Journal of Nematology 26(3):260–270. 1994.

Characterization of *Heterorhabditis* Isolates by PCR Amplification of Segments of mtDNA and rDNA Genes[1]

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Abstract: Restriction digests of amplified DNA from the mitochondrial genome and the nuclear ribosomal internally transcribed spacer region have been evaluated as genetic markers for species groups in *Heterorhabditis*. Six RFLP profiles have been identified. These profiles supported groupings determined by cross-breeding studies and were in agreement with less definitive groupings based on other biochemical and molecular methods. Digestion patterns of both amplification products provided strong evidence for the recognition of species groups, which include Irish, NW European, tropical, and a *H. bacteriophora* complex. The *H. bacteriophora* complex could be further resolved into three genotypes represented by *H. zealandica*, the *H. bacteriophora*, Brecon (Australian) type isolate for *H. bacteriophora*, and a grouping composed of isolates NC1, V16, HI82, and HP88. All cultures obtained of the *H. megidis* isolate were identical to the NW European group. These results could be used to aid monitoring of field release of *Heterorhabditis* as well as allowing a rapid initial assessment of taxonomic grouping.

Key words: entomopathogenic nematode, *Heterorhabditis*, molecular diagnostics, mtDNA, nematode, ribosomal DNA.

The potential of entomopathogenic nematodes of the genus *Heterorhabditis* (family Heterorhabditidae Poinar 1975) as biological control agents has stimulated much interest in the ecology, biology, and distribution of these nematodes and their bacterial symbiont *Photorhabdus* (2,10). Currently four species of *Heterorhabditis* are recognized on the basis of morphology: *H. bacteriophora* Poinar 1975; *H. megidis* Poinar, Jackson, and Klein 1987; *H. zealandica* Poinar 1990; and *H. indicus* Poinar, Karunaker and Hastings 1992. A proposal for a fifth species from China has been presented in a conference abstract (7). Undescribed species have been identified in the literature (4,16) and genetic variation among several common laboratory isolates suggests that additional genetically differentiated taxa exist in the genus (1,6,9). Characterization of these taxa has included morphological criteria (8–12), allozyme electrophoresis (1), isoelectric focusing of soluble proteins (6), DNA analysis by hybridization with repetitive probes (3), and restriction endonuclease digestion of genomic DNA (16).

No single method has allowed unambiguous placement of an individual nematode into the various *Heterorhabditis* taxa. Crossbreeding studies have confirmed the reproductive isolation of *H. bacteriophora, H. megidis, H. zealandica,* and an undescribed species from Ireland (4). A widely distributed laboratory strain, HP88 originally isolated from Utah (9), has been grouped with *H. bacteriophora,* although crossbreeding between the HP88 and the Brecon, Australia, isolate of *H. bacteriophora* (11) did not produce fertile offspring (4). A cluster analysis of dissimilarity values derived from allozyme patterns (1) supports three broad groupings, "bacteriophora group," a tropical group that included isolates from China, Australia, and Cuba, and a third group that contained isolates from New Zealand, Australia, Lithuania, and Russia. The New Zealand isolate (19) was later recognized as a distinct species *H. zealandica* Poinar 1990 (10). Restriction endonuclease digestion patterns of genomic DNA also supported three groupings; however, this method could not discriminate between the *H. bacteriophora* group and a New Zealand isolate (16). The

Received for publication 30 September 1993.

[1] Journal Series Number 10618, Agricultural Research Division, University of Nebraska. Research was funded in part by the European Community (ECLAIR contract AGRE0002).

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We acknowledge the assistance of the researchers who provided us with nematodes from their collections.

Brecon type isolate, USA isolates from North Carolina, California, Utah (HP88), and isolates from mid and southern Europe were also included in the *H. bacteriophora* group by these authors. The other groupings supported in the study consisted of NW European isolates and an Irish group. Isoelectric focusing and SDS PAGE (6) confirmed the genetic distinction of the Irish, NW European, tropical, and *H. bacteriophora* groups but could not distinguish between *H. bacteriophora* and New Zealand isolates. The taxonomic affinity of *H. megidis* is unclear, although protein patterns of several laboratory cultures indicated conspecific status with the undescribed NW European species (6,16). None of the comparative studies described above has included *H. indicus* or the Chinese isolate reported by Liu (1992).

In the present study, we report the application of a PCR method to evaluate *Heterorhabditis* taxonomic groupings based on the amplification of both nuclear and mitochondrial DNA (mtDNA) from individual infective juveniles (IJ) and from DNA isolated from bulked samples of IJ. Two PCR primer sets were used. The first set amplified the internal transcribed spacer region, their flanking 18 S and 28 S coding sequences, and presumably, the 5.8 S gene of the nuclear ribosomal gene cluster (17). The second set amplified the 3′ portion of the mitochondrial cytochrome oxidase subunit II gene and approximately 800 base pairs of the large ribosomal subunit (16 S rRNA) gene (13). Restriction digestion of the amplification products provided a rapid method for the determination of species grouping.

TABLE 1. Source and origin of *Heterorhabditis* isolates, grouped according to biological species (4,5,6).

Isolate	Biological species	Location	Source
K122	Irish	North Slobs, Wexford, Ireland	Dr. C. T. Griffin[1]
M170	Irish	Rosses Point, Sligo, Ireland	Dr. C. T. Griffin
M217	Irish	Corballis, Dublin, Ireland	Dr. C. T. Griffin
M266	Irish	White Strand Bay, Donegal, Ireland	Dr. C. T. Griffin
M288	Irish	Ballyhiernan Bay, Donegal, Ireland	Dr. C. T. Griffin
M244	Irish	Benone, Derry, Northern Ireland	Dr. C. T. Griffin
M385	Irish	Killibegs, Donegal, Ireland	Dr. C. T. Griffin
S159	Irish	Fraserburgh, Scotland	Dr. C. T. Griffin
S29	Irish	Balinakeil, Scotland	Dr. C. T. Griffin
W9	Irish	Pendine, Wales	Dr. C. T. Griffin
W18	Irish	Tenby, Wales	Dr. C. T. Griffin
W30	Irish	Fresh Water East, Wales	Dr. C. T. Griffin
W31	Irish	Fresh Water East, Wales	Dr. C. T. Griffin
W70	Irish	Cornelly, Wales	Dr. C. T. Griffin
UK462	Irish	Norfolk, England	Dr. W. Hominick[2]
UK211	North West European (NWE)	Dorset, England	Dr. W. Hominick
HL81	North West European (NWE)	Netherlands	Dr. P. Westerman[3]
HF85	North West European (NWE)	Netherlands	Dr. P. Westerman[3]
HP88	NC1	Utah, U.S.A.	Dr. P. Westerman
HI82	NC1	Italy	Dr. P. Westerman[3]
NC1	NC1	North Carolina, U.S.A.	Dr. W. Brooks[4]
V16	Not determined	Victoria, Australia	Ms. Tracey Nelson[5]
HSH1	NWE	Kiel, Germany	Dr. P. H. Smits[6]
HSie	NWE	SiedIce, Poland	Dr. P. H. Smits
P_2M	Tropical	Havana province, Cuba	Dr. Z. Mracek[7]
H. bacteriophora	*H. bacteriophora*	Brecon, Australia	Dr. R. J. Akhurst[8]
H. zealandica (NZH_3)	*H. zealandica*	New Zealand	Dr. R. J. Akhurst
H. megidis	NWE	Ohio, U.S.A.	Dr. R. J. Akhurst

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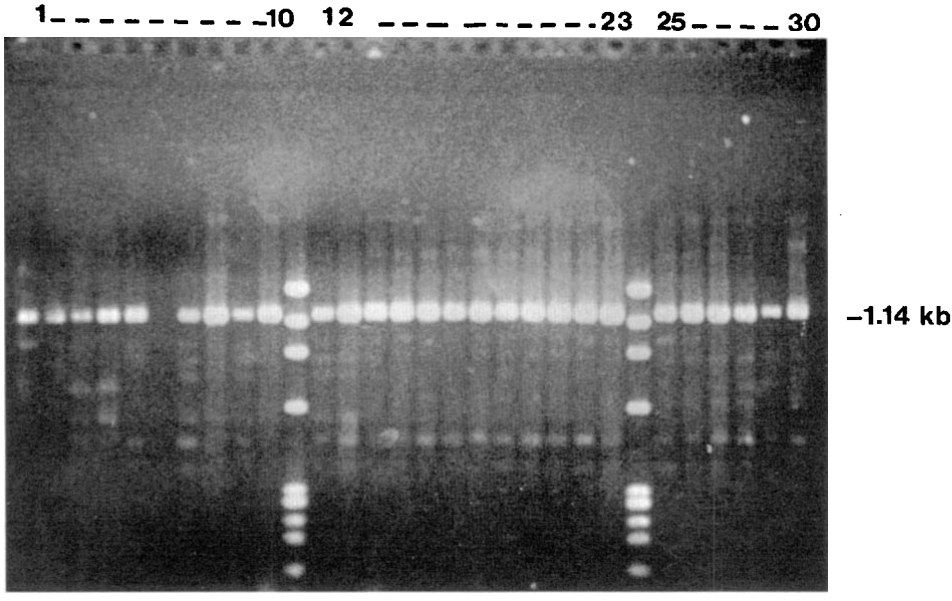

FIG. 1. Amplification of DNA from individual crushed infective juveniles from 26 *Heterorhabditis* isolates using primers (C_2F_3/LRNB1R) for the COII-LrRNA region fractionated on an ethidium-bromide-stained, 1.5% agarose gel. Lane 6 indicates a failed reaction, and lanes 11 and 24 contain *Hae*III cut 0 × 174 (Gibco) as a size marker.

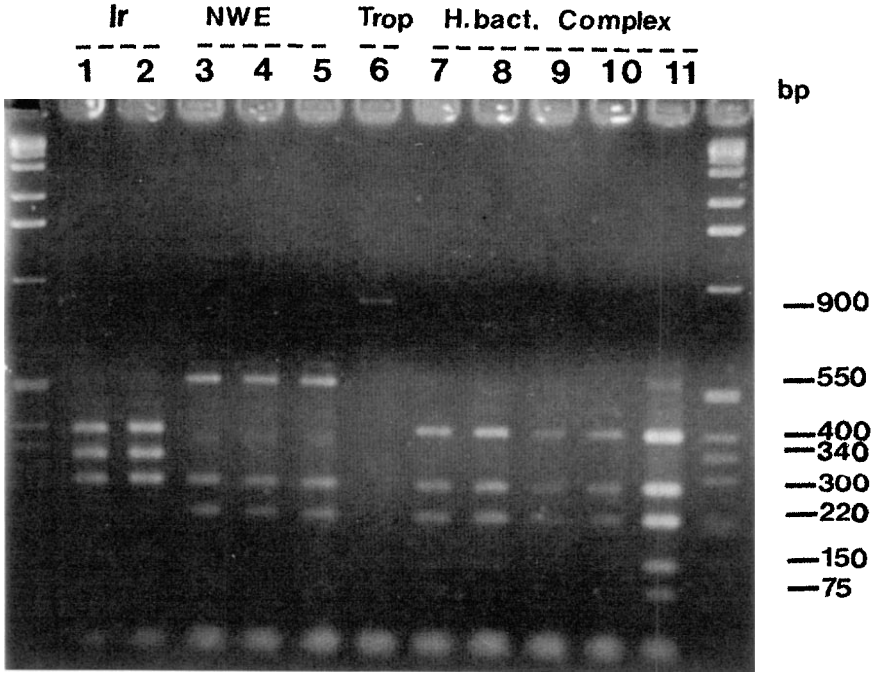

FIG. 2. *Ssp* I restriction digest of the C_2F_3/LRNB1R PCR fragment separated on an ethidium-bromide-stained, 2% agarose gel. 1 kb size marker. Strains: 1:K122, 2:M244, 3:HSH1, 4:*H. megidis*, 5:HF85, 6:P_2M, 7:HP88, 8:*H. zealandica*, 9:*H. bacteriophora*, Brecon, 10:NC1, 11:V16, 1 kb size marker. The Irish isolates (Ir, lanes 1 and 2) and the NW European isolates (NWE, lanes 3–5) share a 300-bp digestion product; however, characteristic restriction fragments of 400 and 340 bp occur among Irish isolates. P_2M (Trop) gives a distinctive profile with characteristic restriction fragments of which only the 900-bp fragment is visible here. All the members of the *H. bacteriophora* species complex (H. bact. complex; lanes 7–11) show the same restriction fragment pattern. The bright band at the front of the gel represents unincorporated primer. Less intense bands in lanes 3–5 and 11 are due to incomplete digestion.

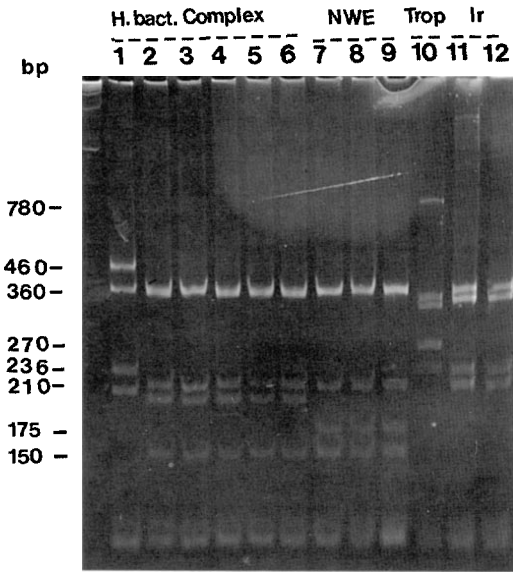

FIG. 3. *Dra* I restriction digest of the C_2F_3-LRNB1R amplified fragment separated on an ethidium-bromide-stained, 8% polyacrylamide gel. 1 kb marker. Strains: 1:*H. zealandica*, 2:NC1, 3:*H. bacteriophora*, Brecon, 4:HI82, 5:V16, 6:HP88, 7:*H. megidis*, 8:HF85, 9:HSH1, 10:P_2M, 11:M244, 12:K122. Characteristic restriction fragments of 340 and 230 bp occur in the Irish isolates (Ir), and 175 and 150 bp fragments characterize the NW European (NWE) group. *H. zealandica* can be distinguished from the other members of the *H. bacteriophora* group (H. bact. complex) by unique restriction fragment of 460-bp.

MATERIALS AND METHODS

Nematode isolates: The isolates used in this study are maintained in the Department of Biology, St. Patrick's College, Maynooth. The sources from which the isolates were originally obtained are listed in Table 1. Each isolate in Table 1 was examined by restriction analysis. A representative subset of these isolates was included in Figures 1–7. All isolates were cultured in vivo in *Galleria mellonella* larvae (18).

DNA isolation: Approximately 1 g of IJ was homogenized in liquid nitrogen and DNA was isolated from the homogenate by incubation with proteinase K, followed by phenol/chloroform extractions and ethanol precipitation (15) (Fig. 4). DNA template was also obtained by crushing individual IJ suspended in a 15-μl drop of sterile water (13). The IJ were crushed using a sterile translucent micropipet tip while viewing through a dissecting microscope.

PCR primers: We have used two sets of "universal" nematode primers: one set was designed by Vrain et al. (17) to flank and amplify the internal transcribed spacer region (ITS) of the rDNA cistron of *Xi-*

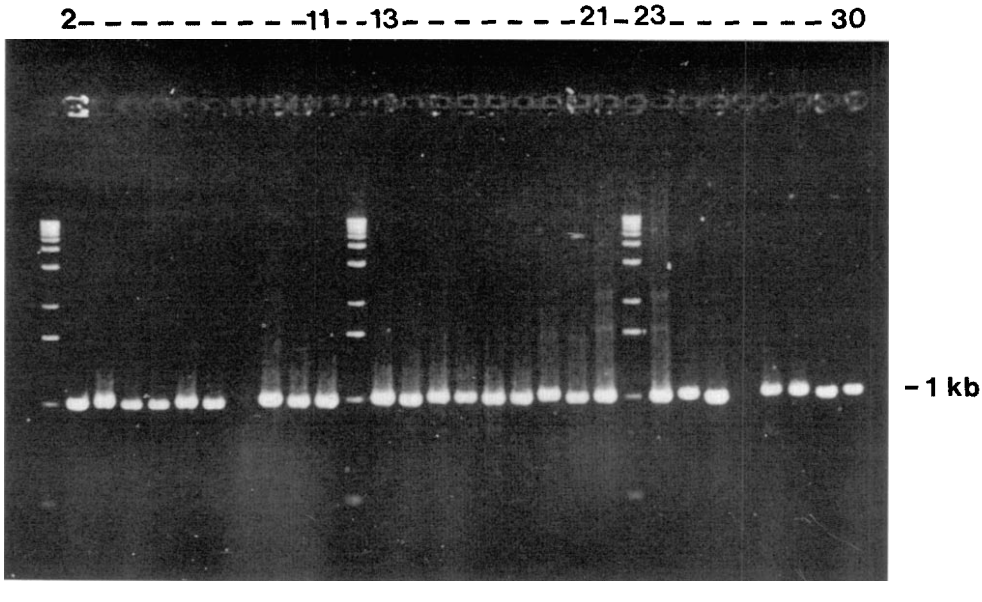

FIG. 4. Amplified Internal Transcribed Spacer region of the rDNA cistron from 27 *Heterorhabditis* isolates using purified DNA as a template. Ethidium-bromide-stained, 1.5% agarose gel. Empty lanes (7 and 26) represent failed reactions. Lanes 1,12 22; 1 kb size markers (Gibco).

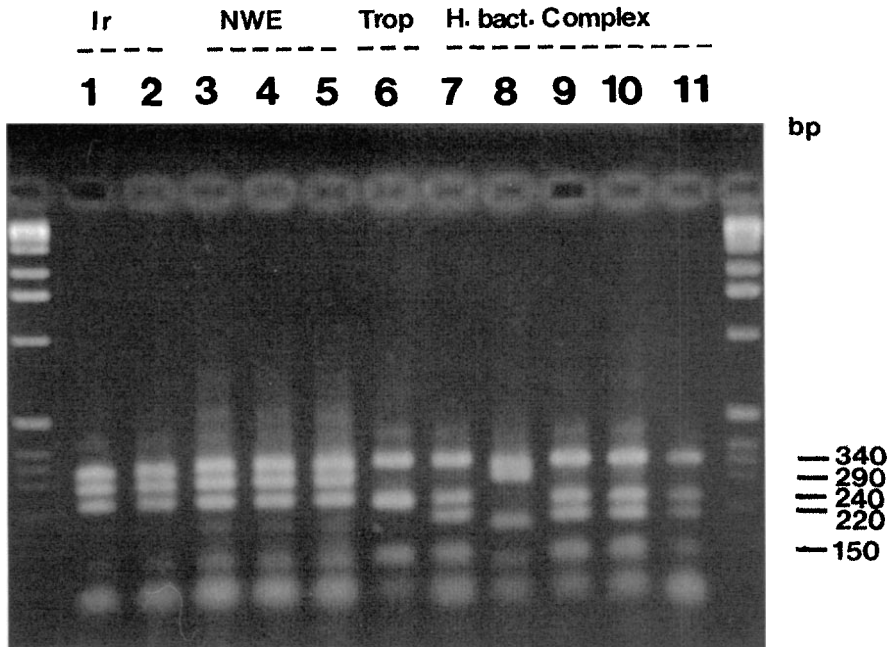

FIG. 5. *Alu* I restriction digest of Amplified Internal Transcribed Spacer region rDNA of *Heterorhabditis* isolates separated on an ethidium-bromide-stained, 2% agarose gel; 1 kb size marker. Strains: 1:K122, 2:M244, 3:HSH1, 4:*H. megidis*, 5:HF85, 6:P_2M, 7:HP88, 8:*H. zealandica*, 9:*H. bacteriophora*, Brecon, 10:V16, 11:NC1, 1 kb size marker. *H. zealandica* can be distinguished from the other members of the *H. bacteriophora* species complex (H. bact. complex) by the presence of 290- and 210-bp fragments and by the absence of the 240-bp fragment found in other members of the complex. The Irish isolates (Ir) and the NW European (NWE) isolates exhibit similar patterns. Less intense variable bands above 340 bp are due to incomplete digestion.

phinema and the second set was designed to amplify the mtDNA cytochrome oxidase subunit II (COII) gene and the 16 S gene. Primer C_2F_3, 5′-GGTCAATGTTCAGAAATTTGTGG-3′ (13) and primer #LRNB1R 5′-ATAATTTTCCTTTCGTACT-3′ were designed by nucleotide sequencing and alignment of mtDNA sequence data from *Meloidogyne incognita* (14) with available sequence in Genbank. Primers were synthesized at the DNA synthesis facility of the University of Nebraska Center for Biotechnology and by Operon Technologies Inc., Alameda, California.

PCR amplification: Identical conditions were used for both sets of primers. PCR amplifications were performed in 25-µl volumes containing 50 mM KCl, 2 mM $MgCl_2$ 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.25 mM of each dNTP, 0.8 mM of each primer, 15 µl nematode lysate and 2.5 units of *Taq* DNA polymerase under a mineral oil overlay. This mixture was placed in a thermal cycler (Prem™, Lep Scientific) already heated to 94 C and subjected to a "hot start" of 5 minutes at 94 C followed by 35 cycles of denaturation at 94 C for 1 minute, reannealing at 44 C for 2 minutes and extension at 72 C for 3 minutes. A 5-minute incubation period at 74 C followed the last cycle in order to complete any partially synthesized second strands. When purified DNA was used as the template, the PCR reaction conditions were identical to those described above, except that the nematode lysate was replaced by 15 ng of purified DNA. Amplification products were stored at −20 C until utilized.

Restriction digestion and electrophoresis of PCR amplified DNA: Following PCR, 6 µl from each reaction was fractionated on a 1.5% agarose gel in TBE, pH 8.0 (14) at 5 V/cm for 1.5 hour and stained with ethidium bromide. To increase the resolution of smaller restriction fragments, 8% poly-

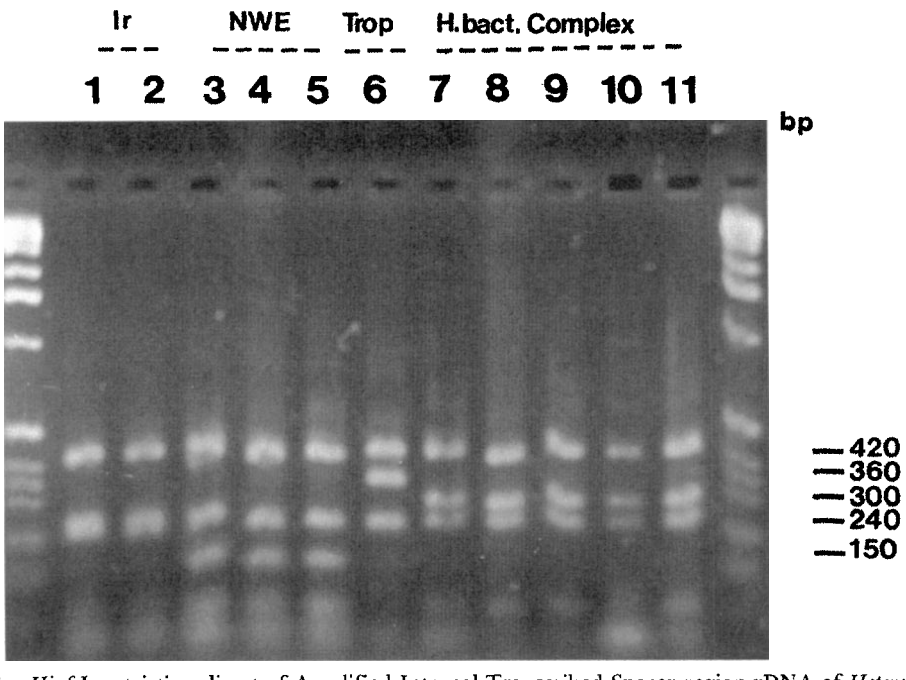

FIG. 6. *Hinf* I restriction digest of Amplified Internal Transcribed Spacer region rDNA of *Heterorhabditis* isolates separated on an ethidium-bromide-stained, 2% agarose gel; 1 kb size marker, 1:K122, 2:M244, 3:HSH1, 4:*H. megidis*, 5:HF85, 6:P_2M, 7:HP88, 8:*H. zealandica*, 9:*H. bacteriophora*, Brecon, 10:NC1, 11:V16, 1 kb size marker. The NW European (NWE) isolates and *H. megidis* are differentiated from the Irish isolates (Ir), K122, and M244, by a characteristic restriction fragment at ca. 150 bp. All members of the *H. bacteriophora* complex (H. bact. complex; lanes 7–11) share the same restriction pattern. A 360-bp fragment distinguishes P_2M (Trop) from the other isolates.

acrylamide minigels (Mini Protean II, Bio Rad) were occasionally run at 8 V/cm for 3–4 hours and stained with 5 mg/ml ethidium bromide.

RESULTS

Amplification and restriction of the mtDNA PCR product: Although suitable DNA template was obtained from the lysate of an individual crushed IJ (Fig. 1), we have observed that changing to different batches of *Taq* and of PCR primer sometimes resulted in a dramatic reduction in the number of successful amplifications from crushed IJ. With such reagents, successful amplification was always achieved when purified total DNA was used as the template. The PCR amplification products obtained using crushed nematode suspension (Fig. 1) were the same size as those obtained when purified DNA was used as the template. All six *Heterorhabditis* species and all isolates investigated yielded a ca. 1.14-kb fragment following PCR amplification. A series of restriction digests was performed on the amplification products, and the results obtained are summarized in Table 2. Identical restriction products were obtained whether purified DNA or crushed nematode suspension was used as the template. Restriction patterns were consistently reproducible, allowing further isolates to be easily identified. Two of the ten enzymes tested provided species-specific restriction patterns.

Restriction with *Ssp* I (Fig. 2) distinguished between the Irish isolates (lanes 1 and 2) and the NW European isolates (lanes 3 and 4). The restriction pattern obtained for the NW European isolates was identical to that of *H. megidis* (lane 4) and a distinct pattern characterized by the presence of an approximately 900-bp fragment and two small (<150 bp) fragments was observed for the tropical isolate P_2M (lane

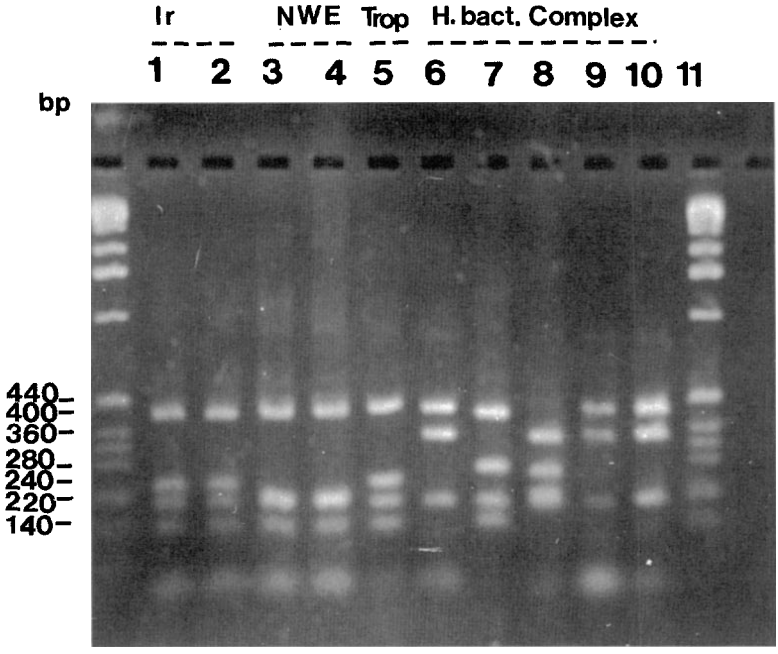

FIG. 7. *Mbo* I restriction digest of Amplified Internal Transcribed Spacer region rDNA of *Heterorhabditis* isolates separated on an ethidium-bromide-stained, 2% agarose gel; 1 kb size marker, 1:K122, 2:M244, 3:HSH1, 4:*H. megidis*, 5:P_2M, 6:HP88, 7:*H. zealandica*, 8:*H. bacteriophora*, Brecon, 9:NC1, 10:V16, 1 kb size marker. The NW European (NWE) isolates are distinguishable from the Irish (Ir) isolates by the presence of a restriction site in the 240-bp fragment. HP88, NC1, and V16 share the same restriction fragment pattern with 440-, 390-, and 220-bp fragments. *H. zealandica* and *H. bacteriophora*, Brecon, both exhibit unique digestion patterns permitting differentiation among the *H. bacteriophora* species complex.

6). Restriction of the mtDNA fragment with *Ssp* I did not, however, distinguish among isolates of the *H. bacteriophora* species complex (lanes 7–11). Five different digestion profiles were obtained upon *Dra* I digestion (Fig. 3). Each of the isolates of the *H. bacteriophora* complex (lanes 2–6) produced an identical pattern, with the exception of *H. zealandica* (lane 1), which was characterized by unique 460-bp fragment and which appeared to share two small fragments of 236 and 210 bp with the Irish isolates. *H. megidis* (lane 7) and the NW European isolates (lanes 8 and 9) shared the same digestion profile, and P_2M (lane 10) and the Irish isolates (lanes 11 and 12) gave distinct patterns from each other and from other isolates.

Amplification and restriction of the rDNA PCR product: All the *Heterorhabditis* species and isolates investigated yielded a ca. 1-kb fragment upon PCR amplification with the ITS rDNA primers (Fig. 4). The results obtained from a series of restriction digests of these amplified ITS fragments are summarized in Table 3. The enzyme *Alu* I (Fig. 5) did not discriminate among the Irish isolates (lanes 1 and 2) and the NW European isolates including *H. megidis* (lanes 3–5). The tropical isolate P_2M (lane 6) produced a unique digestion pattern, and *H. zealandica* (lane 8) was distinct from the other members of the *H. bacteriophora* complex (lanes 7, 9–11). The Irish isolates and the NW European isolates were distinct in *Hinf* I restriction fragment patterns (Fig. 6, lanes 1–5); however, *Hinf* I did not separate *H. zealandica* (lane 8) from the other members of the *H. bacteriophora* complex (lanes 7, 9–11). The tropical isolate P_2M (lane 6) again produced a unique pattern. *Mbo* I digestion (Fig. 7) distinguished the Irish isolates (lanes 1 and 2) from the NW European isolates (lanes 3 and 4) and also *H. zealandica* (lane 7) from the other members of the *H. bacteriophora* complex (lanes

TABLE 2. Differentiation of *Heterorhabditis* isolates using restriction fragment analysis of a PCR-amplified mtDNA fragment.

Enzyme	Recognition sequence	No. bands (Range)	Diagnostic value	Biological species[a] restriction pattern (bp)					
				Irish	NWE	Tropical	*H. bacteriophora*, Brecon	NC1	*H. zealandica*
Ssp I	AAT/ATT	3–5	Distinguishes Irish, NWE, and P_2M. All members of the *H. bacteriophora* species complex (*H. zealandica*, V16, NC1, HP88, & *H. bacteriophora*, Brecon) exhibit identical patterns	400 340 300 140	550 300 220	900 †	400 300 220 150 75	400 300 220 150 75	400 300 220 150 75
Dra I	TTT/AAA	4	Distinguishes Irish from NWE isolates. *H. bacteriophora*, Brecon, NC1, V16, & HP88 show an identical profile easily discernible from other isolates *H. zealandica* is distinctly different. P_2M exhibits a unique profile.	360 340 230 210	360 210 175 150	340 320 270 230	360 210 200 150	360 210 200 150	460 360 230 210
Taq I	T/CGA	3	Does not distinguish isolates	—	—	—	—	—	—
Mbo I	N/GATC	3	Does not distinguish isolates	—	—	—	—	—	—
Alu I	AG/CT	4	Does not distinguish isolates	—	—	—	—	—	—
Hpa I	GTT/AAC	1	Does not cut	—	—	—	—	—	—
Hpa II	C/CGG	1	Does not cut	—	—	—	—	—	—
Tha I	CG/CG	1	Does not cut	—	—	—	—	—	—
Hinf I	G/ANTC	1	Does not cut	—	—	—	—	—	—
Rsa I	GT/AC	1	Does not cut	—	—	—	—	—	—

Irish = Irish species representatives; NWE = North West European species representatives.

[a] Biological species as indicated by cross-breeding (4). Fragment sizes are approximate, and some small fragments (<150 bp) and superimposed fragments (doublets) may have been missed in these analyses.

† Small fragments present.

TABLE 3. Differentiation of *Heterorhabditis* isolates using restriction fragment analysis of a PCR-amplified rDNA fragment.

Enzyme	Recognition sequence	No. bands (range)	Diagnostic value	Biological species[a] restriction pattern (bp)					
				Irish	NWE	Tropical	*H. bacteriophora*, Brecon	NC1	*H. zelandica*
Hinf I	G/ANTC	2–3	Distinguishes Irish, NWE, P_2M, and *H. bacteriophora* group	420 240 (D) †	420 240 150 †	420 360 240	420 300 240 †	420 300 240 †	420 300 240 †
Ssp I	AAT/ATT	1–3	Distinguishes P_2M from the other isolates only.	—	—	—	—	—	—
Alu I	AG/CT	3–4	Distinguishes P_2M and *H. zealandica* from other members of the *H. bacteriophora* group. The Irish and NWE isolates share the same distinct profile.	340 290 240 150	340 290 240 150	340 240 (D) 150	340 240 220 150	340 240 220 150	340 290 210 150
EcoR I	G/AATTC	1	Does not cut.	—	—	—	—	—	—
Sau 3A/*Mbo* I	N/GATC	3–4	Distinguishes NWE, *H. zealandica*, HP88, and *H. bacteriophora*, Brecon. HP88, NC1, and V16 exhibit the same profile, which is distinct from *H. bacteriophora* Brecon type species. Irish and P_2M have identical patterns.	440 240 220 150	440 220 150 †	440 240 220 150	390 290 220 (D)	440 390 220	440 300 220 150

Irish = Irish species representatives; NWE = North West European species representatives. *(D)* = *suspected doublet.*

[a] Biological species as indicated by cross-breeding (4). Fragment sizes are approximate, and some small fragments (<150 bp) and superimposed fragments (doublets) may have been missed in these analyses.

† Small fragments present.

6–10). The tropical isolate, P_2M (lane 5), is identical in profile to the Irish isolates. A *Mbo* I restriction site in the 420-bp fragment further distinguished the *H. bacteriophora* Brecon type isolate (lane 8) from the NC1, HP88, and V16 isolates. Thus, digestion of the ITS region of the rDNA cistron recognizes three groupings within the *H. bacteriophora* species complex as follows: 1) *H. zealandica,* 2) *H. bacteriophora* Brecon, and 3) the HP88, NC1, and V16 isolates.

Discussion

The PCR-based RFLP method described here allows the rapid categorization of different *Heterorhabditis* groups based on an analysis of amplified DNA from individual infective juvenile nematodes. Identifications are made by an evaluation of restriction endonuclease digestion patterns following electrophoresis. The six *Heterorhabditis* groups revealed by the digestion patterns of the two PCR primer sets used in this study appear to be concordant with species groupings as previously recognized by morphology (8–12), reproductive isolation (4), protein analysis (1), and other molecular data (3,16). The method also provides the first molecular means to discriminate among members of the *H. bacteriophora* species complex. Several taxa were readily identified by both primer sets and by more than one restriction enzyme. These results suggest that the genetic distance among the Irish, NW European, tropical and *H. bacteriophora* species groups is substantial. Within the *H. bacteriophora* complex of biological species, *H. zealandica* can be discriminated by rRNA digestion patterns using two restriction enzymes (*Alu* I and *Mbo* I), and the Brecon isolate can be identified by digestion of the same product with *Mbo* I. Thus, among heterorhabditids, each biological species that exhibits partial or complete reproductive incompatibility (4,5) is also recognizable by a unique rDNA digestion profile (summarized in Table 3).

No effort was made to assess the phylogenetic relationship of these isolates. Our inability to account for all the restriction site changes due to unobserved small fragments, and to the possible presence of superimposed fragments following digestion made assessment of homology inadvisable. We are currently sequencing these amplified products for a complete determination of polymorphism at these two loci.

This diagnostic technique has other applications. Using the PCR-RFLP method, it will be possible to monitor the release of isolates selected for biological control purposes in the presence of a genetically distinct indigenous *Heterorhabditis* species. Also, newly discovered isolates can be rapidly assessed for taxonomic affinities. Finally, the extensive characterization of *Heterorhabditis* provides a basis for an examination of genetic differentiation that occurs among well-defined and incipient species.

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Note Added In Proof

Recent analyses of the DNA of *H. indicus* have shown that the RFLP profiles of this species are identical to those of the tropical isolate P_2M, and we have also found that both strains are interfertile.