Characterization of *Heterorhabditis* isolates using DNA restriction fragment length polymorphism

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SUMMARY

The total DNA of 33 *Heterorhabditis* isolates from Europe, Australia, New Zealand and the USA was analysed on restriction fragment length differences of repetitive sequences. On that basis the isolates could be classified in three homogeneous groups : 1. *H. bacteriophora* and *H. heliothidis* group, 2. North-West European group and 3. Irish group. Based on the number of common bands the Irish and NW-European groups seem more closely related with each other than with the forst group.

RÉSUMÉ

Caractérisation des isolats de Heterorhabditis *par analyse du polymorphisme des fragments de restriction du DNA*

Le DNA total de 33 isolats d'*Heterorhabditis* originaires d'Europe, d'Australie, de Nouvelle-Zélande et des USA est analysé sur la base des différences de longueur des fragments de restriction des séquences de DNA. Les isolats se répartissent en trois groupes homogènes : 1 : *H. bacteriophora* et *H. heliothidis;* 2 : Groupe Nord-Ouest Europe; 3 : Groupe Irlande. En se fondant sur le nombre de bandes communes, les groupes irlandais et N.-O. européen paraissent plus proches l'un de l'autre que du premier groupe.

Insect parasitic nematodes of the genus *Heterorhabditis* have been collected in Australia, New Zealand, the USA and Europe. Of the many isolates very few have been identified and described at species level. Most of them only have a number referring the date and site of collection. Many of the isolates may even be identical. In practice, collected insect parasitic nematodes are named *Heterorhabditis* when they colour parasitized insect larvae red and have a life cycle and size which roughly corresponds with published data. Morphological characters are difficult to see and interpret. Interbreeding experiments, often used to determine species in the genus *Steinernema,* are not possible because the first generation in the insect host is self-fertilising hermaphroditic.

Five *Heterorhabditis* species have been described, namely : *H. hambletoni* (Pereira, 1937); *H. hoptha* (Turco, 1970); *H. heliothidis* (Khan, Brooks & Hirschmann, 1976); *H. bacteriophora* Poinar, 1975 and *H. megidis* Poinar, Jackson & Klein, 1987. The first three species were placed in the genus *Heterorhabditis* by Poinar (1975, 1979). Though numerous *Heterorhabditis* isolates have been collected in Europe none has been identified. All the described species originate from America and Australia.

With the increasing scientific and commercial use of *Heterorhabditis* spp. as biological control agents for insect pests and with the increasing number of isolates collected the need for a good identification method becomes urgent. Analysis of the total DNA based on comparison of restriction fragment length patterns of repetitive DNA elements has shown to be a useful method for identification of insect parasitic nematodes (Curran, Baillie & Webster, 1985; Curran & Webster, 1987, 1989).

This paper aims at comparing *Heterorhabditis* isolates from various European countries with each other and with the described species *H. heliothidis* and *H. bacteriophora* from the USA and Australia, in order to determine their relationships.

Materials and methods

NEMATODE ISOLATES

Some of the *Heterorhabditis* isolates were collected by ourselves but most were obtained from collections of other researchers. Table 1 gives a summary of the origin and the names of the isolates. The name or coding of the 39 isolates corresponds with that of our immediate source. In a number of instances this may not be the coding of the original collector. Some isolates, supposed to be identical, were obtained from more than one source. For these we have added the immediate source name to the isolate name or coding.

Table 1

Origin of the nematode isolates

Isolate code or name	*Original place of isolation*	*Obtained from the collection of*
1. HL-81	Limburg, The Netherlands	Groene Vlieg
2. HF-85	Flevoland, The Netherlands	Westerman/Simons
3. HFR-86	Friesland, The Netherlands	Westerman/Simons
4. HNH1-87	N-Holland, The Netherlands	Westerman/Simons
5. HW-79	Wageningen, The Netherlands	Westerman/Simons
6. HNB-87	N-Brabant, The Netherlands	Westerman/Simons
7. HB-87.1	Bergeyk, The Netherlands	Smits
8. HB-87.2	Bergeyk, The Netherlands	Smits
9. HE-87.3	Eindhoven, The Netherlands	Smits
10. HK-3	Darmstadt, W-Germany	Bathon
11. HK-6	Darmstadt, W-Germany	Bathon
12. HDA-1	Darmstadt, W-Germany	Ehlers (1)
13. HSH-1	Kiel, W-Germany	Ehlers
14. HSH-2	Kiel, W-Germany	Ehlers
15. HI-23	Italy	Ehlers (2)
16. HI-127	Italy	Ehlers (2)
17. HI-82	Italy	Westerman/Simons (2)
18. HSie	Siedlce, Poland	Bednarek
19. HPB	Puszcza Blata, Poland	Bednarek
20. *H. bacteriophora* W	Warsaw, Poland	Bednarek
21. HMol	Moldavia, USSR	Bednarek
22. HIR-K 122	Ireland	Griffin
23. HIR-M 145	Ireland	Griffin
24. HIR-M 170	Ireland	Griffin
25. HSP-1	Barcelona, Spain	del Pino
26. HSP-2	Barcelona, Spain	del Pino
27. HNZ	New Zealand	Ehlers (3)
28. HT-327	Australia	Ehlers (4)
29. *H. bacteriophora* K	Brecon, S-Australia	Kaya
30. *H. bacteriophora* NJ	Brecon, S-Australia	Bednarek (5)
31. *H. heliothidis*	N-Carolina, USA	Kaya
32. HPTI	California, USA	Kaya
33. HP-88	Utah, USA	Kaya

Groene Vlieg Company, Nieuwe Tonge, The Netherlands; P. Westerman, Friesland Agricultural College, Leeuwarden, The Netherlands; R.-U. Ehlers, Christian-Albrechts-University, Kiel, W-Germany; H. Kaya, University of California, Davis, USA; A. Bednarek, University of Warsaw, Poland; C. T. Griffin, St. Patricks College, Dublin, Ireland; F. G. del Pino, University of Barcelona, Spain; H. Bathon, Institute for Biological Control, Darmstadt, W-Germany.
( ) Isolates originally from : (1) H. Bathon, W-Germany. (2) K. V. Deseö, Italy. (3) W. Wouts, New Zealand. (4) D. H. Molyneux, Australia. (5) R. Gaugler, USA.

Nematode propagation

The nematodes were propagated in our institute on greater waxmoth larvae, *Galleria mellonella*, at 25 °C. Twenty fifth instar waxmoth larvae were exposed to 1000 infective juveniles, applied in 2 ml of water in a Petri dish (9 cm diam.), provided with two layers of filter paper. After 3-5 days the red-coloured infected waxmoth larvae were placed on a layer of humid filterpaper in a small Petri dish (5 cm diam.) without lid floating on a thin layer of water in a larger Petri dish (15 cm diameter) with lid. After *ca* 3 weeks the infective juveniles had moved to the water and were harvested for a period of 1 week. The nematodes were stored in *ca* 30 ml tap water at 5 °C in 250 ml tissue-culture bottles at densities up to 20 000 nematodes per ml.

DNA extraction

DNA was extracted by grinding 1 million infective juveniles in a mortar with liquid nitrogen until a fine white powder was produced. Before transferring the nematodes to the mortar they were first concentrated on filter paper in a vacuum filter holder connected with a water-jet pump. The powder was collected and 1 ml of extraction buffer (10 mM Tris-Cl pH = 8, 1 % SDS, 0.4 M NaCl, 5 mM EDTA) was added. Then 50 µl proteinase K solution (0.01 g/ml) was added and the mixture was incubated for 30 min at 37 °C and subsequently for 60 min at 65 °C. The solution was twice extracted with equal volumes phenol-chloroform-isoamylalcohol (25:24:1) and finally with chloroform-isoamylalcohol (24:1). DNA was precipitated from the final aqueous phase by adding 2 volumes of 96 % ethanol and 0.1 volume of 3 M ammoniumacetate and placing the mixture at — 70 °C for 30 min. The precipitated DNA was pelleted in a microcentrifuge and dried under vacuum at room temperature. The pellet was resuspended in 50 µl of TE-buffer (0.01 M Tris-Cl pH = 7.5, 0.001 M EDTA) and stored at 4 °C. The nucleic acid concentration was measured in a spectrophotometer at 260 nm. Generally 200-300 µg of nucleic acids was extracted from a million infective juveniles.

DNA digestion

The extracted total DNA was digested with restriction enzymes according to the manufacturers instructions by adding restriction enzyme EcoRI or MspI (Gibco/BRL), the appropriate enzyme reaction buffer and DNase-free RNase to 25 µg nucleic acids in TE-buffer. This mixture was incubated at 37 °C for 3 h.

Gel electrophoresis

Digested DNA samples (10 µl) were mixed with 2 µl loading buffer (0.1 % bromophenol blue, 0.25 % xylene cyanol, 40 % w/v sucrose in water) and run on a 1.1 % agarose gel, containing 1 µg/ml ethidium-bromide, at room temperature and 60 V for 3 h in TAE running buffer (0.04 M Tris-Acetate, 0.001 M EDTA pH = 8). A 1 Kb DNA marker (Gibco/BRL) was used as reference. The gel was photographed under UV-light.

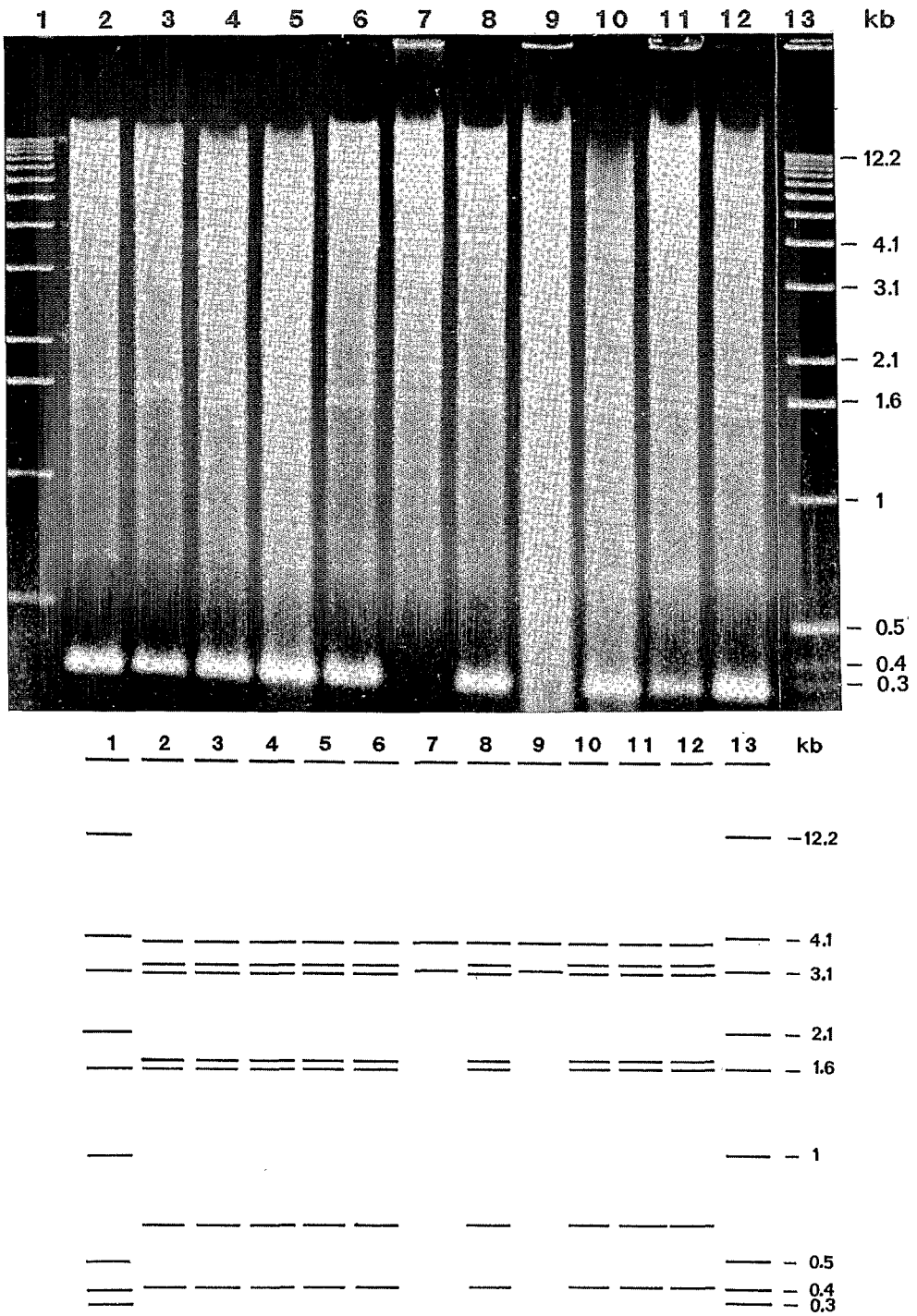

Fig. 1. EcoR1 digestion patterns of total DNA showing repetitive DNA fragments of the listed *Heterorhabditis* isolates from the Netherlands (Neth.), Australia (Aust.) and the USA. Photograph of the ethidium-bromide stained 1.1 % agarose gel (above). Line drawing showing only the repetitive DNA bands (below).

1. 1 kb DNA-marker, 2. HF-85, Neth., 3. HB-87.2, Neth., 4. HNB-87, Neth., 5. HB-87.1 Neth, 6. HFR-86, Neth., 7. *H. bacteriophora* K, Aust., 8. HE-87.3, Neth., 9. *H. heliothidis*, USA, 10. HL-81, Neth., 11. HNH1-87, Neth., 12. HW-79, Neth., 13. 1 kb DNA-marker.

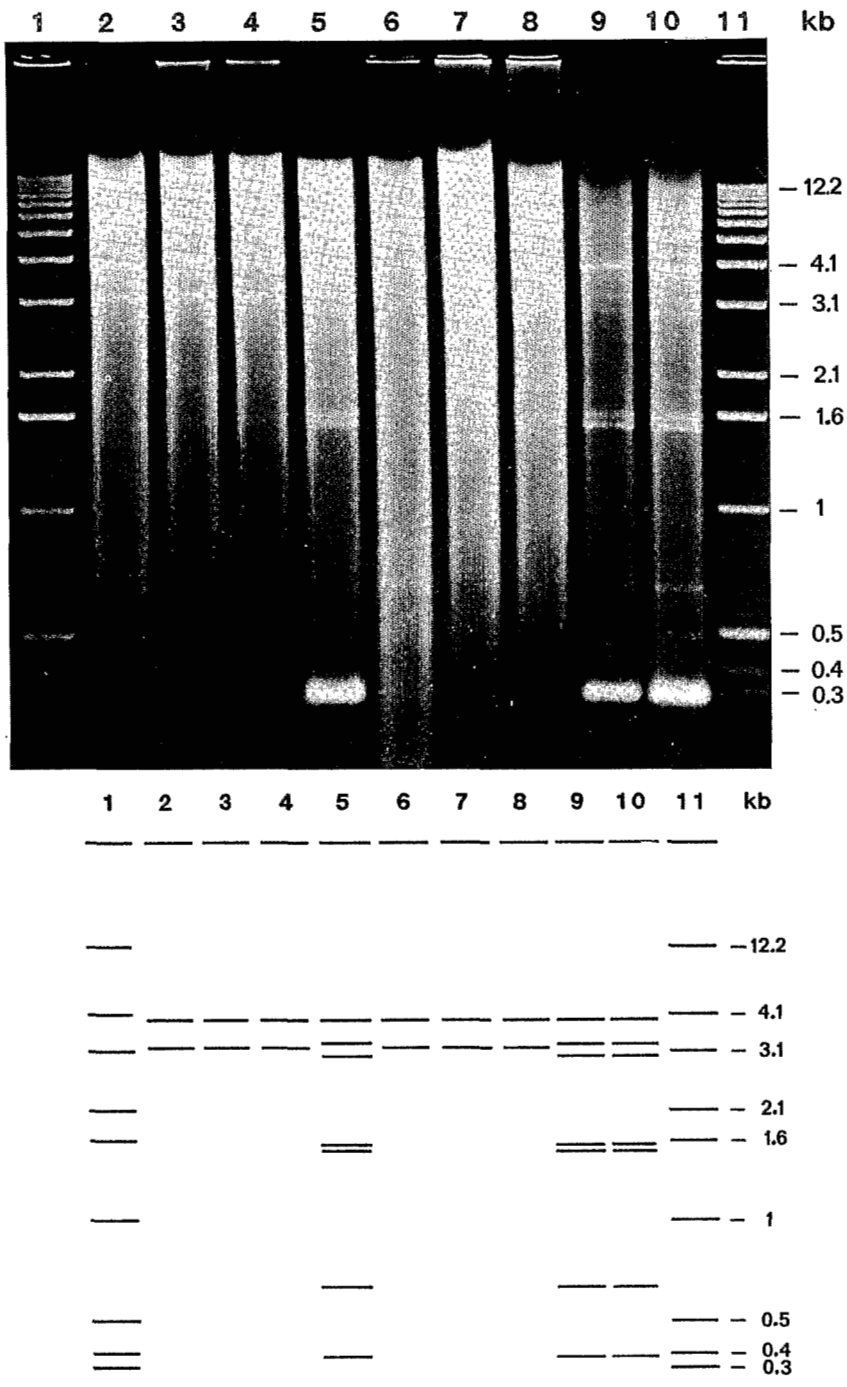

Fig. 2. EcoR1 digestion patterns of total DNA showing repetitive DNA fragments of the listed *Heterorhabditis* isolates from the USA, Australia (Aust.), Italy, Poland, Moldavia (Mold.) and the Netherlands (Neth.). Photograph of the ethidium-bromide stained 1.1 % agarose gel (above). Line drawing showing only the repetitive DNA bands (below).

1. 1 kb DNA-marker, 2. HPTI, USA, 3. HP-88, USA, 4. *H. bacteriophora* K, Aust., 5. HE-87.3, Neth, 6. *H. heliothidis*, USA, 7. HI-82, Italy, 8. HMol, Moldavia, 9. HSie, Poland, 10. HPB, Poland, 11. 1 kb DNA-marker.

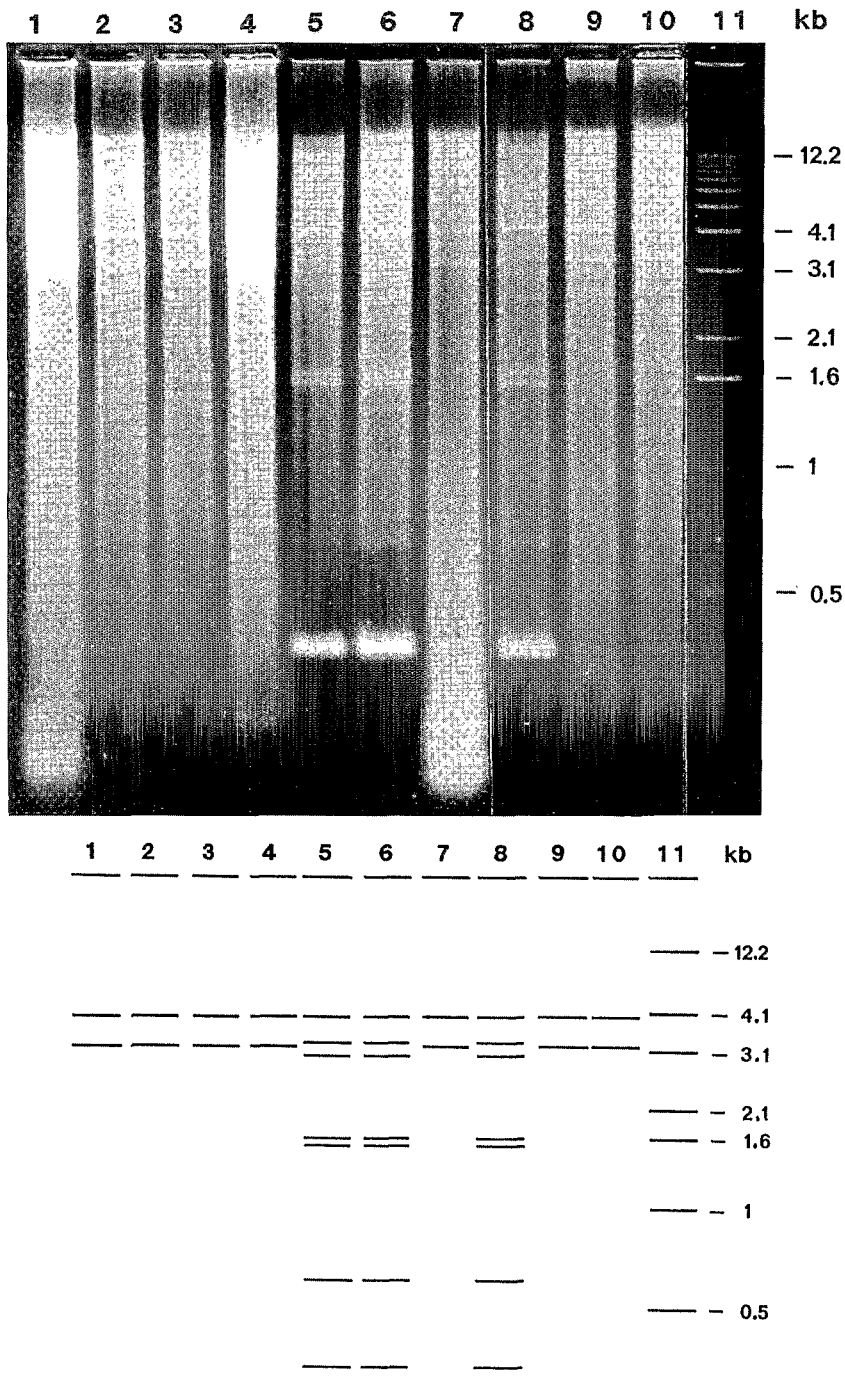

Fig. 3. EcoR1 digestion patterns of total DNA showing repetitive DNA fragments of the listed *Heterorhabditis* isolates from the USA, Australia (Aust.), New Zealand, Italy, W-Germany (W-Ger.) and The Netherlands (Neth.). Photograph of the ethidium-bromide stained 1.1 % agarose gel (above). Line drawing showing only the repetitive DNA bands (below).

1. *H. bacteriophora* NJ, Aust., 2. HI-23, Italy, 3. HI-127, Italy, 4. HNZ, New Zealand, 5. HSH-1, W-Ger., 6. HSH-2, W-Ger., 7. HT-327, Aust., 8. HE-87.3, Neth., 9. *H. bacteriophora* K, Aust., 10. *H. heliothidis*, USA, 11. 1 kb DNA-marker.

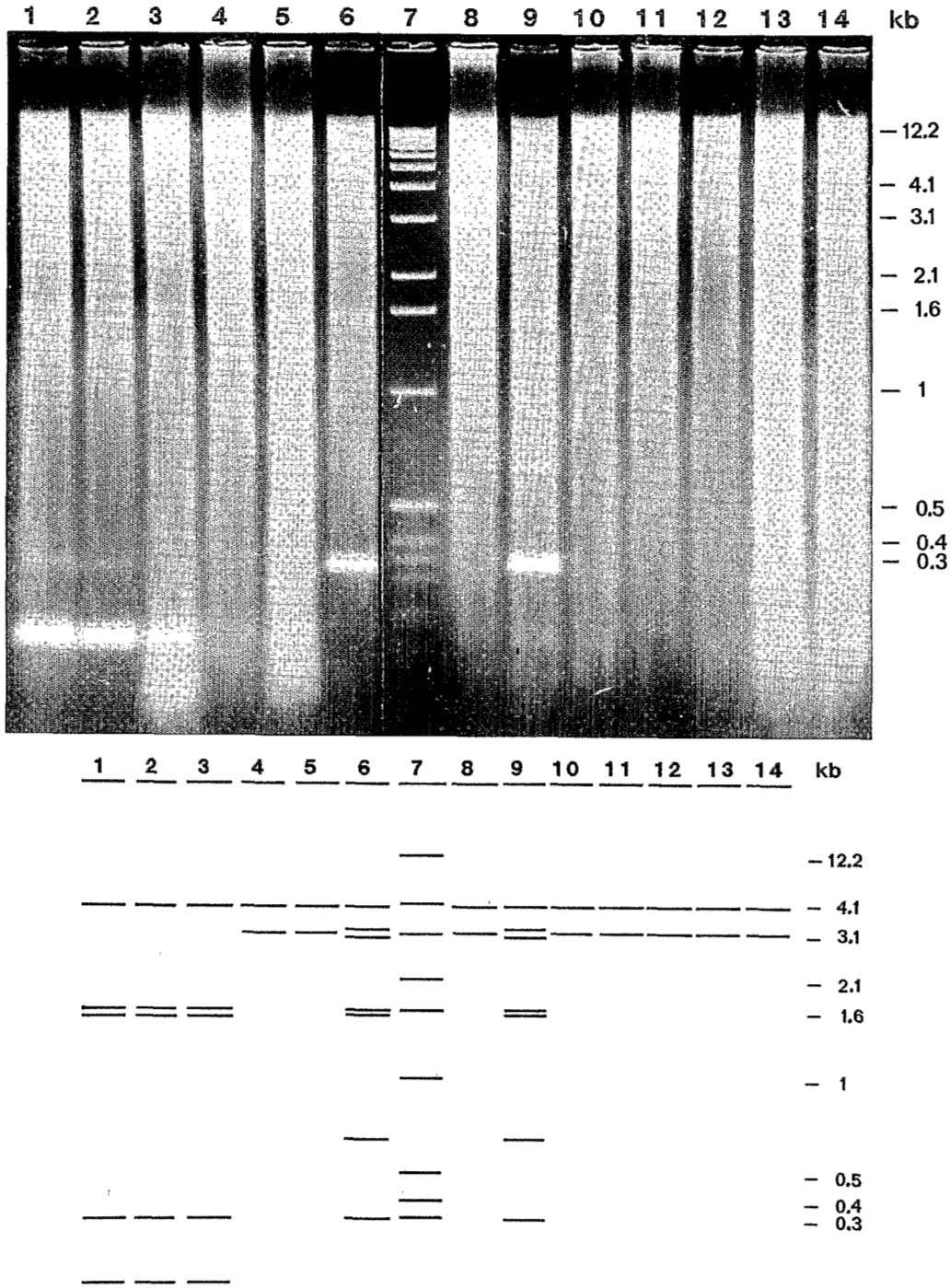

Fig. 4. EcoR1 digestion patterns of total DNA showing repetitive DNA fragments of the listed *Heterorhabditis* isolates from Ireland, Spain, The Netherlands (Neth.), W-Germany (W-Ger.), Poland, Australia (Aust.) and the USA. Photograph of the ethidium-bromide stained 1.1 % agarose gel (above). Line drawing showing only the repetitive DNA bands (below).

1. HIR-K122, Ireland, 2. HIR-M145, Ireland, 3. HIR-M170, Ireland, 4. HSP-1, Spain, 5. HSP-2, Spain, 6. HE-87.3, Neth., 7. 1 kb DNA-marker, 8. *H. bacteriophora* NJ, Aust., 9. *H. bacteriophora* W, Poland, 10. *H. bacteriophora* K, Aust., 11. *H. heliothidis*, USA, 12. HK-3, W-Ger., 13. HK-6, W-Ger., 14. HDA-1, W-Ger.

Results

Digestion of the total DNA of 34 *Heterorhabditis* isolates with restriction enzymes revealed three groups of isolates with different banding patterns. The bands represent repetitive DNA sequences. Digestion of DNA by the restriction enzyme MspI lead to patterns with a higher number of bands than digestion by EcoRI but comparison of the patterns obtained by both enzymes showed no difference in the grouping of the isolates.

In Figure 1 the banding patterns resulting from EcoRI digests of a number of Dutch isolates, so far not identified or described as a species, are compared with those of *H. bacteriophora* and *H. heliothidis*. The Dutch isolates clearly show a banding pattern very different from that of the two species. The banding patterns of *H. bacteriophora* and *H. heliothidis* are identical. The size of the fragments for the Dutch isolates is 3.9, 3.3, 2.9, 1.6, 1.5, 0.7 and 0.35 kilobasepairs (kb) and 3.9 and 3.2 kb for *H. bacteriophora* and *H. heliothidis*. Characteristic for the pattern of the Dutch isolates is the high intensity of the lowest 350 basepair band.

The isolates HPTI and HP-88 from the USA, HI-82 from Italy and HMol from the USSR have patterns identical to those of *H. bacteriophora* and *H. heliothidis* (Fig. 2). The two Polish isolates, HSie and HPB, have a pattern identical with the Dutch isolate HE-87.3 and also show the characteristic intense 350 basepair band.

The gel presented in Figure 3 shows that HSH-1 and HSH-2, two isolates collected in the North of W-Germany, have patterns identical to the Dutch and the Polish isolates. The Australian *H. heliothidis* isolate HT-327, two Italian isolates HI-23 and HI-127 as well as the New Zealand isolate HNZ have patterns identical to those of *H. bacteriophora* and *H. heliothidis*. The MspI digestion gave the same grouping as the EcoRI digestion.

The three isolates from Ireland, HIR-K122, -M145 and -M170 (Fig. 4), have DNA digestion patterns different from those of the Dutch isolates (HE-87.3) and of *H. bacteriophora* and *H. heliothidis*. There are some similarities with the patterns of the Dutch and Polish isolates with which there are three common bands. Characteristic for the Irish isolates is the intense band of *ca* 150 basepairs, lower in the gels than the intense band of the Dutch isolates of *ca* 350 basepairs. The other isolates belonging to the *H. bacteriophora* and *H. heliothidis* group show no intense band in their banding patterns. The pattern of the Irish isolates further shows bands of 0.35 kb, 1.5 kb, 1.7 kb and 3.9 kb.

The two Spanish isolates, HSP-1 and HSP-2, have patterns identical with *H. bacteriophora* and *H. heliothidis* (Fig. 4). The isolate *H. bacteriophora* Warsaw, collected in Poland, clearly shows the pattern of the Dutch isolates and the two other Polish isolates, HPB and HSie (Fig. 4), and therefore should not be named *H. bacteriophora*.

The three West-German isolates from Darmstadt, HDA-1, HK-3 and HK-6, have patterns identical to *H. bacteriophora* and *H. heliothidis* and the isolates from Italy, Spain and Moldavia. Their patterns differ from those of the two isolates from the north of Germany. As in the previous gels the MspI digestion patterns showed the same grouping of the isolates.

The typical banding patterns with fragment sizes of the three groups of isolates obtained after digestion of the total DNA by the restriction enzyme MspI are presented in Figure 5. The patterns show more bands than the EcoRI digestion patterns but characteristic intense bands are not present. All bands in the pattern of the NW-European group are also present in the pattern of the Irish group, which has three extra bands. Only two or three of the nine bands that are present in the pattern of the *H. bacteriophora* and *H. heliothidis* group are common with the NW-European and Irish groups.

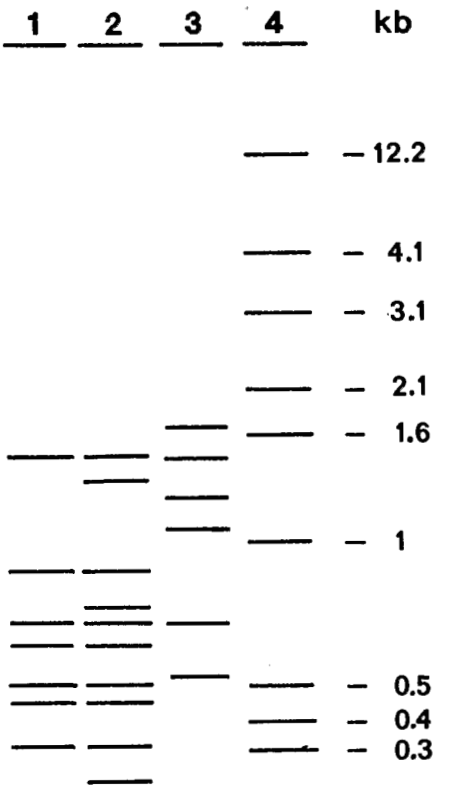

Fig. 5. Line drawing of typical MspI digestion patterns of total DNA showing repetitive DNA fragments of three groups of *Heterorhabditis* isolates. 1 : NW-European group; 2 : Irish group; 3 : *H. bacteriophora* and *H. heliothidis* group; 4 : 1 kb DNA-marker.

Discussion

The analysis of restriction enzyme digestion patterns showed that the 33 isolates tested in this study could be divided into three different groups. The first group consists of *H. bacteriophora*, *H. heliothidis* and all the isolates from the USA, Australia and New Zealand. In Europe the isolates from Italy, Spain, Moldavia and the middle of West-Germany also belong to this group. The second group consists of all the Dutch isolates together with two isolates from the North of West-Germany and the three Polish isolates. The third group consists of the three Irish isolates. Differences in restriction fragment patterns of repetitive DNA sequences are indicative of geneflow barriers and therefore of species boundaries (Curran, Baillie & Webster, 1985). Although these patterns cannot be the only distinguishing characters and morphological studies have to be done as well, it is likely that the three different groups represent three different species.

The number of common bands in the patterns of the three groups suggest that the Irish and NW-European groups are closer related to each other than with the *H. bacteriophora* and *H. heliothidis* group. The latter group should probably be given the species name *H. bacteriophora* Poinar, 1975. In the NW-European group no species has been described yet but work has already been started and the results will be published soon (R.-U. Ehlers & P. H. Smits, pers. comm.). HW-79, the first isolate of this group that was found, was isolated from an *Otiorhynchus sulcatus* larva in Wageningen, but it has been maintained too long in laboratory cultures to serve as a good type species. HSH-1 was collected recently from the same host in the neighbourhood of Kiel and therefore would be a better choice. Most other NW-European isolates were collected using the *Galleria*-trap method. Still one could argue that an analysis of the total DNA gives sufficient information to serve as the basis for the description of a new species, especially when morphological characters have shown to give insufficient clues.

The repetitive DNA-patterns found in this study for the *H. bacteriophora* group seem identical to those published by Curran and Webster (1989) for *Heterorhabditis* isolates group 1 from North Carolina. Surprisingly, however, these authors stated that the pattern of their group 1 differs from that of *H. bacteriophora*. Unfortunately they do not show the pattern of this species nor indicate the size of the fragments that are different. In an earlier study Curran, Baillie and Webster (1985), however, presented repetitive DNA patterns of three isolates from North Carolina, two of which were later placed in group 1. These patterns show more bands with smaller fragments than the patterns presented in their recent paper. It is possible that these smaller fragments are actually present on the gels in their recent study but that they were not visible in the published figures.

The group 2 isolates of Curran and Webster (1989), to which they state the isolate on which the description of *H. heliothidis* was based belongs, shows a very small difference to group 1, with the presence of a 3.1 kb band instead of the 3.2 kb band. We considered whether a similar difference between *H. heliothidis* and *H. bacteriophora* may be visible on Figures 1 and 2 of our paper and on one of the MspI digested gels (not shown), but finally decided also on the basis of Figures 3 and 4 that there is no difference between the patterns of these species. If there is a difference, it must be minimal and the two species must be very closely related. The third group of Curran and Webster (1989) has a EcoRI digestion pattern with bands at 3.9, 1.7 and 1.5 kb. Bands of approximately these sizes were also found in the Irish isolates and in the NW-European group and may point to some relationship.

Curran and Webster (1989) showed by positive hybridization with a ribosomal DNA probe from *Caenorhabditis elegans* that the 3.9, 3.2 and 1.7 kb bands in the ECoRI digestion of total DNA of *Heterorhabditis* isolates from North Carolina, USA, represent 28 s, 18 s and 5.8 s ribosomal DNA genes.

All insect-parasitic nematodes in the genus *Heterorhabditis* carry symbiotic bacteria of the genus *Xenorhabdus luminescens* (Akhurst, 1983). It has also been found that these bacteria may differ between species and isolates of *Heterorhabditis*. It is encouraging that R.-U. Ehlers (pers. comm.) while testing the symbiotic bacteria from the same *Heterorhabditis* isolates with a species specific DNA-probe, came to almost the same grouping of isolates as we did in our study. He did, however, find a difference between the symbiotic bacteria of *H. bacteriophora* and *H. heliothidis* and also further subgrouping within the three groups that are described in our paper. Analysis of the fatty acid patterns (Janse & Smits, 1990) also separated *X. luminescens* of the Dutch *Heterorhabditis* isolates from those of the *H. bacteriophora* group and placed the *X. luminescens* of *H. bacteriophora* and *H. heliothidis* in different groups.

The analysis of restriction fragment length patterns of repetitive DNA has been shown to be a useful tool in the identification of groups of *Heterorhabditis* isolates. However with this method we are not able to discriminate strain differences, which are very important in the use of these nematodes for the biological control of insect pests. For instance, it is known that a number of Dutch isolates differ in their activity at low temperatures (Griffin, Simons & Smits, 1989). In order to be able to identify *Heterorhabditis* isolates at subspecies or strain level strain-specific DNA probes will now be developed.

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Note added in proof

Recent analysis of the DNA of *H. megidis* has shown that the restriction fragment patterns of this species are identical to those of the NW-European group.

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