The identification of biological species in the genus *Heterorhabditis* (Nematoda: Heterorhabditidae) by cross-breeding second-generation amphimictic adults

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(*Received 3 August 1991; revised 23 November 1991; accepted 24 November 1991*)

SUMMARY

Entomopathogenic nematodes of the genus *Heterorhabditis* are morphologically conservative, consequently the majority of isolates remain unassigned at the species level. When a *Heterorhabditis* infective juvenile infects an insect host, it develops into a protandrous hermaphrodite female. These first-generation females give rise to a second generation which contains both males and females. Because of this complex life-cycle and also because of uncertainty as to whether second-generation females are amphimictic, cross-breeding studies to facilitate species determination have not been carried out previously. We demonstrate here that second-generation *Heterorhabditis* females are amphimictic. Because of this finding, we have been able to develop a successful cross-breeding technique for the purposes of species determination in *Heterorhabditis*. Interstrain crosses using second-generation males and females from the appropriate strains have been successfully set up in *Xenorhabdus luminescens*-treated *G. mellonella* cadavers and also on agar plates. Using the techniques described here we confirm that *H. bacteriophora* (Brecon strain), *H. megidis* and *H. zealandica* are distinct biological species, we note that the *H. bacteriophora* group contains at least 2 species and we provide evidence for the existence of a new Irish species of *Heterorhabditis*.

Key words: *Heterorhabditis*, amphimictic, cross-breeding, biological species.

INTRODUCTION

Entomopathogenic nematodes of the genus *Heterorhabditis* and *Steinernema*, together with their symbiotic bacterium *Xenorhabdus*, are becoming increasingly important as biological control agents for soil dwelling insect pests (see reviews in Gaugler & Kaya, 1990). The infective juveniles (IJs) of these nematodes actively seek insect hosts in the soil. Having gained entry into the insect haemocoel, the IJs release cells of their bacterial symbiont which multiply rapidly and usually kill the insect within 48 h. The growth of bacteria in the haemolymph provides suitable conditions for nematode growth and reproduction and within approximately 2 weeks of infection up to half a million IJs/gram of insect are produced (Akhurst & Bedding, 1986). These non-feeding stages emerge into the soil where they may survive for several months in the absence of a suitable host. The genus *Steinernema* was first described by Travassos in 1927 (Wouts *et al.* 1982) and the family Steinernematidae was erected by Chitwood & Chitwood (1937). The genus *Heterorhabditis* and family Heterorhabditidae were erected by Poinar (1975) with *H. bacteriophora*, which was isolated from a noctuid moth larva (*Heliothis punctigera*) in Brecon, South Australia, as the type species.

Many isolates of entomopathogenic nematodes have been collected from around the world, but it has been difficult, particularly for *Heterorhabditis*, to find suitable diagnostic characters for species identification and thus a large majority of *Heterorhabditis* isolates remains unassigned at the species level. In *Steinernema*, it has been possible to identify species on the basis of morphological characters in combination with cross-breeding tests and at present 9 species of *Steinernema* are recognized (reviewed by Poinar, 1990). Although 3 named species of *Heterorhabditis* were recognized by Poinar (1990), the extent of overlap in morphometric characters is such that, with the possible exception of *H. megidis*, no one individual from a population can be reliably assigned to a particular species (see for example the data reported by Poinar, Jackson & Klein (1987) and Wright (1990)).

Cluster analysis of isozyme electrophoretic data obtained using 22 isolates of *Heterorhabditis* from Australia, Asia, Europe, North America and Cuba, but not including *H. megidis*, identified 3 genetically separate groups (Akhurst, 1987). Akhurst also established that *H. bacteriophora* (Poinar, 1975) was probably conspecific with *H. heliothidis* (Khan, Brooks & Hirschmann, 1976; Poinar, 1979), that a New Zealand isolate (NZH) (Wouts, 1979) clustered with Tasmanian and USSR isolates, and that tropical isolates from Australia and Cuba and a Chinese

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isolate were clustered together and were only distantly related to the other isolates. On the basis of Akhurst's study and of certain morphometric characters (length of IJs and distance from the head to the pharynx base), Poinar (1990) considered that the NZH isolate represents a separate species, *H. zealandica*. At present, then, 4 *Heterorhabditis* species are usually recognized: *H. bacteriophora* Poinar 1975; *H. megidis* Poinar *et al.* 1987; *H. zealandica* Poinar 1990 and the unnamed species which contains the tropical and Chinese isolates of Akhurst's study.

Akhurst (1987) noted that, depending on the degree of genetic dissimilarity which delineates species in *Heterorhabditis* and which is at present unknown, there may be as many as 8 species among the 22 isolates which he studied. The extent of genetic divergence which is required to delineate a species, however, varies both between and within animal phyla. Allozyme electrophoresis indicates that the genetic distance observed between some species seems to be little more than that observed in local populations in other species (see King & Wilson (1975) and reviews by Avise & Aquadro (1982) and by Singh (1990)). Thus, where possible, the application of the biological species concept (Mayr, 1963) to test whether two populations can cross-breed and produce fertile F_1 progeny remains the most objective criterion by which species may be identified.

In *Steinernema*, where the IJs develop into amphimictic females and males, cross-breeding studies can be successfully carried out by placing individual IJs in hanging drops of *Galleria mellonella* haemolymph. After the nematodes reach adulthood, appropriate male–female crosses can be set up in the hanging drops (Poinar, 1967). Alternatively, two IJs may be injected into a single *G. mellonella* larva (Akhurst & Bedding, 1978). In *Heterorhabditis*, by contrast, when an IJ infects an insect host, it develops into a protandrous hermaphrodite female. These first-generation females give rise to a second generation which contains both males and females. However, it is not clear whether the second-generation females are exclusively amphimictic or whether they may also be facultative hermaphrodites (Akhurst, 1987; Curran & Webster, 1989; Poinar, 1990; Glaser, Gaugler & Segal, 1991). Because of the more complex life-cycle of *Heterorhabditis* which would necessitate the setting up of crosses using second-generation adults rather than the more robust IJs (as is possible for *Steinernema*), cross-breeding studies to facilitate species determination have not been carried out for *Heterorhabditis*. In the study reported here, we describe the technique which we have used successfully to carry out cross-breeding tests for species determination in *Heterorhabditis*, we provide evidence for the existence of new Irish species of *Heterorhabditis* and we confirm that *H. bacteriophora* (Brecon strain), *H. megidis* and *H. zealandica* are distinct biological species.

MATERIALS AND METHODS

Source and maintenance of the nematode and bacterial isolates

The *Heterorhabditis* isolates used in this study were obtained from the following sources: Dr R. J. Akhurst, CSIRO, Canberra – *H. megidis* (Ohio, USA), *H. bacteriophora* (Brecon, South Australia) and D1 (Darwin, Australia); Ir. P. Westerman, Agarische Hogeschool Friesland, The Netherlands – *H. zealandica* (strain NZH3), HI82 (Italy) and HP88 (Utah, USA); Dr Z. Mracek, Institute of Entomology, Czechoslovak Academy of Sciences, Branisovska, Czechoslovakia – P_2M (Havana, Cuba). The Irish isolates M170 and K122 are maintained in the authors' laboratories. Nematodes were cultured *in vivo* in *G. mellonella* larvae as described by Woodring & Kaya (1988). Symbiotic bacteria (*Xenorhabdus luminescens*) were isolated from surface sterilized IJs as described by Akhurst (1980). Primary forms of *X. luminescens* were maintained on NBTA agar plates (Akhurst, 1986).

Preparation of G. mellonella *cadavers*

One loop of the primary form of *X. luminescens* isolated from the appropriate nematode strain was suspended in 2 ml of Ringer's solution (Oxoid BR 52). Ten μl of this bacterial suspension was injected into the haemocoel of a *G. mellonella* larva at the base of the last pro-leg using a repeating Hamilton (PB 600) syringe. The *G. mellonella* larvae used for injection had been maintained at 15 °C without food for 1–2 weeks. The injected insect larvae were incubated at 20 °C and after 3 days they were checked for death and bioluminescence. Bioluminescing cadavers were stored at 15 °C and were used for crosses 5–14 days later.

Cross-breeding Heterorhabditis *isolates in* G. mellonella *cadavers*

Second-generation male and female nematodes were obtained from *G. mellonella* which had been incubated at 25 °C for 6–7 days post-infection with IJs of the appropriate strain. The nematode-infected insects were dissected in Ringer's solution using a Wild M5A stereomicroscope at 50 × magnification. Nematodes were collected by aspiration using a microcapillary pipette which was drawn out to give an external diameter of 150–250 μm. Only those female nematodes which had immature gonads in which mature oocytes were not visible were used in the cross-breeding experiments and, as an additional precaution, virgin females were taken only from

cadavers in which not more than 5–10% of the second-generation females had begun oogenesis. A series of *G. mellonella* were infected with IJs at 2-day intervals, to ensure that female nematodes at a suitable stage for cross-breeding were available when required.

For each cross, 5 virgin females and 5 males of the appropriate strains were injected (by aspiration using a microcapillary pipette) into each of 10 *X. luminescens*-treated *G. mellonella* cadavers. The cadavers had been prepared by injecting bacteria isolated from the nematode strain from which the female partner in the cross was derived. Three to five days later, an additional 5 males were injected into each cadaver. Following injection with nematodes, the cadavers were incubated at 25 °C on a damp filter paper in a Petri dish. One week after the crosses were set up 3–5 cadavers were dissected in Ringer's solution and the success of the cross was evaluated using a Wild stereo-microscope at 50× magnification. Where no progeny were found in the cadaver, the females were measured using a micrometer and were dissected to assess the state of development of their eggs. Where progeny did occur, their stage of development was recorded. Further cadavers were dissected 5 days to 2 weeks later, by which time either all the nematodes were dead or progeny had become established in the cadaver. In crosses where progeny were established, 3–4 cadavers were put on water traps to collect emerging infective juveniles.

The following controls were set up for each cross: Virginity/self-fertility test: 5 virgin females were injected without males into each of 10 *X. luminescens*-treated insect cadavers; Mating test/self-cross: 5 virgin females and 5 males of the same strain were injected into each of 10 cadavers and an additional 5 males were injected into the cadavers 3–5 days later. The result of a cross between different isolates was taken as valid only if (1) there were no progeny in the virginity test and (2) there were progeny from the self-cross. A cross was considered positive only if it resulted in the production of infective juveniles which were capable of infecting *G. mellonella* and from which a fertile hybrid line could be established.

Cross-breeding Heterorhabditis *isolates in lipid agar plates*

Lipid agar plates (5 cm diameter), prepared as described by Dunphy & Webster (1989), were inoculated with bacteria of the appropriate strain and incubated at 30 °C for 2 days. Second-generation males and virgin females were obtained by dissection from *G. mellonella* cadavers as described above. For each cross 10 females and 10 males of the appropriate strain were placed on each of 5 lipid agar plates (which had been inoculated and pre-incubated with primary form bacteria isolated from the nematode strain from which the female partner in the cross was derived). The plates were incubated at 25 °C and 3 days later an additional 10 males were added to each plate. Appropriate controls for virginity/self fertility and for self-crossing/mating test were also carried out. One week after the cross was set up, half of the females were removed from those plates where no progeny was observed. These females were measured and dissected to assess the state of development of their eggs. The remaining nematodes were assessed 2 weeks after the cross was set up. Where the progeny of a cross were fertile, IJs were transferred to fresh lipid agar plates and a hybrid line was established.

RESULTS

Self fertility tests of second-generation females

An experiment was set up to determine whether females of the *Heterorhabditis* strains HP88 and K122 were amphimictic or self fertile. Young females with immature gonads were isolated and grouped into 4 size classes. The smallest size class contained nematodes at the earliest stage at which they could be reliably identified as females and the largest size class contained non-gravid females with well-developed gonads, but in which oocytes were not visible (Fig. 1). The female nematodes were injected in groups of 5 into *X. luminescens* K122 or *X. luminescens* HP88-treated *G. mellonella* cadavers.

The results obtained are presented in Tables 1 and 2. In the case of K122, nematode progeny were detected only in 1 cadaver which had been injected with nematodes of the smallest size class. This finding resulted most probably from the misidentification of a male nematode. Nematode progeny were not found in any of the remaining 42 cadavers. In the case of HP88, progeny were detected in 4 cadavers which had been injected with nematodes of the largest size class. None of the HP88 nematodes in the 3 other size classes yielded progeny. These observations suggest that female nematodes in the largest size class can become inseminated before oogenesis has commenced. Adult female nematodes were recovered by dissection from the cadavers after a 7–10 day incubation at 25 °C. The female worms dissected from the sterile cadavers had undergone extensive oogenesis, but there was no evidence of zygote formation and the unfertilized eggs disintegrated after the death of the female.

On the basis of these findings, nematodes which had reached the size two developmental stage were used in subsequent crosses. At this stage it is easy to distinguish between males and females and the females not already inseminated. This developmental stage is reached at 800 μm by K122 females but in the other *Heterorhabditis* strains used in the cross-breeding experiments, the size at which females had reached a comparable developmental state depended

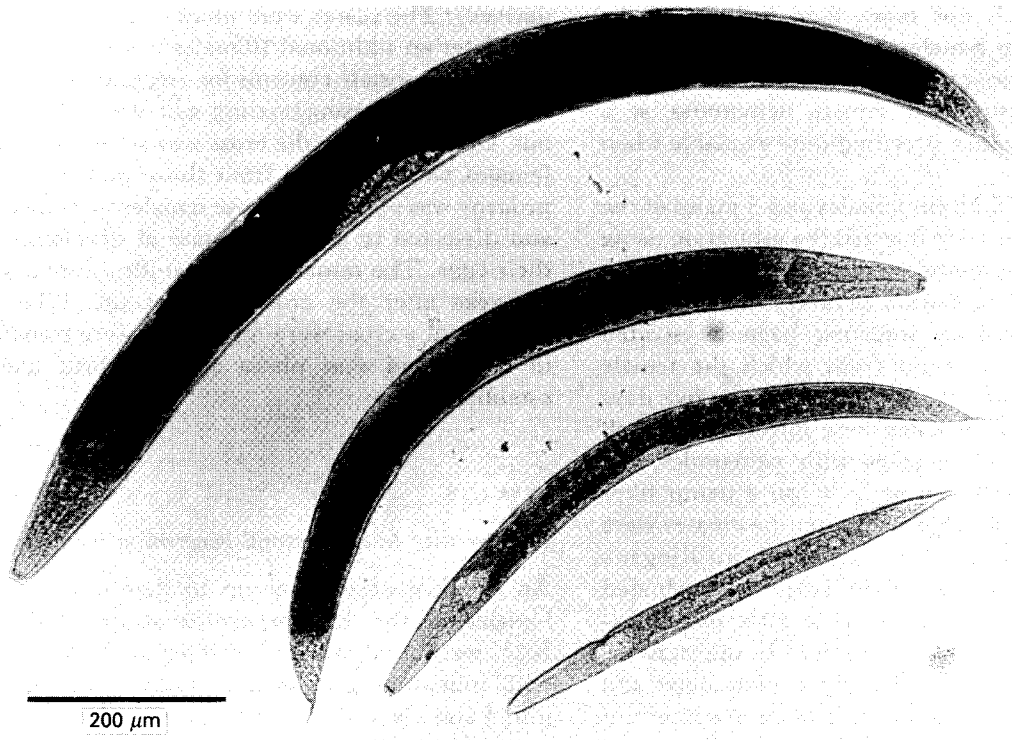

Fig. 1. Second-generation *Heterorhabditis* HP88 females corresponding to the 4 size classes used in the self fertility tests.

Table 1. Virginity/self fertility tests on second-generation *Heterorhabditis* K122 females

(Nematodes have been grouped into 4 classes on the basis of the size at which they were injected into *Xenorhabdus luminescens* K122-treated *Galleria mellonella* cadavers.)

No. of female nematodes injected	Mean length (μm ± S.E.) when injected into *Galleria*	No. of cadavers in which nematode reproduction		No. of injected nematodes recovered subsequently from cadavers
		was established	was not established	
55	497 ± 10·9 range 425–625	1	10	19
55	800 ± 15·2 range 700–925	0	11	23
45	1016 ± 14·0 range 925–1125	0	9	10
60	1266 ± 28·7 range 1100–1425	0	12	23

upon the strain (Table 3). The extent of gonadal development in the female nematodes which we selected for the cross-breeding experiments reported here corresponds to that described for the L4 stages of both *H. zealandica* (Wouts, 1979) and *Caenorhabditis elegans* (Kimble & White, 1981). Immature female nematodes at the developmental stage used for cross-breeding were set up in hanging drops of *G. mellonella* blood to observe their development and although they became gravid, moulting was not observed.

The self-fertility tests which we carried out for K122, HP88 (Tables 1 and 2) and for 5 additional *Heterorhabditis* strains (Table 3), show that when

Table 2. Virginity/self fertility tests on second-generation *Heterorhabditis* HP88 females

(Nematodes have been grouped into 4 classes on the basis of the size at which they were injected into *Xenorhabdus luminescens* HP88 treated *Galleria mellonella* cadavers.)

No. of female nematodes injected	Mean length (μm ± S.E.) when injected into *Galleria*	No. of cadavers in which nematode reproduction		No. of injected nematodes recovered subsequently from cadavers
		was established	was not established	
50	497 ± 10·8 range 396–594	0	10	28
50	785 ± 18·0 range 693–940	0	10	30
50	1057 ± 23·9 range 900–1250	0	10	31
50	1317 ± 20·6 range 1200–1550	4	6	17

Table 3. Self fertility tests on second-generation *Heterorhabditis* virgin females

Strain	No. of female nematodes injected	Mean length (μm ± S.E.) when injected into *Galleria*	No. of cadavers in which nematode reproduction		No. of injected nematodes recovered subsequently from cadavers	Mean length (μm ± S.E.) of recovered adult female nematodes
			was established	was not established		
K122	155	756 ± 8·99	0	31	78	3299 ± 65·2
M170	50	637 ± 13·1	0	10	14	2961 ± 204·8
H. megidis	100	666 ± 26·7	0	20	61	2887 ± 52·7
H. zealandica	60	665 ± 6·4	0	9	35	2531 ± 80·0
HP88	90	714 ± 18·2	0	18	65	3865 ± 66·2
HI82	60	795 ± 21·9	0	12	38	3822 ± 74·2
H. bacteriophora	70	815 ± 28·4	0	14	27	3111 ± 136·3

second-generation females with immature gonads are injected into prepared *G. mellonella* cadavers, zygote formation does not occur. These experiments clearly demonstrate that second-generation *Heterorhabditis* females are amphimictic and are therefore suitable for use in interstrain crosses for the determination of biological species. However, as is illustrated in Tables 1 and 2, care must be taken to ensure that the second-generation nematodes chosen for interstrain crosses are sufficiently well developed that males and females can be reliably distinguished from each other, while the females must still be sufficiently immature to ensure their virginity.

Cross-breeding experiments with Heterorhabditis *isolates in* G. mellonella *cadavers*

(1) *Interstrain crosses with strains from the* H. bacteriophora *group*. Table 4 details the results obtained when HP88 virgin females were crossed with males from the other isolates. Similar interstrain crosses were set up using virgin females from each of the other strains and the data obtained are summarized in Table 5. From Table 4 it can be seen that interstrain crosses between HP88 and HI82 yielded fertile progeny. The resulting hybrid IJs were used to infect *G. mellonella* larvae and a fertile hybrid line was established. Although HP88 is usually considered to be a strain of *H. bacteriophora* (Poinar, 1990; Poinar & Georgis, 1990), in our experiments fertile progeny were not obtained when either HP88 or HI82 were crossed with the Brecon strain of *H. bacteriophora*. The Brecon strain is not very vigorous and we find it difficult to obtain large numbers of progeny from this strain using either *in vitro* or *in vivo* cultivation. Despite this, fertile progeny were obtained from the self-cross between Brecon males and females (11 out of 12 cadavers yielded progeny) and males from the Brecon strain were capable of inseminating HP88 and HI82 females but the F_1 larvae died upon hatching. When Brecon females were inseminated by HP88 and HI82 males, embryonic lethal progeny resulted (Table 5). These results indicate that HP88 and HI82 are conspecific and represent a species which is distinct from *H. bacteriophora*.

Table 4. Virginity test and cross-mating experiments using second-generation *Heterorhabditis* HP88 females in *Xenorhabdus luminescens* HP88 treated *Galleria mellonella* cadavers

Strains crossed	No. of female nematodes injected	No. of cadavers in which nematode reproduction		No. of injected nematodes recovered subsequently from cadavers	
		was established	was not established	Sterile	Fertile
Virginity test: HP88 ♀♀	90	0	18	34	0
Self cross: HP88 ♀♀ × ♂♂	50	10	0	N.D.	N.D.
HI82 ♂♂ × HP88 ♀♀	50	9	1	N.D.	N.D.
H. bacteriophora (Brecon) ♂♂ × HP88 ♀♀	50	8*	2	17	15*
K122 ♂♂ × HP88 ♀♀	50	0	10	31	0
M170 ♂♂ × HP88 ♀♀	50	0	10	29	0
H. megidis ♂♂ × HP88 ♀♀	50	9†	1	N.D.	N.D.
H. zealandica ♂♂ × HP88 ♀♀	50	3‡	7	13	8‡

* Larval lethal in mated female.
† Sterile F_1 in cadaver.
‡ Embryonic lethal.
N.D., Not determined. Where there is an F_1 generation in the cadaver, the parents cannot be easily distinguished from the progeny.

Table 5. Results of cross-mating experiments to determine biological species in strains of *Heterorhabditis*

(In crosses which resulted in embryonic or larval lethals, progeny occurred in very low numbers, relative to the number of progeny observed in successful crosses.)

♂\♀	K122	M170	*H. megidis*	*H. zealandica*	HP88	HI82	*H. bacteriophora*
K122	+	+	−[b]	−[a]	−	−	−
M170	+	+	−[b]	−[a]	−	−	not done
H. megidis	−[c]	−[c]	+	−[c]	−[c]	−[c]	−[c]
H. zealandica	−[a]	−[a]	−[a]	+	−[a]	−[a]	−[a]
HP88	−	−	−	−[a]	+	+	−[a]
HI82	−	−	−	−[a]	+	+	−[a]
H. bacteriophora	−	−	−	−[a]	−[b]	−[b]	+

−, No zygote formed.
[a] Embryonic lethal.
[b] Larval lethal.
[c] Sterile F_1 progeny.
+, Fertile F_1 progeny.

(2) *Interstrain crosses with H. megidis.* None of the interstrain crosses carried out in this study with *H. megidis* males or females yielded fertile progeny. *H. megidis* males were successful in inseminating females from all the *Heterorhabditis* isolates studied, but the resulting F_1 adult progeny were sterile and some displayed an abnormal '*dumpy*' phenotype (Fig. 2). When *H. megidis* females were crossed with males from the other strains, however, zygote formation occurred only when the male partners were from *H. zealandica* (embryonic lethal progeny) or from the Irish strains (larval lethal progeny).

(3) *Crosses with the Irish isolates K122 and M170.* From Table 5 it can be seen that the two Irish isolates M170 and K122 are interfertile. The hybrid

Fig. 2. Sterile adult F_1 progeny obtained from a *Heterorhabditis megidis* male × *Heterorhabditis* HP88 female cross. Two of these F_1 progeny display a '*dumpy*' phenotype.

Table 6. Virginity test and cross-mating experiments using second-generation *Heterorhabditis* HP88 females on lipid agar plates containing *Xenorhabdus luminescens* HP88

Strains crossed	No. of female nematodes tested	No. of plates on which nematode reproduction		No. and status of nematodes recovered subsequently from plates	
		was established	was not established	Sterile	Fertile
Virginity test: HP88 ♀♀	60	0	6	30	0
Self cross: HP88 ♀♀ × ♂♂	50	5	0	4	30
HI82 ♂♂ × HP88 ♀♀	50	5	0	0	28
K122 ♂♂ × HP88 ♀♀	50	0	5	27	0
M170 ♂♂ × HP88 ♀♀	50	0	5	32	0
H. megidis ♂♂ × HP88 ♀♀	50	5*	0	0	16

* Sterile F_1 progeny.

IJs which resulted from the crosses between the Irish isolates were capable of infecting and killing *G. mellonella* and fertile hybrid lines were established. When K122 and M170 females were crossed with *H. megidis* males, the F_1 progeny developed into sterile adults, with the exception of three F_1 females in which a few hatched larvae were observed within the mother. These 3 females were transferred to lipid

agar plates but the F_2 larvae did not survive. When males from either of the Irish strains were crossed with *H. megidis* females, the progeny died as larvae. When the Irish strains were crossed with either males or females from *H. zealandica* embryonic lethal progeny resulted. None of the other interstrain crosses with the Irish isolates yielded F_1 zygotes. These results indicate that the Irish *Heterorhabditis* isolates are not conspecific with *H. megidis*, *H. bacteriophora* or *H. zealandica* and we have also confirmed that they are not interfertile with the tropical isolates D1 and P_2M (data not presented). These Irish isolates thus represent a distinct undescribed species of *Heterorhabditis*. A full description of this new species is in preparation.

Cross-breeding experiments with Heterorhabditis *isolates in lipid agar plates*

Since the progress of a cross-breeding experiment could be monitored more easily on agar plates than in cadavers, an experiment was set up in which second-generation HP88 females were cross-mated with males from four different strains of *Heterorhabditis*. Preliminary experiments had demonstrated that second-generation males and females obtained by dissection from *G. mellonella* cadavers were easier to collect and appeared to be more robust than those obtained from agar plates which had been inoculated with IJs. The results presented in Table 6 demonstrate that cross-mating experiments can be successfully carried out on agar plates. The results obtained in this series of cross-breeding experiments are also in agreement with the data obtained when cross-breeding experiments were carried out in *G. mellonella* cadavers.

DISCUSSION

The genus *Heterorhabditis* has a widespread distribution in tropical and temperate regions of the world. Because of the increasing importance of entomopathogenic nematodes in biological control, a number of surveys have been undertaken in different parts of the world in attempts to isolate promising new isolates of *Heterorhabditis* and *Steinernema* (Akhurst & Brooks, 1984; Arteaga-Hernandez & Mracek, 1984; Akhurst & Bedding, 1986; Blackshaw, 1988; Deseo, Fantonni & Lazzari, 1988; Hominick & Briscoe, 1990; Downes & Griffin, 1991; Griffin, Moore & Downes, 1991; Hara *et al.* 1991). Since heterorhabditids are morphologically conservative, the vast majority of isolates from this genus remain unassigned at the species level, resulting in a confusing variety of strain designations. The apparent lack of distinctive morphological features by which species of *Heterorhabditis* might be delimited, coupled with the inability to assign organisms to a given taxonomic level on the basis of genetic distance alone, has made the development of a suitable cross-breeding technique a matter of priority. The results which we present here are the first demonstration of successful cross-breeding experiments between *Heterorhabditis* isolates for the purposes of species determination. With the identification of a new Irish species of *Heterorhabditis* and the observation that the *H. bacteriophora* group contains at least 2 species, our results clearly demonstrate the value of interstrain crosses in delimiting species of *Heterorhabditis*.

Poinar (1990) noted that there are a number of unpublished accounts of second-generation females of *Heterorhabditis* being autotokous (i.e. producing progeny in the absence of males), but he suggested that these observations may be the result of the formation of infective juveniles from the progeny of first-generation females, since such IJs would be expected to give rise to hermaphrodite adults. The data which we present here for 7 strains and 5 species of *Heterorhabditis* demonstrate clearly that the initial progeny of first-generation hermaphrodite females are not autotokous. We did not investigate whether the second-generation progeny which arise later, and which frequently develop within the mother nematode, are amphimictic or autotokous. However, the observation that early second-generation *Heterorhabditis* females are amphimictic means that such females can be used in interstrain crosses for the determination of biological species.

A survey of Irish soil samples for the purposes of finding new cold-tolerant *Heterorhabditis* isolates yielded 48 Irish isolates of *Heterorhabditis* (Downes & Griffin, 1991). In this survey, positive *Heterorhabditis* sites were restricted to sand dunes and other sandy soils in coastal areas. All of the Irish isolates which we have tested so far are interfertile, but as the data which we report here indicate, the Irish isolates K122 and M170 are not conspecific with any of the *Heterorhabditis* type species nor within the undescribed tropical species D1 or P_2M. Further support for the genetic isolation of the Irish *Heterorhabditis* has been obtained by Smits, Groenen & de Raay (1991). In a comparison of the RFLP patterns obtained from total genomic DNA of 33 *Heterorhabditis* isolates from Europe, Australia, New Zealand and the US, these authors identified 3 genotypic types, namely the *H. bacteriophora* group; the North West European group; and the Irish group.

Poinar & Georgis (1990) presented a morphometric analysis of strain HP88. Although some quantitative differences (in particular in the size of males and amphimictic females) occurred between HP88 and the *H. bacteriophora* Brecon strain, these authors concluded that HP88 is qualitatively similar morphologically to the *H. bacteriophora* type species. In his (1990) review of the taxonomy of the Steinernematidae and Heterorhabditidae, Poinar

placed 23 *Heterorhabditis* isolates from Australia, North and South America, China and Europe in *H. bacteriophora*. Detailed morphometric analysis failed to identify HP88 as a distinct species (Poinar & Georgis, 1990), yet the cross-breeding results presented here indicate that HP88 and the Brecon isolate of *H. bacteriophora* are not conspecific. Thus the strains grouped at present within *H. bacteriophora* represent a complex of at least 2 species. Further cross-breeding studies, in conjunction with techniques of molecular systematics are being carried out at present in our laboratories in order to resolve the systematic relationships of the *H. bacteriophora* complex.

In addition to their usefulness in identifying biological species of *Heterorhabditis*, the cross-breeding techniques which we describe in this report will also be of use in genetic and applied studies (e.g. in generating genetic variability in founder strains for selective breeding programmes). We feel, however, that these techniques will be especially valuable in clarifying the taxonomic status of the many unassigned isolates in the genus *Heterorhabditis*.

This research was carried out with the generous support of the European Community (ECLAIR Grant 151). Our thanks to the researchers who provided us with nematodes from their collections, for their courtesy and co-operation. We also wish to thank Dr Paul Rodgers for his helpful comments on a draft of this paper.

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