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Novel application of PhastSystem polyacrylamide gel electrophoresis using restriction fragment length polymorphism ± internal transcribed spacer patterns of individuals for molecular identification of entomopathogenic nematodes

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Novel application of PhastSystem polyacrylamide gel electrophoresis using restriction fragment length polymorphism – internal transcribed spacer patterns of individuals for molecular identification of entomopathogenic nematodes

A relatively rapid and economic way of identifying and assigning nematodes to taxons, which had already been determined either by comparative sequence analysis of nuclear rDNA internal transcribed spacer (ITS) region or by other methods of molecular or conventional taxonomy, is provided. Molecular identification of entomopathogenic nematodes (EPN) can be upgraded by basing it on PhastSystem polyacrylamide gel electrophoresis (PAGE) analysis of restriction fragment length polymorphism (RFLP) patterns of polymerase chain reaction (PCR)-amplified DNA derived from single nematodes of *Steinernema* or *Heterorhabditis* spp. Although analysis from single worms has previously been made on agarose gel, the resolution on PhastSystem PAGE gel is much higher. The DNA sequences selected for analysis were those constituting the internal transcribed spacer region between the 18S and 26S rDNA genes within the rRNA operon. RFLP analysis was carried out by gel electrophoresis on the PhastSystem (Pharmacia) as detailed elsewhere (Triga *et al., Electrophoresis* 1999, *20*, 1272–1277. The downscaling from conventional agarose to PhastSystem gels resulted in pattern of DNA fragments differing from those obtained with agarose gel electrophoresis under conventional conditions by increasing the number of detected fragments. The approach supported previous species identifications and was able to identify several unclassified isolates, such as those from Hungary and Ireland, and provides a method for identification of previously unclassified strains. We confirmed that *Heterorhabditis "Irish Type"*, represented by two strains of different geographical origin, comprise a species different from *H. megidis*. We also confirmed that strain IS5 belongs to the species *H. indicus* rather than to *H. bacteriophora*, as had been suggested previously.

Keywords: Molecular diagnostics / Identification / Entomopathogenic / Nematode / *Heterorhabditis / Steinernema*
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1 Introduction

Entomopathogenic nematodes (EPN) [1] belonging to the genera *Heterorhabditis* (the prokaryotic symbiont of which is *Photorhabdus luminescens* [2–4]) and *Steinernema* (the prokaryotic symbiont of which is *Xenorhabdus* spp., [2–4]) possess great potential as bioinsecticides and are attractive from a biological and commercial viewpoint. Phylogenetic analysis of Phylum Nematoda [5], based upon 18S rDNA sequence homology, indicates that steinernematids and heterorhabditids are phylogenetically relatively far from each other within Rhabditida [6]. On the basis of the 18S rDNA sequences, genera could be well separated within Phylum Nematoda [5]. EPN strains can be found all over the world [8]. The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA [7, 9] appears to be a reliable marker for identifying species within each EPN genera.

Nematode taxonomy has been mainly based upon morphological and morphometric characters. Since the steinernematids are amphimictic, cross-breeding between virgin females and males of good fertility is sufficient to establish true biological species, and allow identification. For the *Steinernema*, an international team led by W. Hominick presented a revised taxonomy based on unam-

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Abbreviations: EPN, entomopathogenic nematodes; **ITS,** internal transcribed spacer; **NWE,** North Western European

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biguously standardized morphological and molecular identification criteria. This revision had consensus since the morphological/morphometric and molecular characters fitted perfectly and the following species are recognized: *S. kraussei* [14], *S. glaseri* [15], *S. feltiae* [16], *S. affinis* [1], *S. carpocapsae* [17], *S. anomali* [18], *S. intermedium* [19], *S. rarum* [20, 21], *S. kushidai* [22, 23], *S. scapterisci* [24], as well as *S. riobravum, S. bicornutum, S. ritteri, S. serratum, S. cubanum, S. caudatum*, and *S. longicaudatum* [25].

The diagnosis of *Heterorhabditis* needs a more sophisticated molecular tool kit because of the more complicated situation. Although only one genus (*Heterorhabditis*) belongs to the family *Heterorhabditidae* [26], each species exists in alternating automictic and amphimictic cycles [27], and the researcher needs some practice to be able to distinguish between self progeny and cross progeny. That is probably the main reason why the crossbreeding technique [28] has rarely been used for identifying biological species in this genus. Instead, new species have been determined by morphology, electrophoretic and electrofocusing analyses of polypeptides [29, 30] and DNA fingerprinting [1, 10, 33, 34]. The first species in the taxon was *H. bacteriophora* [31]. More and more *Heterorhabditis* isolates were identified morphologically and morphometrically on the basis of genetic, cytogenetic and protein polymorphism, with the ultimate aim of diagnosing phenetic or biological species [8, 12, 13, 32–42]. However, no evolutionary or phylogenetic species concept has been considered until recently [12, 13]. To avoid confusion, we refer to the literature describing *Heterorhabditis* strains and aligning them to different species [27, 31–33, 43–45].

Accumulated evidence [10, 11, 34, 35] suggests that DNA sequences of the internally transcribed region of the rRNA tandem repeat (ITS) could provide the requisite heritable characteristic for a thorough phylogenetic analysis. The advantages and taxonomic suitability of this marker include the potential for PCR amplification and sequencing by universal primers, forced uniformity of paralogues *via* rapid concerted evolution, variation due primarily to point mutations, apparent independence of variable sites, and phylogenetic information appropriate for species level investigations [12]. In a comprehensive study by Adams *et al.* [13], phylogenetic relationships among nine representative strains of *Heterorhabditis*, each considered as distinct species, were determined by using DNA sequences of the ITS1 region of the ribosomal tandem repeat unit. The authors demonstrated that this region performs better at resolving relationships among closely related sister taxa than among inclusive clades [13].

The studies based upon comparison of ITS as well as mitochondrial DNA sequences made it possible to separate species of "phylogenetic" *H. bacteriophora, H. megidis, H. argentinensis, H. indicus, H. zealandica*, and *H. marelatus*. The latter was represented by two strains of different geographical origin, *H. hepialius* (Bodega Bay isolate) and *H. marelatus* (Oregon strains). Though initially thought to be separate species, they were later synonymized [42]. The *H. bacteriophora* phylogenetic species includes several "biological species" [12, 27]. These studies provide a frame within which unclassifed strains could be assigned, on condition that proper and accurate tools of molecular identification were available. In our study we focused on strains not involved in the previous study [13] but that should be unambiguously assigned, such as IS5 [46] and EU349/HIT (Griffin *et al.*, submitted; [8]).

First we demonstrate that the new method for molecular identification of EPN [48] provides comparable results to those obtained by comparative sequence analyses [13]. The study presented here provides a new technique, the PhastSystem PAGE, as a contribution to the molecular tool kit of EPN taxonomy. We analyzed several digestion patterns of PCR-amplified ITS sequences from different *Steinernema* and *Heterorhabditis* species and strains. We show that the patterns obtained by the new technique are identical to the already published patterns [25] obtained by agarose electrophoresis for the genus *Steinernema*. The new method provides better resolution in the range of 100 bp DNA length.

2 Materials and methods

2.1 EPN strains

The EPN strains used in this study as well as their source are listed in Table 1.

2.2 Molecular techniques

The techniques consisted of extraction of DNA, PCR amplification of the ITS region, restriction endonuclease digestion of PCR products, and gel electrophoresis of the DNA fragments on the PhastSystem (Pharmacia, Uppsala, Sweden). DNA was extracted after lysis of individual EPN, using standard proteinase K digestion and DNA purification methods [8]. PCR amplification of the ITS region was carried out as described previously [49]. For the *Steinernema* species, the two primers were 18S (forward) and 26S (reverse) [9]. For the *Heterorhabditis* species, the primers were AB 28 (18S forward) and TW 81 (26S reverse) (John Curran, personal communication). PCR products were identified by electrophoresis on 1% agarose gel in TBE buffer. For the restriction endonuclease digestion of PCR products *Alu*I, *Dde*I, *Hin-f*I and *Rsa*I enzymes were used. Gel electrophoresis of the DNA

Table 1. EPN strains and origin

Genus	Species	Strain	Notes	Origin
Steinernema	*carpocapsae*	Mexicana		James E. Lindegreen, 1986
Steinernema	*intermedium*	224-35-11	BIOSYS	Lonne Gerritsen, 1994
Steinernema	*anomali*	Azores		Nelson Simoes, 1996
Steinernema	*scapterisci*	Scapterisci		Harry Kaya, 1997
Steinernema	*glaseri*	NC 513		Lonne Gerritsen, 1994
Steinernema	*feltiae*	IS6		Itamar Glazer, 1995
Steinernema	*oh-sp*	KMD 15	Unpublished	Attila Lucskai and Michael G. Klein, 1997
Heterorhabditis	*bacteriophora*	Heliothidis		Lonne Gerritsen, 1995
Heterorhabditis	*argentinen-sis*	Argentinen-sis	or *bacterio-phora*	Byron Adams, 1997
Heterorhabditis	*megidis*	HE87.3	NWE	Lonne Gerritsen, 1994
Heterorhabditis	*marelatus*	Hepialius		Byron Adams, 1997
Heterorhabditis	*zealandica*	NZH3		Ann Burnell, 1997
Heterorhabditis	*indicus*	LN2	Coimbatore	Ann Burnell, 1997
Heterorhabditis	*indicus*	IS5	or *bacterio-phora*	Itamar Glazer, 1994
Heterorhabditis	*Irish Type*	K122	or *megidis* from Ireland	Christine Griffin, 1994
Heterorhabditis	*Irish Type from Hun-gary (HIT)*	EU349/HIT isolated by C. Griffin, M. Downes	or *megidis* from Hungary	Christine Griffin, 1994, 95

IS5 [46] was believed to be *H. bacteriophora*, since it can successfully be crossed with *H. bacteriophora* [50] strain HP88 [47].

fragments was carried out on the PhastSystem at 15°C, using stacking gels of 5%T, 3%C (Bis) and resolving gels of 7.5%T, 5%C (Bis). The stacking and resolving gel buffer was 0.112 M Na acetate, 0.112 M Tris, pH 6.4, and the catholyte and anolyte were 0.2 M tricine, 0.2 M Tris, 0.55% SDS, pH 8.1, contained in 2.8% IsoGel agarose strips [48, 49]. Data processing was carried out as described elsewhere [49].

3 Results

3.1 Identity between *Heterorhabditis* "Irish Type" strains K122 and EU349/HIT

The identical nature of strain K122 isolated in Ireland, and EU349/HIT isolated in Hungary was demonstrated by the indistinguishable RFLP patterns in PhastSystem gels of digests prepared with four different restriction enzymes (Fig. 1).

3.2 Distinction between phylogenetic species *H. megidis* and *Heterorhabditis* "Irish Type" (comparison of "Irish Type" strains K122 to a North Western European (NWE) strain of *H. megidis*, HE87.3)

A comparison of four RFLP patterns derived from different restriction enzymes of strain K122 and *H. megidis* NWE demonstrates that these two strains are not identical (Fig. 2).

3.3 Identity of *Heterorhabditis* species *H. argentinensis* and *H. bacteriophora*

The RFLP patterns obtained for four restriction digests of strains Argentinensis and Heliothidis are indistinguishable, demonstrating their identity (Fig. 3).

3.4 Assignment of Israeli strain IS5 to species *H. indicus*

The assignment of strain IS5 to either *H. indicus* or *H. bacteriophora* was resolved in favor of the former, based on the identical RFLP patterns of IS5 and LN2 (Coimbatore) strain of *H. indicus* and the lack of similarity between IS5 and *H. bacteriophora* (Heliothidis) gel patterns (Fig. 4).

3.5 Distinction of *H. zealandica* from any other *Heterorhabditis* strain

The RFLP pattern of *H. zealandica* (NZH3) differs from that of any of the other strains depicted in Fig. 5. The patterns of Fig. 5 derive from digestion with restriction

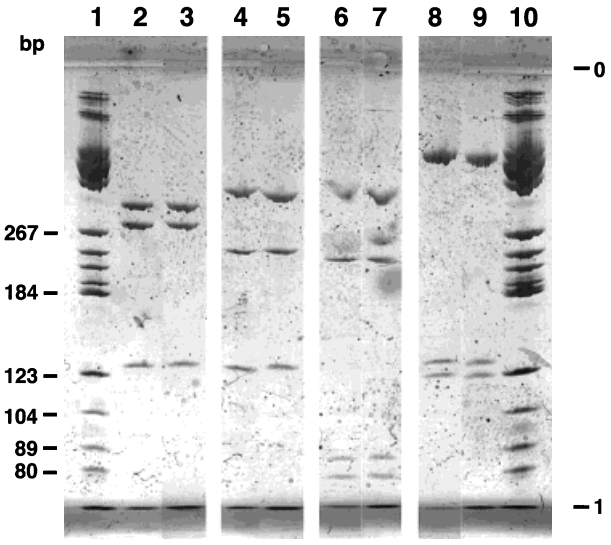

Figure 1. Identity between *Heterorhabditis "Irish Type"* strain K122 and Hungarian *"Irish Type"* EU349/HIT. RFLP analysis on the PhastSystem at 15°C, using 7.5%T, 5%C (Bis) resolving gel in 0.112 M Tris/acetate buffer, pH 6.4. Digests of restriction endonucleases *Alu*I, *Dde*I, *Hin*fI and *Rsa*I are depicted in this order in gel lanes (2), (4), (6), and (8) in the case of strain K122, and restriction fragments of strain EU349/HIT are shown in (3), (5), (7), and (9). DNA fragments of the size standard ladder migrated as shown in (1) and (10).

enzyme *Hin*fI. The same distinction of the *H. zealandica* pattern from all the others under consideration was also demonstrated for three other restriction digests (data not shown).

3.6 KMD 15, a new *Steinernema* isolate representing a novel species

An isolate from Ohio being described as a new species (Lucskai and Klein, in preparation) exhibits RFLP patterns in three restriction digests which are different from those of all other strains under consideration. This distinction is exemplified in Fig. 6.

4 Discussion

The following *Heterorhabditis* species were recognized by molecular identification: *H. bacteriophora, H. indicus, H. marelatus, H. megidis, H. zealandica*, and *H. argentinensis* [13]. Four pairs of sister species were finally determined in the genus, namely: *H. megidis* and *Heterorhabditis "Irish Type"; H. Zealandica; H. hepialius* and *H. marelatus; H. bacteriophora* and *H. argentinensis*; and *H. indicus* and *H. hawaiiensis*. On the basis of molecular analysis, some of these sister taxa may be conspecific, namely, *H. hepialius* and *H. marelata; H. indicus* and *H. hawaiiensis; H. bacteriophora* and *H. argentinensis*

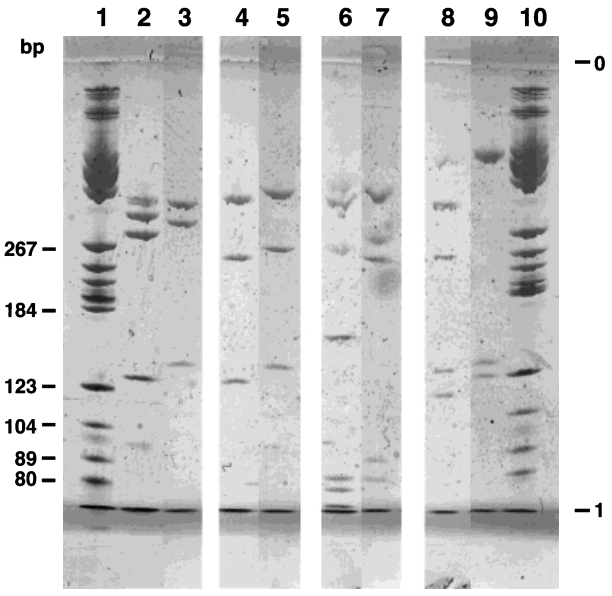

Figure 2. Comparison of *Heterorhabditis "Irish Type"* strain K122 to an NWE strain of *H. megidis* HE87.3. RFLP analysis on the PhastSystem using the same conditions as in Fig. 1. Digests of restriction endonucleases *Alu*I, *Dde*I, *Hin*fI and *Rsa*I are depicted in this order in gel lanes (2), (4), (6), and (8) in the case of strain HE 87.3, and restriction fragments of strain K122 are shown in (3), (5), (7), and (9). Lanes (1) and (10) depict the patterns of DNA size standards.

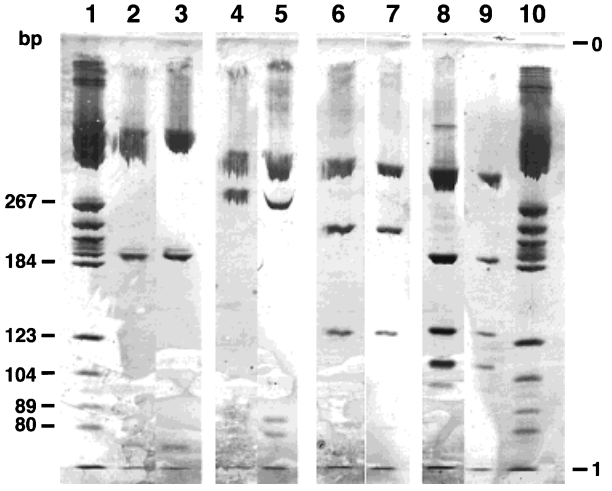

Figure 3. Identity between *Heterorhabditis* strains *H. argentinensis* and *H. bacteriophora*. RFLP analysis on the PhastSystem using the same conditions as in Fig. 1. Order of the restriction endonucleases: *Rsa*I, *Hin*fI, *Dde*I and *Alu*I. Digests of PCR product of strain *H. argentinensis* are shown in gel lanes (2), (4), (6), and (8); lanes (3), (5), (7), and (9) depict the restriction fragments of strain *H. bacteriophora*; (1) and (10), patterns of DNA size standards.

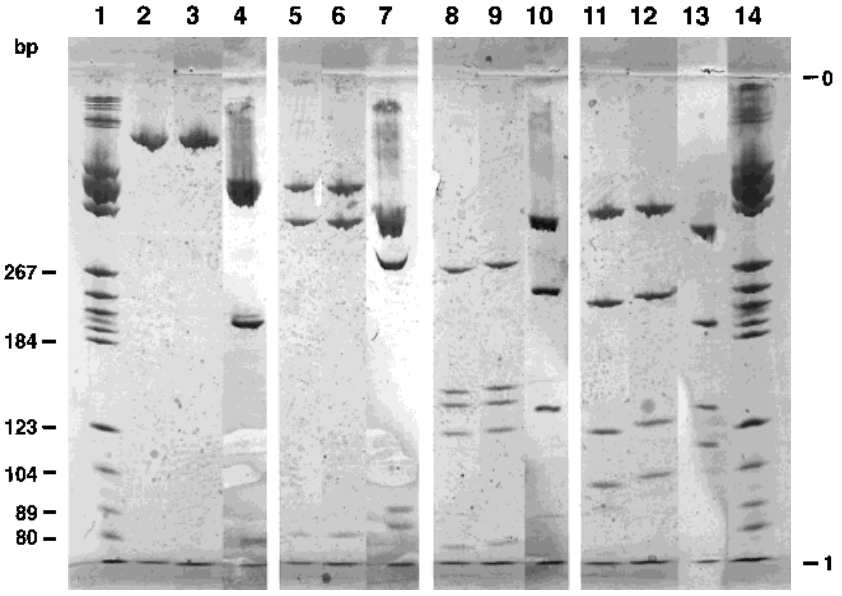

Figure 4. Assignment of Israeli strain IS5 to species *H. indicus*. RFLP analysis on the PhastSystem using the same conditions as in Fig. 1. Order of the restriction endonucleases: *Rsa*I, *Hin*fI, *Dde*I and *Alu*I. Lanes (3), (6), (9), and (12), restriction fragments of PCR product of IS5 strain of *H. indicus*; (2), (5), (8), and (11); restriction fragments of PCR product of LN2 strain of *H. indicus*; (3), (7), (10), and (13); restriction fragments of PCR product of Heliothidis strain of *H. bacteriophora*; (1) and (14), patterns of DNA size standards.

[13]. In fact, phylogenetic relationships between these pairs were also resolved but are less well supported. A high degree of sequence identity and lack of apomorphic characters suggest that sister species pairs within three distinct lineages may be mutually conspecific [13].

To date, all *H. bacteriophora* strains examined by us (data not presented) exhibit identical patterns to that of strain Heliothidis (Fig. 4). None of the strains seemed different from the EPN *H. argentinensis*. Thus our data provide more evidence for the conclusion of conspecificity of different strains assigned to *H. bacteriophora* and *H. argentinensis*, as suggested [13]. *Heterorhabditis* strains belonging to the so-called NWE group were considered as *H. megidis* when compared to the original OHI *H. megidis* strain [43]. Natural bacterial symbionts of OHI and NWE strains definitely belong to different subclusters (IV and II, respectively) of *Photorhabdus luminescens* [4] but they can grow on and are capable of utilizing each others' symbionts (Böszörményi *et al.*, in preparation).

It was reported that *Heterorabditis* strains isolated in Ireland and classified as *Heterorhabditis "Irish Type"* (represented by strains such as K122 or M145 [10, 28, 35, 45]) differ from both *H. bacteriophora* and *H. megidis*. The most convincing evidence of the phylogenetic separation of the Irish K122 and *H. megidis* OHI strain was provided by Adams *et al.* (Fig. 3 in [13]), reporting differences of the ITS sequences of OHI and K122. Our results presented in Fig. 2 demonstrate that K122 is also completely different from the *H. megidis* NWE strain HE 87.3. On the other hand, an *"Irish Type" Heterorhabditis* strain (EU349/HIT) isolated in Hungary (Griffin *et al.*, submitted; [8]), shows a gel pattern identical to K122, but different from HE 87.3. Our data (presented in Fig. 1) unambiguously indicate that K122 and EU349/HIT are identical. Using polyacrylamide gel electrophoresis in the PhastSystem, no differences between the two *"Irish Type"* strains, K122 and EU349/HIT, could be found and their patterns were similar to those of strain M145 *"Irish Type"* in Reid's data (Fig. 5), but differed characteristically from any *megidis* species, as well as from the *H. marelatus* (Hepialius) pattern (Fig. 2). Our data confirm that *Heterorhabditis "Irish Type"* strains comprise a distinct phylogenetic species [13] and contradict the hypothesis of conspecificity of *H. megidis* and *"Irish Type" Heterorhabditis*. This would support previous work from the Maynooth group (Griffin *et al.*, submitted; [8, 10, 28, 30, 35]). Definitely, there are similar and dissimilar digestion patterns of *H. megidis* (OHI + NWE) and *Heterorhabditis "Irish Type"* isolates, depending upon the enzymes used. Joyce *et al.* [30] could also distinguish between them using protein IEF. On the other hand, in the rDNS ITS region, 35 substitutions were found between *Heterorhabditis "Irish Type"* K122 and *H. megidis* OHI, in contrast with one substitution between *H. marelatus* and *H. hepialius*; two substitutions between *H. indicus* and *H. hawaiiensis* [35] and one substitution between *H. bacteriophora* and *H. argentinensis* [13].

Our results on IS5, supporting Reid's previous (unpublished) findings, suggest that it is identical to *H. indicus* (Fig. 4). Due to cross-breeding data [50], IS5 was previously assigned to *H. bacteriophora*. In fact, the problem of the contradiction of the biological and phylogenetic species concept has not yet been solved: IS5 and HP88 strains belonging to different phylogenetic species could be crossed successfully [46, 47, 50]; in spite of that, neither *H. bacteriophora* nor *H. megidis* can utilize or retain

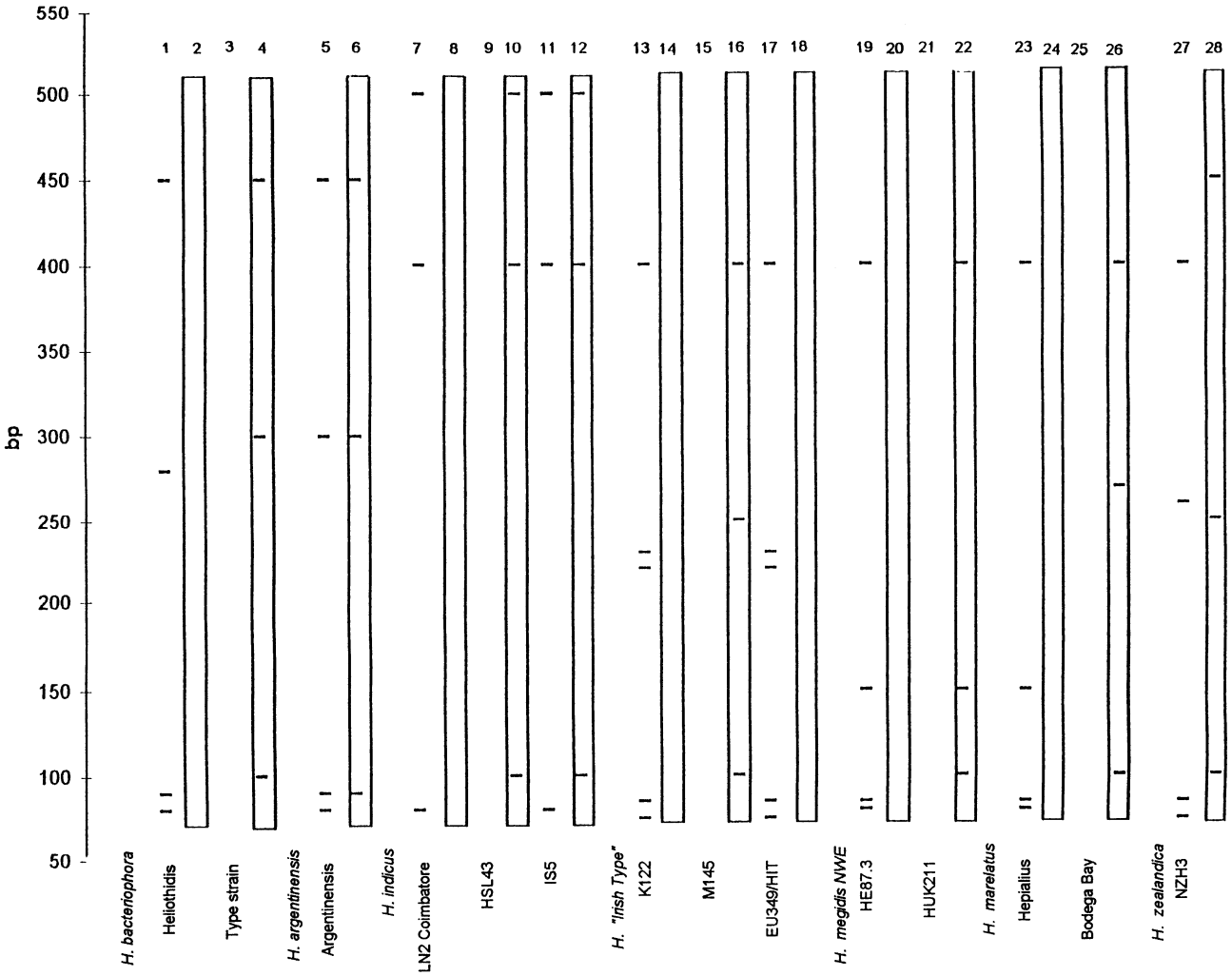

Figure 5. Improved resolution of the PhastSystem RFLP patterns compared to agarose gel electrophoresis patterns of DNA from individuals of strains representing different *Heterorhabditis* species. RFLP analysis on the PhastSystem using the same conditions as in Fig. 1. Gel electrophoresis patterns on agarose gels derived from Reid *et al.* [25]. The patterns derive from digestion with restriction endonuclease *Hin*fI. The patterns of the NZH3 strain, lanes (27) and (28), of *H. zealandica* are distinct from any other *Heterorhabditis* strain. The 28 gel lanes represent RFLP patterns on acrylamide (odd numbers) and agarose gels (even numbers, boxed lanes). There is no data in the empty lanes.

the bacterial symbiont of IS5 as its own (Böszörményi *et al.*, in preparation). IS5 can grow only on a few *H. bacteriophora* symbionts, such as HP88. *H. zealandica* differs from any other strain examined (Fig. 5), supporting its status as a separate species, in agreement with [13]. Two other strains (*H. hepialius, H. marelatus*) [37, 40, 41] were reported to belong to the same species, indistinguishable from the others. This was also proved in a previous molecular study [13]. Both *H. marelatus* and the *"Irish Type"* strains seem to be closer to *H. megidis* than to either *H. bacteriophora* or *H. zealandica* or *H. indicus*, on the basis of satellite sequence homology (unpublished data).

Steinernematids present a rather clear picture. Each species examined, including the new KMD 15 strain from Ohio, provides a characteristic pattern (Fig. 6). Comparing the patterns of different steinernematids obtained from restriction digests of *Alu*I, *Dde*I, and *Hin*fI, to those previously determined [25] on agarose gels, it was found that the two methods provide the same identification. Patterns were similar except that resolution in the lower molecular weight region of the gel (100 bp) was superior in the PhastSystem gels. In fact, some of the single bands seen on the agarose gels proved to the double the size of small molecular species. For instance, when one compares *S. intermedium* patterns obtained from an *Alu*I digest (Fig. 6) with the corresponding agarose gel pattern, it appears that the thick band on the agarose gel (200 bp) can be separated into distinct bands of 180 and 200 bp. Other bands appear similar.

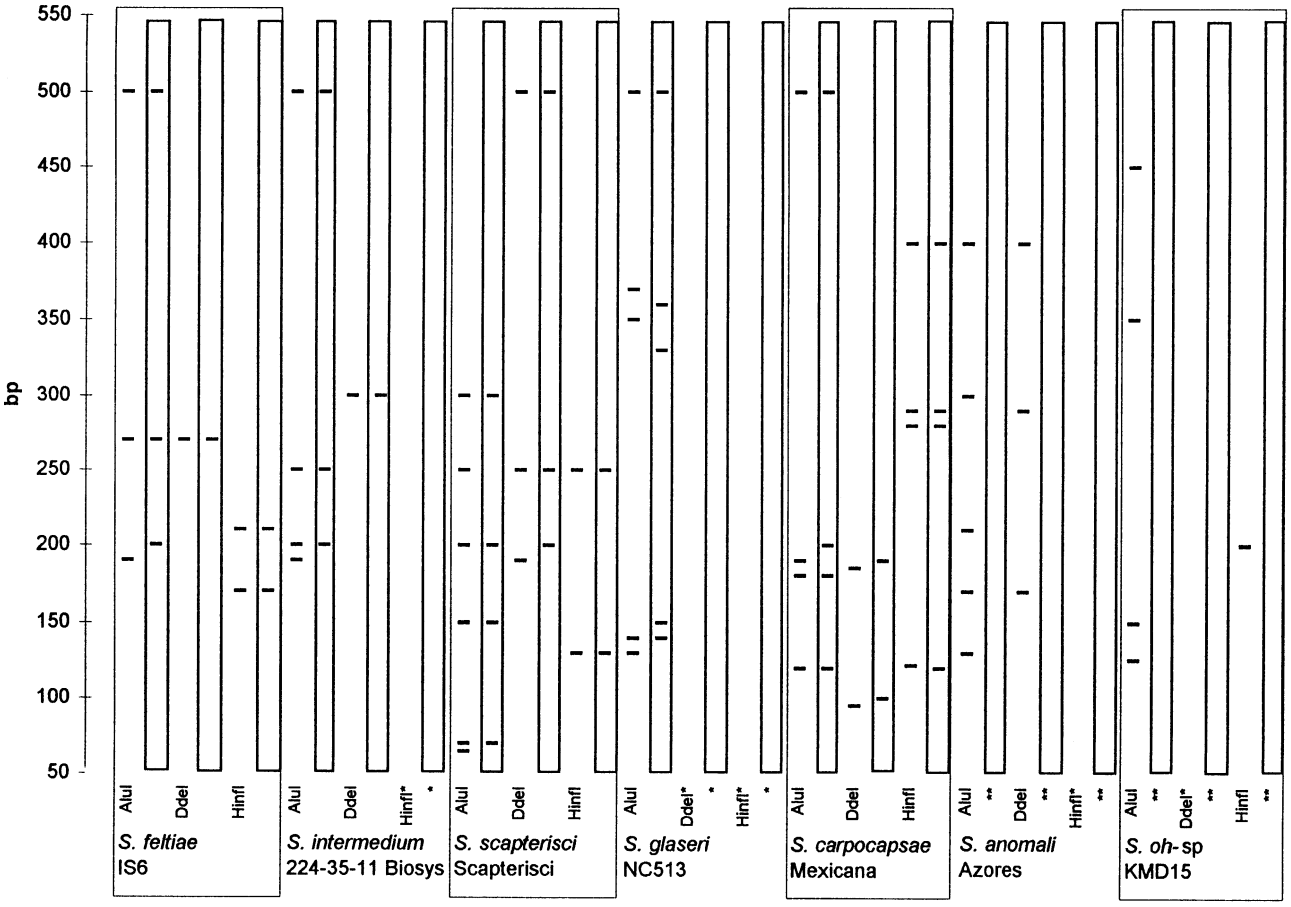

Figure 6. Improved resolution of the PhastSystem RFLP patterns compared to agarose gel electrophoresis patterns of DNA from individuals of strains representing different *Steinernema* species. RFLP analysis on the PhastSystem using the same conditions as in Fig. 1. Gel electrophoresis patterns on agarose derived from Reid *et al.* [25]. The patterns derive from digestions with restriction enzymes *Alu*I, *Dde*I, and *Hin*fI. The patterns of the KMD15 strain of *S. oh*-sp are distinct from any other *Steinernema* strain. The 42 gel lanes represent RFLP patterns on acrylamide (odd numbers) and agarose gels (even numbers, boxed lanes); *, no restriction fragment in this DNA size range; **, no data.

Corresponding results were obtained on the basis of comparisons of the *Heterorabditis* patterns between PhastSystem and agarose (Reid, A., in preparation) gels. For instance, the comparison of the *Alu*I, *Dde*I, *Hin*fI and *Rsa*I restriction patterns of *H. argentinensis* obtained on agarose and PhastSystem gels shows the identity of all corresponding patterns. Considering the advantages and the reproducibility of the results provided by the technique presented here, the use of PhastSystem electrophoresis as an alternative for the molecular identification of EPN strains can be recommended.

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