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Molecular comparisons of entomopathogenic nematodes using Randomly Amplified Polymorphic DNA (RAPD) markers

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Summary – The two entomopathogenic nematode genera *Heterorhabditis* and *Steinernema* were examined for genetic relatedness by random amplified polymorphic DNA (RAPD) markers. Species of these genera have great potential for biological control of insect pests, and rapid and accurate identification of the many isolates of these nematodes is important for their implementation. Eighty ten-mer random primers were screened for use in determining genetic variability among isolates of *Heterorhabditis bacteriophora, H. megidis, Steinernema glaseri, S. carpocapsae,* and *S. feltiae.* A similarity of 0-6 % was observed between the members of different genera, and less than 20 % similarity was observed among congeneric species. Genetic analysis using seven primers of different *Heterorhabditis* isolates from various parts of the world showed 24-96 % similarity. These results agreed with other reports which showed many genetic differences among isolates. RAPD analysis described here can be used for the assessment of genetic variability among species and isolates of entomopathogenic nematodes.

Résumé – *Comparaison moléculaire entre nématodes entomopathogènes grâce à des marqueurs polymorphes d'ADN amplifiés au hasard (RAPD)* – Les relations génétiques des deux genres de nématodes entomopathogènes *Heterorhabditis* et *Steinernema* ont été étudiées grâce à des marqueurs d'ADN polymorphes amplifiés au hasard (RAPD). Les espèces appartenant à ces genres sont en effet dotées d'un fort potentiel de contrôle biologique des insectes nuisibles et leur identification rapide et sûre est importante pour leur utilisation. Quatre-vingts amorces de séquence aléatoire de dix bases ont été testées en vue de leur utilisation pour déterminer la variabilité génétique existant chez des isolats d'*Heterorhabditis bacteriophora, H. megidis, Steinernema glaseri, S. carpocapsae* et *S. feltiae.* Une similarité de 0 à 6 % est observée entre les membres des différents genres; cette valeur est de 20 % pour les espèces congénères. Une analyse génétique utilisant sept amorces de différents isolats d'*Heterorhabditis* d'origines variées a révélé une similarité de 24-96 %. Ces résultats confirment d'autres observations montrant de nombreuses différences génétiques entre les isolats. L'analyse en RAPD décrite ici peut être utilisée pour l'évaluation de la variabilité génétique chez les espèces et isolats de nématodes entomopathogènes.

Key words : Entomopathogenic nematodes, genetic variations, *Heterorhabditis,* RAPD, *Steinernema.*

Entomopathogenic nematodes, *(Heterorhabditis* and *Steinernema)* are efficient parasites of several economically important insect pests (Kaya, 1985; Gaugler, 1988; Gaugler & Kaya, 1990). To date, five species have been described for *Heterorhabditis* (Gardner *et al.,* 1994) and seventeen for *Steinernema.* These two nematode genera are comprised of numerous isolates and new isolates are continuously being added in nematode culture collection in various laboratories all over the world. These isolates often differ in their potential as a biological control agent. Knowledge about the systematic relationships among these isolates is a prerequisite for field implementation, and also for proprietary rights. Efforts to collect, preserve and use for biological control of new isolates are impaired by the inability to effectively document their uniqueness or taxonomic relationships. The lack of the understanding of the genetic diversity within species and within populations also dampened these efforts. There has been considerable debate about the proper identification of entomopathogenic nematodes (Curran & Webster, 1989; Gaugler & Kaya, 1990; Poinar, 1990). Taxonomic relationships of these nematodes are usually based on morphological characters for *Heterorhabditis* species while for *Steinernema* species morphological characters are combined with cross breeding data (Akhurst & Bedding, 1978; Poinar, 1986, 1990). Morphological characters can not be used unambiguously to place new isolates into a particular species. The feasibility of using these nematodes as biological control agents depends upon the resources required for a rapid and accurate means to determine the genetic diversity among existing populations of entomopathogenic nematode species. These methods can also be used for the development of identification tools.

The use of molecular approaches to study systematic relationships among entomopathogenic nematodes have been emphasized (Curran, 1990). Several examples of these methods to determine variability among species

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and strains of *Heterorhabditis* and *Steinernema* are available. Isozyme banding patterns were used to detect variability among both *Heterorhabditis* and *Steinernema* species (Akhurst, 1987; also see for review Curran, 1990). Use of restriction fragment length polymorphism (RFLP) for the genetic characterization of *Heterorhabditis* and *Steinernema* species, and for the development of species specific probe has also been reported (Curran & Webster, 1989; Smith *et al.*, 1991; Reid & Hominik, 1992; Joyce *et al.*, 1994 *b*).

Various methods are available to study the genetic divergence and polymorphism. One of these methods, random amplified polymorphic DNA (RAPD), detects polymorphisms using a single primer of arbitrary nucleotide sequence (Williams *et al.*, 1990; Welsh & McClellan, 1990). Polymorphisms are caused by base changes in the primer binding site or by chromosome length mutations within the amplified sequence. These types of polymorphisms makes RAPD markers well suited for studies for genetic diversity, genetic relationships, genetic mapping, DNA fingerprinting and population genetics (Vierling & Nguyen, 1992). RAPD has already been used for the genetic variability and phylogenetic studies of different organisms including insects, animals and plants (Hu & Quiros, 1991; Kambhampati *et al.*, 1992; Black *et al.*, 1992; Woodward *et al.*, 1992; Puterka *et al.*, 1993; Landry *et al.*, 1993; Fondrk *et al.*, 1993). This technique has also been used to study genetic diversity in bacterial feeding and plant parasitic nematodes (Caswell-Chen *et al.*, 1992; Knaap *et al.*, 1993; Hahn *et al.*, 1994; Pinochet *et al.*, 1994).

Although RAPD analysis was used for the description of a new species of *Heterorhabditis* from Hawaii (Gardner *et al.*, 1994), these markers have not been used to determine the extent of polymorphisms within species and populations of entomopathogenic nematodes. We explore the use of RAPD analysis to study genetic diversity among various species and isolates of entomopathogenic nematodes *(Heterorhabditis,* and *Steinernema)* from different geographical regions of the world. Genetic similarities were compared by generating similarity matrix on the basis of DNA banding patterns produced with arbitrary primers.

Materials and methods

NEMATODE CULTURE

The nematode species and strains used in this study and their geographical origin are listed in Table 1. Nematodes were grown on lipid agar media (Dunphy & Webster, 1989), seeded with their symbiotic bacteria *(Photorhabdus luminescens* for *Heterorhabditis* spp., *Xenorhabdus nematophilus* for *Steinernema carpocapsae, X. bovienii* for *S. feltiae, X. poinarii* for *S. glaseri).* All stages of nematodes were collected by centrigugation at 3000 rpm for 10 min in EN buffer (100 mM NaCl, 10 mM EDTA) followed by three washes with distilled sterile water (Sulston & Hodgkin, 1988). Finally, the nematodes were collected in 1.5 ml Eppendorf tubes and stored frozen at – 20 °C until used for DNA extraction.

Table 1. *List of* Heterorhabditis *and* Steinernema *species with their isolate designation and geographic origin, used in the genetic analysis.*

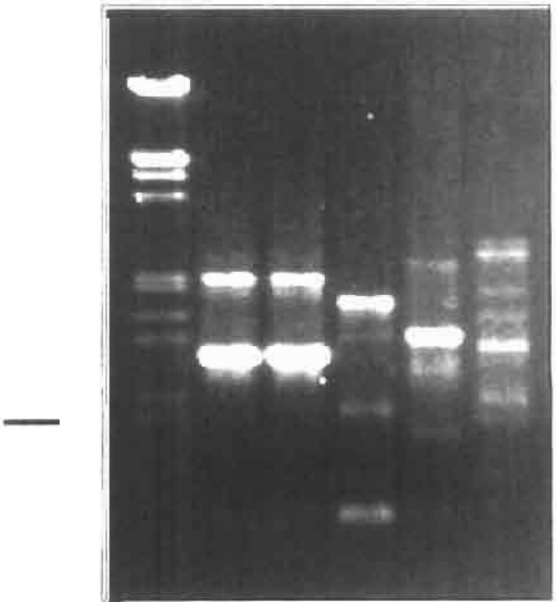
Biological species	Isolate designation	Geographic origin
H. bacteriophora	HP88	USA
H. bacteriophora	HB1	South Australia
H. bacteriophora	C1 (St. François)	Guadeloupe
H. bacteriophora	IH-273	Italy
Heterorhabditis sp.	32.5	USA
Heterorhabditis sp.	Moldavia	Moldavia
Heterorhabditis sp.	E1	Barcelona, Spain
Heterorhabditis sp.	IH-127	Italy
H. megidis	HSH2	Germany
S. carpocapsae	All	Georgia, USA
S. feltiae	AB (Australian)	Australia
S. glaseri	NC	North Carolina, USA

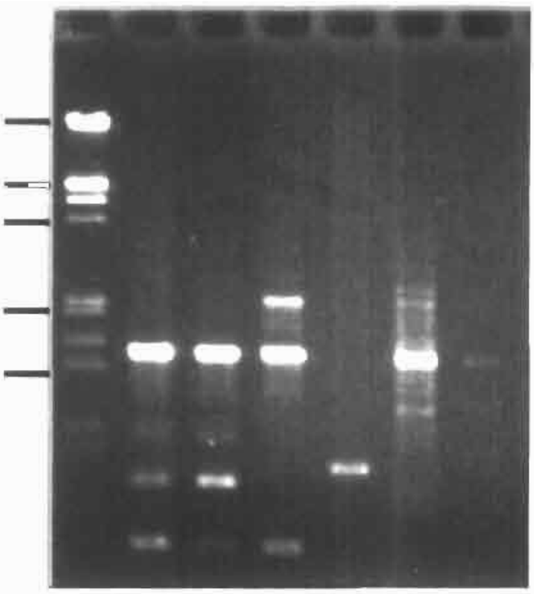
DNA EXTRACTION

DNA extraction from all stages of nematodes was performed according to the method of Sulston and Hodgkin (1988). The nematode pellet was ground in liquid nitrogen and treated with proteinase K buffer containing 100 mM NaCl, 100 mM Tris HCl at pH 8.5, 50 mM EDTA pH 7.4, 1 % SDS, 1 % β mercaptoethanol, and 100 μg/ml proteinase K, for 1 h at 65 °C. The DNA was extracted with equal volumes of phenol, phenol-chloroform, and chloroform-isoamyl alcohol (24 : 1) and was precipitated in 100 % ethanol over night at – 20 °C. DNA pellet was then dissolved in 1X TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, pH 8.0). DNA concentration was determined by spectrophotometer.

RAPD ANALYSIS

The reaction mixture (20 μl) contained 10 mM Tris HCl at pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, .001 % (w/v) gelatin, 200 μM each of dNTPs, 0.5 μM primer, 50 ng of template DNA and 0.5 units of Taq DNA polymerase (Perkin Elmer Cetus), overlaid with mineral oil (United States Biochemicals). Eighty random primers (kit A, C, H, S) used in this study were obtained from Operon Technologies Inc., Alameda, CA. Amplifications were performed in a Stratagene Robocycler^{TM} 40 temperature cycler, programmed for one cycle of 1 min at 94 °C, 2 min at 40 °C, 3 min at 72 °C, 39 cycles of 1 min at 94 °C, 1:30 min at 40 °C, and 3 min at 72 °C (modified from Joyce *et al.*, 1994 *a*). The amplification products were electrophoresed in 0.8-1 % w/v agarose gel in 1X TBE buffer at 1 V/cm for 12-16 h and visualized by staining with ethidium bromide. Reproducibility





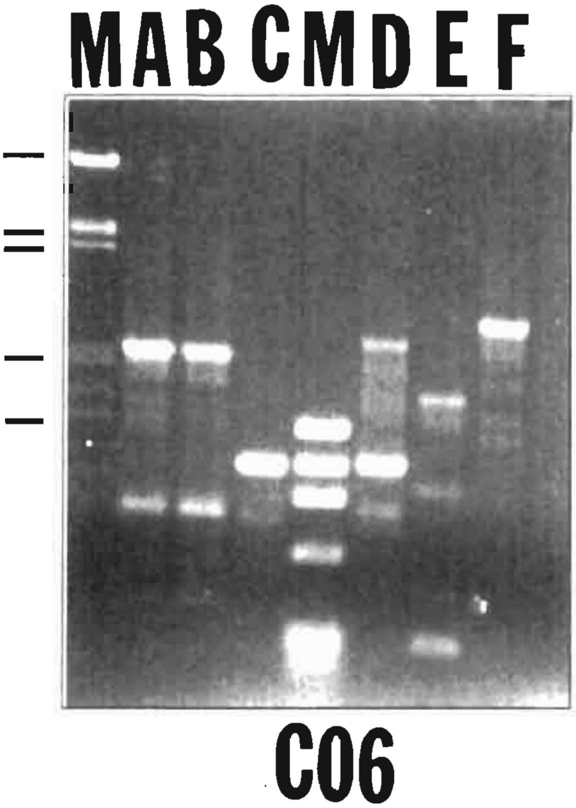


Fig. 3. *RAPD amplifications with primer OP-C06, where M = molecular weight marker, lambda DNA cut with* HindIII/EcoRI, *molecular weight sites as in Fig. 1. A :* Heterorhabditis bacteriophora *HP88; B :* H. bacteriophora *HB1; C :* H. megidis *HSH2; D :* Steinernema glaseri *NC; E :* S. carpocapsae *All; F :* S. feltiae *AB.*

were found among *Heterorhabditis* isolates, *H. megidis* always produced a DNA banding pattern that distinguished it from all other isolates (Figs 5, 6). Many primers produced sufficient polymorphism to distinguish all isolates from each other by at least one band difference. Percent similarity was calculated based on shared DNA fragments per primer and the results were averaged (Table 5). Similarity between all *Heterorhabditis* isolates and *H. megidis* was less than 10 %. Two *H. bacteriophora* isolates HP88 and HB1 shared a high percent similarity of DNA fragments (96 %). High similarity was also found between HP88 and El, 32.5 (73.8 and 77.4, respectively) and E1 and 32.5 (100 %). There was a similarity of 45.3 % between two Italian isolates (IH-127, IH-273). Different levels of similarities were observed between isolate C1 and others, for example, C1 was found 24 % similar to IH-273, 37.9 % similar to IH-127, 45 % similar to HP88, HB1, and 64.4 % similar to isolate from Moldavia.

UPGMA analysis of eight isolates of *Heterorhabditis* is shown in Figures 7. The average linkage method used in

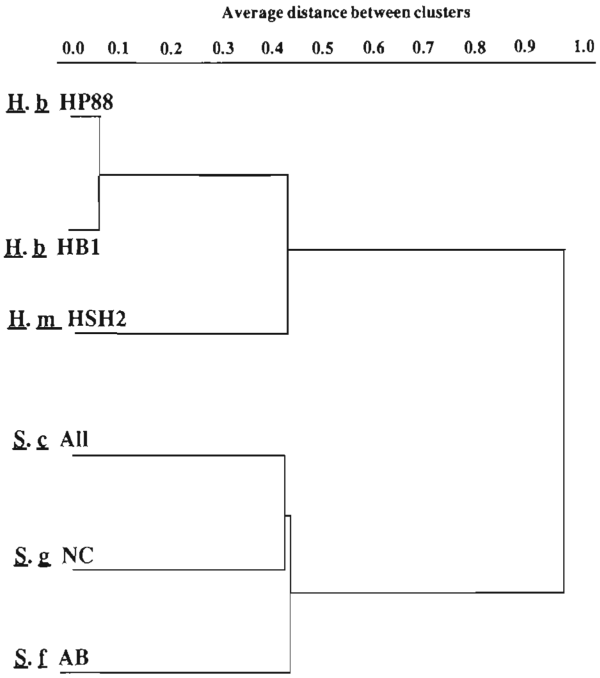


Fig. 4. *UPGMA dendrogram showing estimated average genetic distance among different species of entomopathogenic nematodes based on polymorphisms generated by RAPD-PCR. This dendrogram was generated using 108 polymorphic bands produced with twelve random primers. Similarity matrix was generated on the bases of shared DNA fragments as described in materials and methods. Average linkage cluster analysis was performed on the values of 1-F.*

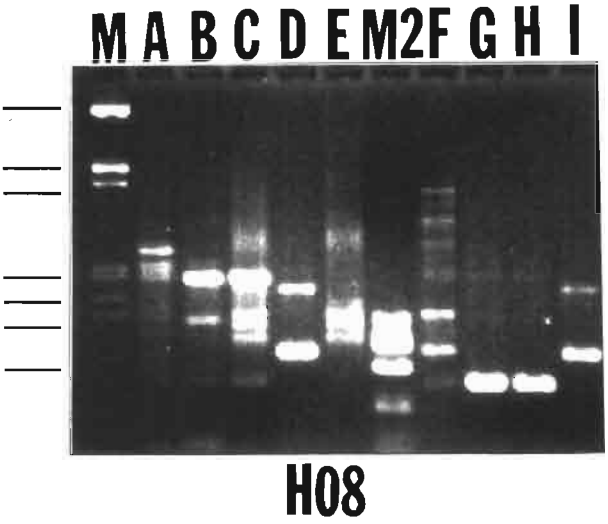
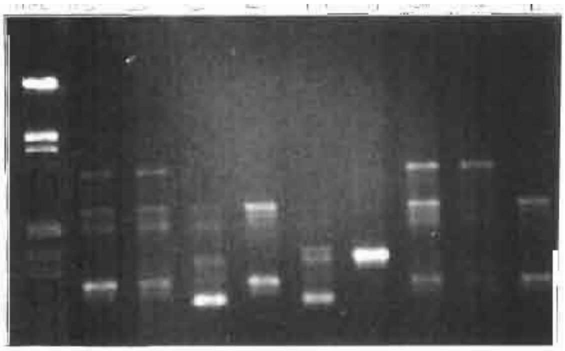


Fig. 5. *RAPD amplifications of nine different* Heterorhabditis *isolates amplified with primer OP-H08, where M = lambda DNA cut with* HindIII/EcoRI, *molecular weight sites as in Fig. 1, except for the smallest fragment which is 0.56 kb. A :* Heterorhabditis bacteriophora *HP88; B : HB1; C : IH-273; D : C1; E : IH-127; F :* H. megidis *HSH2; G : 32.5; H : E1; I : isolate from Moldavia.*



within species. Analysis of genetic variability using RAPD technique described here may be used in routine genetic analysis of new isolates. Molecular methods can also help to clarify the taxonomic status of new isolates as also suggested by Curran (1990). However care must be taken in using these markers for the determination of phylogenetic relationships, because of the dominant nature of RAPD markers (Williams *et al.*, 1990).

In our RAPD analyses polymorphims were scored for the presence *versus* absence of a particular band. The proportion of DNA fragments shared between two populations is expected to be correlated with the degree of genetic divergence of DNA. This proportion declines as the organism's DNA sequences diverge (Nei & Li, 1979). Our data demonstrate low levels of percent similarity (0-6 %) between two genera, *Heterorhabditis* and *Steinernema*, indicating high level of genetic divergence, whereas similarity among species of the same genus was less than 20 %. Cluster analysis correlated with the presumed taxonomic relationship of all individuals. The percent divergence of two *Heterorhabditis* species *(H. bacteriophora* and *H. megidis)* was similar to that of two *Steinernema* species *(S. carpocapsae* and *S. glaseri)*, whereas *S. feltiae* was less similar (Fig. 4). These results show that discrimination among different genotypes is possible with a RAPD assay.

In the studies of genetic diversity among nine *Heterorhabditis* isolates from different parts of the world, intermediate similarities were observed. Similarity of these isolates with *H. megidis* was only 4-10 %. On an average there was less than 50 % similarity among these isolates with several exceptions. Although these isolates showed intermediate similarities, each one showed a banding pattern with at least one marker difference from other isolates when screened with a particular primer. Two *Heterorhabditis* isolates 32.5 and E1 that showed 100 % similarities with each other did show slightly different levels of similarities with other isolates. This difference was due to the presence or absence of a particular fragment that was not used in certain comparisons because of their low intensity/reproducibility.

RAPD-PCR has already been used to assess the genetic variability of different organisms including bacterial feeding and plant parasitic nematodes (Caswell-Chen *et al.*, 1992; Knaap *et al.*, 1993; Hahn *et al.*, 1994). RAPD markers may become an efficient and rapid way to characterize the species and isolates of entomopathogenic nematode. Genetic information obtained from RAPD analysis can also be used to generate more specific probes for the identification of different genotypes. RFLP analysis with different Steinernematids isolated from British soils (Reid & Hominick, 1992), has been used for the development of a species-specific clone for *S. feltiae* (Reid & Hominick, 1993). Several species-specific DNA fragments were also identified during present studies. Efforts are now in progress in our laboratory to use RAPD markers for the development of more specific probes for the identification of entomopathogenic nematode species and isolates.

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