiveness as biological control agents of insects. These differences have been well documented for predatory insects, parasitoids, and a number of entomopathogens (9). The importance of such interspecific and intraspecific variation has been recognized and demonstrated for insect-parasitic steinernematids and heterorhabditids (3). An appreciation of the potential benefits of screening different species and strains of nematode for a particular pest insect on a given crop is necessary in order to optimize the value of collecting and maintaining a large number of nematode isolates from around the world for use as biological control agents. Heterorhabditid nematodes have been collected from insects and soil samples from Australasia, the Americas, and Europe (2,10). Four species have been described: namely, *Heterorhabditis heliothidis* (Khan, Brooks, and Hirschmann, 1976) Poinar, Thomas, and Hess, 1977; *H. bacteriophora* Poinar, 1976; *H. poptha* (Turco), 1970; and *H. hambletoni* (Pereira), 1937. Numerous isolates of *Heterorhabditis* from North Carolina have been tentatively identified on the basis of morphology as *H. heliothidis* (2). The possibility existed that there were intraspecific variants (strains) and sibling species among these isolates.

In the genus *Steinernema*, species determinations have been confirmed by interbreeding studies (1,10). Similar interbreeding data cannot be readily obtained for *Heterorhabditis* isolates because reproduction in the first parasitic generation is by self-fertilizing hermaphrodites. The resulting progeny develop into dauers, and subsequently into self-fertilizing hermaphrodites, or into obligatorily out-crossing females and males. The practicality of using these in interbreeding studies is questionable because of the difficulty of obtaining synchronous cultures and virgin females.

Evidence for reproductive isolation and genetic divergence between morphologically similar nematode populations has been obtained for species in other genera by the detection and analysis of restriction fragment length differences (RFLD) in genomic DNA (5–7). Such genotypic "fingerprinting" can be used to identify interspecific and intraspecific isolates of *Heterorhabditis*. Given the biological control potential of this nematode, a rapid screening technique to distinguish isolates genotypically would be useful. Strains so distinguished could be assessed for variability in virulence for any insect pest management situation. The aim of this study was to determine the genotypic variability of some North Carolina, South Carolina, and California isolates of *H. heliothidis*. It was hoped this would help clarify the relationships between these isolates, identify different genotypes, and suggest divergent isolates for assessment as biological control agents of insects.

Infective juveniles of North Carolina *Heterorhabditis* isolates NC 1, 2, 3, 60, 103,

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124, 162, 171, 183, 210, 224, 403, 447, 480, and 504 were obtained from Dr. W. M. Brooks, North Carolina State University, and Dr. R. J. Akhurst, CSIRO, Tasmania. The South Carolina isolate was obtained from C. S. Creighton, USDA Vegetable Laboratory, Charleston, South Carolina, and the California isolate from Dr. H. K. Kaya, University of California at Davis. Each isolate was passaged separately in larvae of the greater wax moth, *Galleria mellonella,* and the emerging infective juveniles were collected in water traps (10). The infective juveniles of each isolate were washed at least three times with distilled water to remove excess contaminating bacteria, pelleted in a microcentrifuge, and frozen in liquid nitrogen, and the genomic DNA was extracted (7). One-microgram aliquots of total DNA were digested with 10 units of EcoRI (ICN Nutritional Biochemicals) in 0.1 M Tris-HCl, pH 7.2 buffer containing 5 mM $MgCl_2$, 50 mM NaCl, and 2 mM 2-mercaptoethanol; incubated at 37 C for 1 hour; and heated to 65 C for 10 minutes to stop the reaction.

Restriction endonuclease digested DNA samples were mixed with loading buffer (to a final concentration of 5% glycerol, 0.025% bromophenol blue) and placed in slots (5 × 1 mm) of a 0.7% agarose gel containing 0.5 μg/ml ethidium bromide. Gels were electrophoresed at 20 V for 16 hours in 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA running buffer. EcoRI/Hind III cut C1857 lambda DNA was used as size marker. The gel was photographed by 260 nm transmitted irradiation.

Transfer of DNA fragments from the agarose gel to nitrocellulose filters was performed using a bidirectional method (14). DNA filters were pretreated (12); hybridized to ^{32}P labeled cloned 28s, 18s rDNA probe; washed; and exposed to Kodak Blue Brand Film under the following conditions: hybridized at 62 C, 5× SSPE, 0.3% SDS; washed at 62 C, 2× SSPE, 0.3% SDS; exposed overnight to autoradiographic film with intensifying screen.

The cloned rDNA probe was identified and constructed as follows: A repetitive EcoRI fragment, lambda Ces 55, was picked at random from a Charon 4 partial EcoRI library of *Caenorhabditis elegans* strain N2 constructed by T. P. Snutch (Simon Fraser University) and identified as part of the 7 kb EcoRI 28s, 18s ribosomal DNA gene cluster by restriction mapping and northern analysis of total RNA of *C. elegans* (unpubl.). The 7 kb EcoRI fragment was subcloned into pUC19 and used as a ribosomal DNA probe after being nick-translated to a specific activity of 10^7–10^8 cpm/μg using ^{32}PdATP (11).

The *Heterorhabditis* isolates tested had a highly repetitive 3.9 kb EcoRI restriction fragment and one or more smaller equimolar fragments. From examination of these latter highly repetitive DNA fragments, it was possible to divide the North Carolina isolates into three groups (Fig. 1): Group 1, with a 3.2-kb repeat (isolates NC 2, 3, 60, 103, 162, 171, 183, 210, 224, 480, 504); Group 2, with a 3.1-kb repeat (NC 1); and Group 3, with 1.7- and 1.5-kb repeats (NC 447). In addition to these prominent highly repetitive DNA fragments, *Heterorhabditis* isolates in Group 1 share middle-repetitive EcoRI restriction fragments not common to Groups 2 or 3. The California isolate was shown to be indistinguishable from Group 1, and the South Carolina isolate was indistinguishable from Group 3. In repeated gel runs and with different restriction enzymes (Bam HI, Hind III), identical groupings of isolates were obtained on examination of repetitive DNA restriction fragment length patterns (data not shown).

The *C. elegans* 7-kb ribosomal DNA probe hybridized to all the highly repetitive DNA fragments in all isolates tested, with the exception of the 1.5-kb repeat of NC 477 (Fig. 2).

Genomic DNA analyses separated the *Heterorhabditis* isolates into three genotypic groups based on restriction fragment length differences between the highly repetitive DNA fragments. These highly repetitive DNA fragments were identified as the 28s, 18s, 5.8s ribosomal DNA genes by

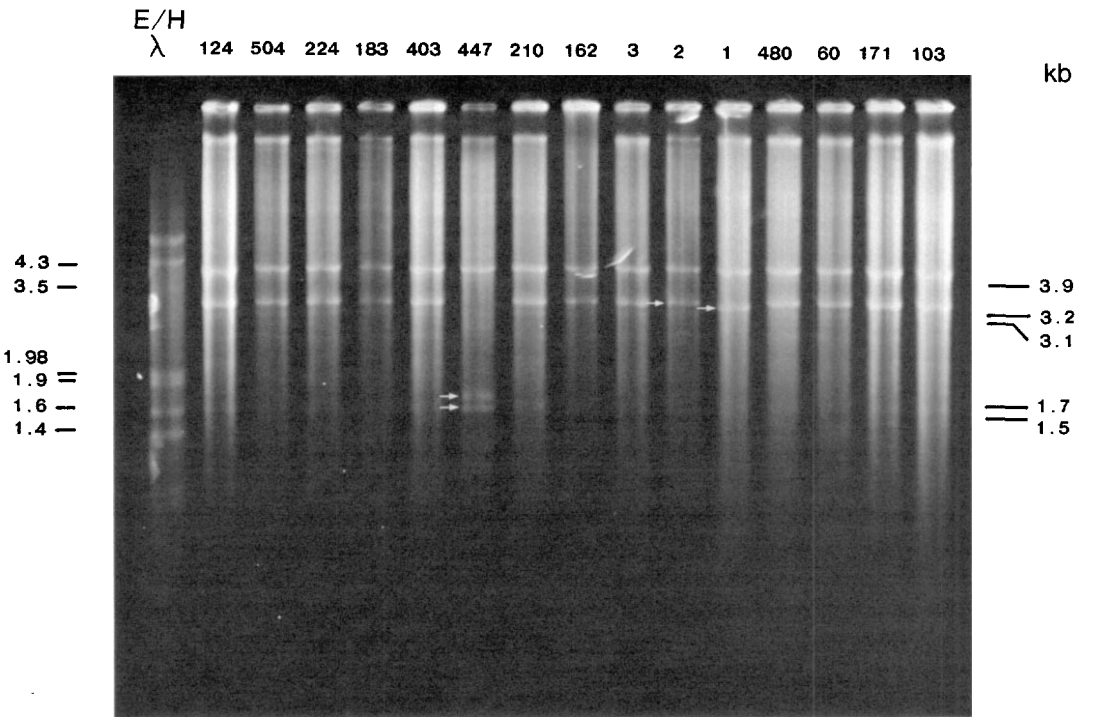

FIG. 1. Agarose gel separation of North Carolina *Heterorhabditis* isolates DNA fragments. Photograph of ethidium bromide stained agarose gel viewed under 260 nm transmitted irradiation showing the size distribution of EcoRI cut total genomic DNA. Lane E/H) EcoRI/Hind III cut C1857 lambda DNA; marker fragment size in kilobases indicated at left. Lanes 124–103) EcoRI digests of total genomic DNA of North Carolina *Heterorhabditis* isolates by accession number. Highly repetitive DNA fragments at 3.9, 3.2, 3.1, 1.7, and 1.5 kilobases are arrowed and marked in the right margin.

positive hybridization to the *C. elegans* ribosomal DNA probe. The restriction fragment length patterns derived from different restriction endonuclease digests indicate that the ribosomal cluster is tandemly arranged in *Heterorhabditis*. These results are the first evidence that these North Carolina isolates are not identical.

In higher eucaryotes, approximately 20% of the genomic DNA is organized as different families of repetitive DNA sequences; e.g., ribosomal genes, histone genes, and centromeric repeats. One of the interesting features of such repetitive DNA sequences is the generally observed pattern, in diverse animal groups, of intraspecific homogeneity and interspecific heterogeneity within a particular repeat family (concerted evolution). This pattern has been observed in a number of nematode groups (5–7). On the basis of empirical evidence, Curran et al. (6) proposed that restriction fragment length differences in repetitive DNA sequences are indicative of a barrier to geneflow between nematode populations and can mark the boundaries between species. Restriction fragment length differences, however, cannot be used as the sole criterion for reproductive isolation and species delimitation. In some nematode species, for example *Trichinella spiralis*, repetitive DNA differences occur between populations that can interbreed in the laboratory (5). In addition, a low rate of intraspecific variation of restriction fragment length differences in ribosomal DNA has been demonstrated between individuals from wild populations of *Drosophila mercatorum* (15). The question arises, therefore, as to the relationship between the three genotypic groups of *Heterorhabditis* demonstrated here: Are they intraspecific variants of a single species, *H. heliothidis* (population NC 1 is the isolate from

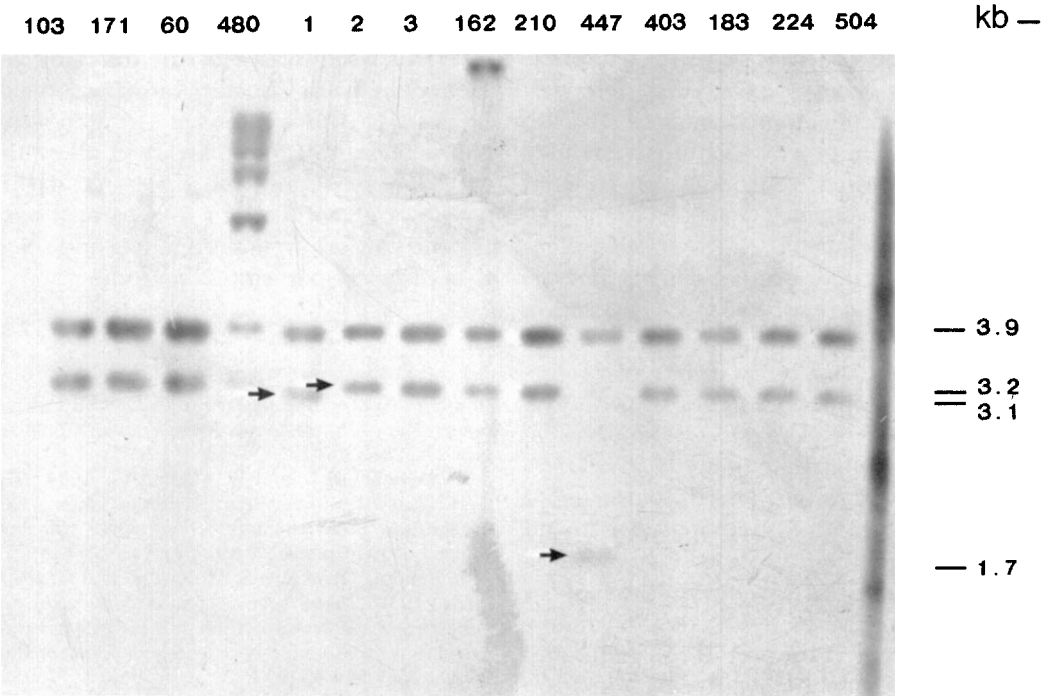

FIG. 2. Autoradiograph of Southern blot showing hybridization of *C. elegans* rDNA probe (pCes 55) to the highly repetitive EcoRI restriction fragments of North Carolina *Heterorhabditis* isolates by accession number. Highly repetitive DNA fragments at 3.9, 3.2, 3.1, and 1.7 kilobases are arrowed and marked on the right margin. Lane 480 contains partial digestion products.

the type locality), or is each group a distinct species? It should be noted that the original isolates were obtained by the *Galleria* trap method (2) and that the initial population size cannot be determined. Subsequent DNA analyses were made on pooled genomic DNA from many thousands of infective juveniles for each isolate. Thus the visualized ribosomal DNA and repetitive DNA restriction fragment length patterns represent a population mean; i.e., low rates of intra-isolate sample variation would not be visualized. In view of this, it is reasonable to claim that there are distinct genotypic differences between the population means of the three groups. There is a slight possibility, however, that the original isolates were started from a single infective nematode that, by chance, had a high frequency of a restriction fragment length variant in its rDNA cluster; as such, isolates represent an isofemale line of an intrapopulation variant.

Consideration of the geographical distribution of the isolates may help resolve this problem. Group I isolates were obtained from widely separated locations within North Carolina (2) and from a single site in California (Kaya, pers. comm.). Group 2 is a single isolate from North Carolina, and the type material for the description of *H. heliothidis* was obtained from this line (NC 1, originally designated NC 19). Group 3 is comprised of NC 447 from North Carolina and the isolate from Charleston, South Carolina (4). It is unlikely, in the case of Groups 1 and 3, that two randomly chosen isolates from different geographic locations would present the same high frequency intraspecific restriction fragment length variant by chance. Furthermore, it is notable that although all three genotypes occur in a small geographic area, no isolates displayed a heterogeneous rDNA restriction fragment length pattern. Indeed, subsequent isolation of nematodes from the original NC 447 sample site has yielded Group 1 ge-

notype only. Thus, despite a sympatric distribution for all three genotypes, no heterogeneous isolates have been detected. The results reported here suggest that the three genotypic groups identified here may represent distinct, reproductively isolated *Heterorhabditis* species.

If this is the case, then the specific identity of the Group 1 and Group 3 isolates now arises. The repetitive DNA restriction fragment length patterns of Groups 1 and 3 differ from that of an isolate of *H. bacteriophora* examined (data not shown). Living specimens of *H. hambletoni* and *H. hoptha* are not available, so the repetitive DNA restriction fragment length patterns of these species cannot be compared as an aid in taxonomic identification. Placing these isolates into distinct groups on the basis of RFLD data may lead to a better understanding of the taxonomy of the genus and encourage morphological re-examination of these nematodes and recognition of distinct morphological characteristics.

One of the original purposes of this study was to identify strains of *Heterorhabditis* species from North Carolina for pest control purposes. It was believed that if many isolates are available, it would be more efficient to screen genetically divergent rather than similar isolates for differences in biological control potential in a given pest situation. The fact that Group 1 isolates from two widely divergent sites are indistinguishable using the RFLD technique is indicative that they are the same species, but these isolates differ in virulence when applied against insects. The two *Heterorhabditis* isolates, NC 1 from Group 2 and NC 447 from Group 3, were used in trials to determine their efficacy as biological control agents of the strawberry root weevil (*Otiorhynchus ovatus*). Significant differences were noted between these two isolates in their biocontrol potential (13). Thus a genotypic difference, as determined by RFLDs, was associated with biological differences in the ability of these nematodes to control the strawberry root weevil. This genotypic "fingerprinting" of isolates has the potential of being a rapid first screen of the genetic divergence of collected isolates but, as indicated by the differing virulence of Group 1 isolates, information may be lost. In the long term the genotypic characterization of isolates will allow the assessment of genetic diversity within species and populations and form a basis for the collection, maintenance, and selection of insect-parasitic nematodes.

Literature Cited

1. Akhurst, R. J., and R. A. Bedding. 1978. A simple cross-breeding technique to facilitate species determination in the genus *Neoaplectana*. Nematologica 24:328–330.

2. Akhurst, R. J., and W. M. Brooks. 1984. The distribution of entomophilic nematodes (Heterorhabditidae and Steinernematidae) in North Carolina. Journal of Invertebrate Pathology 44:140–145.

3. Bedding, R. A., A. S. Molyneux, and R. J. Akhurst. 1983. *Heterorhabditis* spp., *Neoaplectana* spp., and *Steinernema kraussei*: Interspecific and intraspecific differences in infectivity to insects. Experimental Parasitology 55:249–257.

4. Creighton, C. S., and G. Fassuliotis. 1985. *Heterorhabditis* spp. (Nematoda: Heterorhabditidae): A nematode parasite isolated from the banded Cucumber beetle *Diabrotica balteata*. Journal of Nematology 17:150–153.

5. Curran, J., and J. M. Webster. 1984. Reproductive isolation and taxonomic differentiation of *Romanomermis culicivorax* Ross and Smith, 1976 and *R. communensis* Galloway and Brust, 1979. Journal of Nematology 16:375–379.

6. Curran, J., D. L. Baillie, and J. M. Webster. 1985. Use of restriction fragment length differences in genomic DNA to identify nematode species. Parasitology 90:137–144.

7. Curran, J., M. A. McClure, and J. M. Webster. 1986. Genotypic analysis of *Meloidogyne* populations by detection of restriction fragment length differences in total DNA. Journal of Nematology 18:83–86.

8. Maniatis, T., E. J. Fritsch, and J. Sambrook. 1982. Molecular cloning, a laboratory manual. New York: Cold Spring Harbor.

9. Messenger, P. S., F. Wilson, and M. J. Whitten. 1976. Variation, fitness, and adaptability of natural enemies. Pp. 209–231 *in* C. B. Huffaker and P. S. Messenger, eds. Theory and practice of biological control. New York: Academic Press.

10. Poinar, G. O. 1979. Nematodes for biological control of insects. Boca Raton, FL: CRC Press.

11. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labelling deoxyribonucleic acid to high specific in vitro by nick translation with DNA polymerase I. Journal of Molecular Biology 113:237–251.

12. Rose, A. M., D. L. Baillie, E. P. M. Candido, K. A. Beckenbach, and D. Nelson. 1982. The linkage mapping of cloned restriction fragment length dif-

ferences in *Caenorhabditis elegans.* Molecular and General Genetics 188:286–291.

13. Rutherford, T. A., D. Trotter, and J. M. Webster. 1987. The potential of heterorhabditid nematodes as control agents of root weevils. The Canadian Entomologist 119:67–73.

14. Smith, G. E., and M. D. Summers. 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl paper. Analytical Biochemistry 109:123–129.

15. Williams, S. M., R. DeSalle, and C. Strobeck. 1985. Homogenization of geographical variants at the nontranscribed spacer of rDNA in *Drosophila mercatorum.* Molecular Biology Evolution 2:338–346.