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Intraspecific variation among isolates of the entomopathogenic nematode *Steinernema feltiae* from Bull Island, Ireland

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Summary – The application of large numbers of entomopathogenic nematodes (EPN) to control insect pests of agriculture is likely to have an impact on the local EPN fauna, yet little is known about the intraspecific relationships between EPN populations, particularly with regard to phylogeny and outbreeding. Here we assess the fitness, with regards to fecundity, host insect mortality and time taken to produce progeny, of isolates of *Steinernema feltiae* from Bull Island, Ireland. Exon-primed, intron-crossing (EPIC) PCR was used to examine intraspecific phylogenies between *S. feltiae* isolates, and identified up to three possible colonisation events of Bull Island. EPIC-PCR grouped two isolates, 33.D.(2) and 59.F.(2), separately from the remaining ten *S. feltiae* isolates These same two isolates consistently performed poorly in all fitness assessments. Following the crossbreeding of all isolates in *Galleria mellonella*, the number of host cadavers exhibiting emerging infective juveniles was significantly fewer than expected and there were significant differences between isolates in the number of days until progeny were observed. Host insect mortality varied between 40 and 87%. Such intraspecific variation may be a result of adaptation to different microhabitats of Bull Island, which in turn may be accentuated by laboratory culture practices.

Keywords – EPIC-PCR, fitness, insect, intron, progeny, variation.

Due to their apparently large host range, their specificity to insects and their speed of kill, some species of entomopathogenic nematodes (EPN) are traded globally as classical, inundative and augmentative biological control agents (Peters, 1996). The application of these nematodes into local systems is likely to have a great impact on the general soil fauna as well as local populations of EPN (Somasekhar *et al.*, 2002). However, little is known about the interactions of local intraspecific populations of EPN, particularly with regard to phylogenetic relationships and out-breeding potential.

Molecular techniques are often employed for the taxonomic identification of EPN isolates (*e.g.*, Smits *et al.*, 1991; Joyce *et al.*, 1994) as morphological identification is rarely straightforward (Reid *et al.*, 1997). Such techniques are being used with increasing frequency to infer interspecific EPN phylogenetic relationships (*e.g.*, Liu & Berry, 1996; Liu *et al.*, 1997, 2000; Reid *et al.*, 1997; Szalanski *et al.*, 2000; Nguyen *et al.*, 2001). However, it is rare that phylogenetic patterns or heirarchical structures have been inferred for same-species isolates (Blouin *et al.*, 1999; Dillon *et al.*, 2008). The targeting of introns in highly conserved nuclear genes, such as β-tubulin, is useful for identifying high levels of neutral variation within intraspecific populations (Lessa, 1992; France *et al.*, 1999). Exon-primed, intron-crossing (EPIC) primers amplify DNA segments that are usually under lax selective control and so exhibit potentially high rates of sequence divergence that can be informative in studying population structure and genetic diversity, for example, in organisms as diverse as marine shrimp (France *et al.*, 1999), fruit flies (He & Haymer, 1997) and humpback whales (Palumbi & Baker, 1994).

The fitness of EPN infective juveniles (IJ) is frequently measured by virulence and reproductive potential (Fenton & Hudson, 2002; Somasekhar *et al.*, 2002; Shapiro-Ilan *et al.*, 2005) but the ability to survive temperature and desiccation extremes, motility and response to host cues

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are also likely to be important factors (*e.g.*, Grewal *et al.*, 1996; Shapiro *et al.*, 1996; Somasekhar *et al.*, 2002; Wang & Grewal, 2002). Laboratory culture of EPN may have detrimental effects on laboratory populations due to, for example, selection under artificial conditions and inbreeding depression (Stuart & Gaugler, 1996; Bilgrami *et al.*, 2006). Costa *et al.* (2007) state that factors such as the virulence of the symbiotic bacteria, species of host used, concomitant infection with another organism, and the number of penetrated IJ, can affect the multiplication potential of EPN.

Of the three species of EPN found in Ireland (Griffin *et al.*, 1991), *Steinernema feltiae* and *Heterorhabditis downesi* co-exist on Bull Island, Dublin Bay, Ireland, although EPN distribution throughout the dune system is species-dependent (Rolston *et al.*, 2005). This study investigates the intraspecific variation among isolates of *S. feltiae* from Bull Island (Rolston *et al.*, 2005) in terms of fitness, relative infectivity, outbreeding potential and fecundity. As a means of establishing if there is a genetic basis for variance in the sample set, phylogenetic relationships between isolates are also assessed using EPIC-PCR of the β-tubulin gene region.

Materials and methods

Nematode samples and stock isolates

Table 1 lists the isolates of *S. feltiae* used in the experimental analyses. To obtain stock isolates, ten *Galleria mellonella* larvae were placed into a 9 cm diam. Petri dish lined with 9 cm diam. filter paper. A 2 ml suspension of isolate UK76 (1000 nematodes ml^{-1}) was added to the filter paper and the dish stored at 20°C for 5 days. This was repeated for the remaining isolates listed in Table 1. After 5 days, any dead *G. mellonella* were placed onto separate White traps (White, 1927). Twenty-one days after first infection, for each isolate, emerged IJ were pooled and stored as stock isolates (1000 IJ ml^{-1}) at 9°C until further use.

Species identification

Following morphological identification (Gaugler & Kaya, 1990; Griffin, pers. comm.), all isolates listed in Table 1 were confirmed as *S. feltiae* after the production of viable offspring following successful crossing with UK76 as follows. One IJ from each of two isolates to be crossed were removed separately from their container by pooter and placed into 20 μl of distilled water. The two nematodes were then removed using a 5 μl SGE syringe with plunger (SGE Europe, Milton Keynes, UK) and injected into a single late instar *G. mellonella* larva behind the last pro-leg. The syringe was checked to ensure both nematodes had been injected into the insect before being washed once in 70% alcohol and then twice in distilled water. The *G. mellonella* larva was then placed in a 6 cm diam. Petri dish lined with moist 5.5 cm filter paper and stored at 20°C for 5 days. The dead larva was then placed on a White trap and stored at 20°C until emerging IJ were observed in the trap water. There were 20 replicates per cross.

Table 1. *Isolates of* Steinernema feltiae *used in the EPIC-PCR analysis of the β tubulin intron. Asterisks indicate isolates used in all fitness assessment experiments.*

Isolate	Origin
Kildare	*Steinernema feltiae* from Co. Kildare, Ireland
Carlow	*S. feltiae* from Co. Carlow, Ireland
1.E.(1)*	Bull Island, isolated August 2002 (Rolston *et al.*, 2005)
2.G.(1)	Bull Island, isolated August 2002 (Rolston *et al.*, 2005)
4.G.(1)	Bull Island, isolated August 2002 (Rolston *et al.*, 2005)
33.D.(2)*	Bull Island, isolated August 2002 (Rolston *et al.*, 2005)
35.C.(1)	Bull Island, isolated August 2002 (Rolston *et al.*, 2005)
59.F.(2)*	Bull Island, isolated August 2002 (Rolston *et al.*, 2005)
65.D.(1)	Bull Island, isolated August 2002 (Rolston *et al.*, 2005)
74.D.(1)	Bull Island, isolated August 2002 (Rolston *et al.*, 2005)
74.J.(1)	Bull Island, isolated August 2002 (Rolston *et al.*, 2005)
77.J.(1)*	Bull Island, isolated August 2002 (Rolston *et al.*, 2005)
UK76*	Commercial UK *S. feltiae* (Microbio, Cambridge, UK)

Assessment of cross breeding

Four Bull Island isolates were selected for cross breeding studies, two from the extremities of the sample area of Rolston *et al.* (2005), isolates 1.E.(1) and 77.J.(1), and two from the middle region of the same sample area, isolates 33.D.(2) and 59.F.(2). The distances between soil cores from which the Bull Island isolates were obtained are shown in Table 2. The UK isolate, UK76, was also included in this study. These five isolates were crossed with themselves (selfed) and each other (out-crossed) as described above. There were 40 replicates per isolate cross, making 600 crosses in total. Comparisons between actual and expected number of emergences were performed using one-proportion tests. The number of progeny produced, the time taken to produce progeny, and insect mortality were all measured to assess the success of each cross.

Progeny numbers

The numbers of progeny found in the water of each White trap as a result of the above crosses were recorded as follows. The White trap IJ suspension was collected 7 days after emergence was first observed. Each White trap was rinsed twice with water to ensure the collection of IJ. The IJ suspension from a single *G. mellonella* cadaver was placed into individual 100 ml graduated cylinders and inverted three times to ensure thorough mixing. Twenty ml of the suspension was pipetted onto an inverted 6 cm diam. Petri dish lid, and the number of IJ counted. An average number of IJ per 20 μl was calculated after five counts, and the total number of IJ 100 ml^{-1} (or total number of IJ emerged) calculated. This estimated the total emergence of cross-progeny per cadaver per cross after 7 days. This was repeated for each cadaver from which IJ emerged. The totals calculated for each cadaver per cross were combined to produce an overall estimate of the mean number of IJ emerged from each cross.

Table 2. *Calculated straight line distances (m) between cores from which the isolates were obtained on Bull Island.*

Isolate	Isolate			
	1.E.(1)	33.D.(2)	59.F.(2)	77.J.(1)
1.E.(1)	0	320.16	580.09	761.64
33.D.(2)		0	260.77	444.07
59.F.(2)			0	184.39
77.J.(1)				0

Time taken to produce progeny

The isolates used in this study were 1E.(1), 33.D.(2), 59.F.(2), 77.J.(1), UK76, and also IJ produced from their respective crosses. For each isolate or cross, 40 *G. mellonella* were divided equally among four 9 cm diam. Petri dishes lined with moist 9 cm filter paper (10 *G. mellonella* per dish). For each isolate or cross, 2 ml of IJ suspension (1000 IJ ml^{-1}) was pipetted into each of the Petri dishes, and then incubated at 20°C. After 3 days, seven randomly selected *G. mellonella* cadavers per cross progeny were dissected each day in quarter-strength Ringer solution until progeny were observed in a total of seven cadavers per selfing or cross. No attempt was made to identify the juvenile stage of the progeny. A mean time until observation of any progeny was then calculated for each cross or selfing. Differences in the mean time until progeny were observed were calculated using the Kruskal-Wallis test.

Assessment of insect mortality

The ability of all five parent isolates (1.E.(1), 33.D.(2), 59.F.(2), 77.J.(1) and UK76) and their crosses to kill *G. mellonella* larvae was assessed. One-on-one infections were performed in 1.5 ml microcentrifuge tubes as described by Rolston *et al.* (2006). No larva received more than one nematode. The tubes were placed at 20°C for 5 days and then the mortality of *G. mellonella* was scored. The tubes were placed back at 20°C for a further 5 days, when insect mortality was scored again. This was repeated for each parent isolate and each cross progeny. Comparisons between parent isolates and their respective crosses were made using two-proportion tests. Comparisons between rank order of isolates and crosses after 5 and 10 days were made using Spearman Rank Correlation.

Molecular β-tubulin analysis of nematode populations

Nematode isolates (Table 1) were cultured in *G. mellonella* larvae as described above. Seven days after inoculation, the infected cadavers were transferred to White traps to collect emerging infective juveniles. IJ were harvested each day for 7 days following first emergence. Each day's harvest was pooled, washed in distilled water and allowed to settle in their container. The supernatant was discarded and 1 ml of the concentrated nematode suspension was pipetted into a 1.5 ml microcentrifuge tube and centrifuged (Eppendorf centrifuge, 5415D) at 16 000 *g* for 5 min. The supernatant was again discarded and the nematode pellet (approximately 500 mg

wet weight) stored at −20°C until further use. The pellet was semi-defrosted and the nematodes ground in the tube by agitation using a sterilised blunt-ended mounted needle. The DNA of these isolates, plus a negative control, *Caenorhabditis elegans*, was then extracted using a Qiagen Dneasy animal tissue kit (Qiagen, Hilden, Germany).

The PCR reaction was performed in a PTC-200 thermal cycler (Bio-Rad Laboratories, Waltham, MA, USA), using *S. feltiae* β-tubulin EPIC-PCR primers (Boyle *et al.*, unpubl.). The PCR program for DNA amplification was as follows. Ten min of denaturing at 94°C, 3 cycles of 30 s denaturing at 94°C, 30 s annealing at 55°C and 30 s extension at 72°C; 35 cycles of 30 s denaturing at 94°C, 30 s annealing at 58.2°C and 30 s extension at 72°C; 5 min extension at 72°C. Verification of the PCR products was performed in 2.2% agarose gel stained with ethidium bromide (0.5 μg ml^{-1}) in 0.5 TAE buffer. The gel electrophoresis was run at 100 V for 1 h and 10 min and the gel was subsequently visualised using a Kodak DC290 camera, and the imaging program Kodak ID 3.5. Fresh PCR product was cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and plasmid DNA was subsequently isolated using a QIAprep spin miniprep kit (Qiagen). The amplified DNA was sequenced by MWG Biotech (three clones per isolate) and then aligned using CLUSTALX (Thompson *et al.*, 1997), and analysed using the maximum likelihood method, conducted using PAUP* 4.0b 10 (Swofford, 1996).

Statistical analyses

Statistical analyses were conducted using Minitab v13.1 and Statistix 8.

Results

Species identification

All Bull Island isolates were confirmed as *S. feltiae* following morphological studies and the production of viable offspring following cross-breeding with the commercial *S. feltiae* isolate, UK76.

Assessment of cross breeding

All of the insects died as a result of the injection of IJ, with all dead insects showing typical symptoms of EPN infection. Only 50% of cadavers would be expected to have received both a male and a female nematode. However, the number of cadavers that actually resulted in IJ emergence was significantly fewer than that expected, *i.e.*, the number deviated significantly from the expected 50% of cadavers injected (one proportion test, $z = -19.76$, $P < 0.001$). There was great variation between crosses in the number of replicates that resulted in emerging IJ (Table 3). Isolate 1.E.(1) crossed with the greatest number of isolates and produced the highest number of cadavers exhibiting emerging IJ. Isolate 33.D.(2) crossed with the least number of isolates and produced the fewest number of cadavers exhibiting emerging IJ (Table 4). Thirty days post-infection, 82 intact cadavers that had not produced emerging IJ were randomly selected for dissection to examine reasons for their failure. Of the dissected cadavers, 36.6% (30/82) did not contain any adult nematodes (the injected IJ were not found). Of the dissected cadavers that did contain developed first generation adult nematode(s), 50% (25/52) contained only one nematode. Within these lone adults there was a 1 : 1 male to female ratio ($\chi^2 = 0.19$, $df = 1$, $P > 0.05$). Of these lone nematodes, 74.2% of males and 72.2% of females were dead. Live IJ that had failed to emerge were found in five cadavers upon

Table 3. *The number of* Galleria melonella *cadavers that showed infective juvenile (IJ) emergence after various crosses, 30 days after infection. Selfed isolates (isolates crossed with themselves) are italicised.*

Cross	Number of replicates (out of a probable 20*) showing emerging IJ
1.E.(1) × 1.E.(1)	11
1.E.(1) × 77.J.(1)	10
77.J.(1) × UK76	10
UK76 × UK76	9
59.F.(2) × 59.F.(2)	6
1.E.(1) × UK76	6
33.D.(2) × 59.F.(2)	3
33.D.(2) × 33.D.(2)	1
77.J.(1) × 77.J.(1)	1
1.E.(1) × 59.F.(2)	1
1.E.(1) × 33.D.(2)	0
33.D.(2) × 77.J.(1)	0
33.D.(2) × UK76	0
59.F.(2) × UK76	0
59.F.(2) × 77.J.(1)	0
Total	58

* Only half of the replicate insects of any cross would have been expected to have received both a male and a female IJ. On that basis, out of 40 replicates of any cross, 20 replicates would have been expected to show reproduction (emerging IJ).

dissection. The selfing 59.F.(2) × 59.F.(2) resulted in three of these cases. The only instance where all such unemerged progeny were dead was found in a selfing of 33.D.(2) × 33.D.(2). When selfed, the isolates 33.D.(2) and 77.J.(1) performed poorly with only one emergence for each isolate out of an expected 20.

Progeny numbers

There was great variability in the mean number of IJ emerging as a result of successful crosses. When selfed, UK76 and 1.E.(1) produced the highest mean number of emerged IJ, yet crossing these isolates with each other lead to a large decrease in the number of IJ emerging (Fig. 1). On no occasion did outcrossing UK76 or 1.E.(1) with any other isolate result in an increase in numbers of IJ emerging when compared with their respective selfings. Five crosses failed to produce any progeny at all. Three of these failed crosses involved isolate 33.D.(2) and the remaining two involved isolate 59.F.(2). Only crosses that produced emergence could be used to assess both the time taken to produce second generation and insect mortality.

Table 4. *Number of isolates (including selfs) of* Steinernema feltiae *with which each isolate crossed successfully, and cadavers producing emergences.*

Isolate	Number of successful crosses (out of five)	Number (and percentage) of emergences (out of a possible 200)
1.E.(1)	4	28 (14%)
UK76	3	25 (12.5%)
77.F.(1)	3	21 (10.5%)
59.F.(2)	3	10 (5%)
33.D.(2)	2	4 (2%)

Time taken to produce progeny

There was significant difference between isolates for the mean number of days until progeny were observed within host insect cadavers (Kruskal-Wallis test: $F = 42.1$, $df = 97$, $P < 0.001$: Fig. 2). The highest and lowest median values were 7 days (59.F.(2) × 59.F.(2)) and 4 days (the seven crosses with the lowest values shown in Fig. 2), respectively. Progeny of the cross 33.D(2) × 59.F(2) failed to kill any insects and so no second generation juveniles were observed. The overall mean number of days until second generation juveniles were observed was 4.91 ± 0.11 days. Isolate 1.E.(1) and any cross involving it produced second generation juveniles after 4 days, with no measured variation. Outcrosses and selfing involving UK76 produced second generation individuals quicker than the stock UK76 isolate. Except when crossed with 1.E(1), isolates 33.D.(2) and 59.F.(2) consistently took longer than the mean number of days to produce second generation juveniles.

Assessment of insect mortality

Three of the five stock isolates, UK76, 1.E.(1) and 77.J.(1), produced the highest percentage of insect mortality (Fig. 3). When crossed together, 1.E.(1) and 77.J.(1)

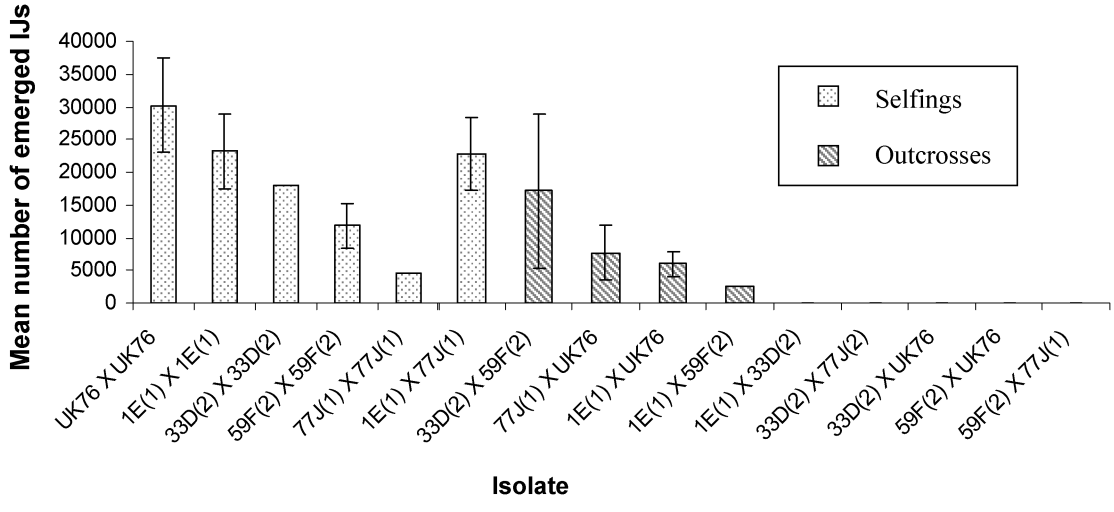

Fig. 1. *Mean number per fertile cadaver (± standard error) of* Steinernema feltiae *infective juveniles emerging from* Galleria mellonella *cadavers after cross-breeding. Data without error bars are the result of only one successful emergence.*

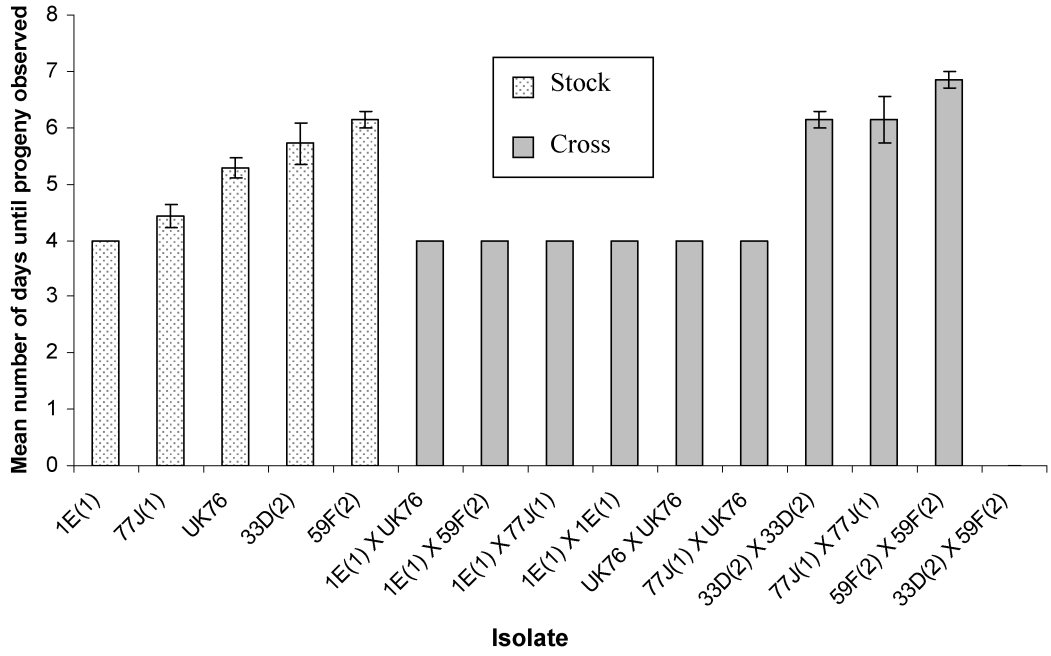

Fig. 2. *The mean (± standard error) number of days until second generation juveniles of various stock* Steinernema feltiae *isolates and their crosses were observed inside* Galleria mellonella *cadavers. Data without error bars showed no variation in the number of days taken for progeny to be observed (n = 7 per cross). No emerging progeny were observed for the cross 33.D.(2) × 59.F.(2).*

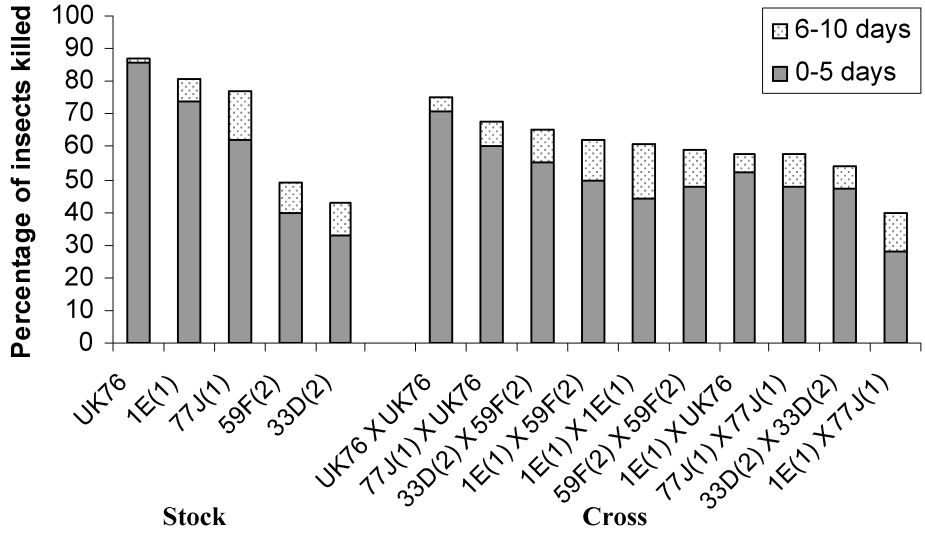

Fig. 3. *Percentage of* Galleria mellonella *killed after 5 and 10 days of one-on-one infection with stock isolates and their various crosses of* Steinernema feltiae *isolates.*

caused the lowest percentage insect mortality. Stock isolates 33.D.(2) and 59.F.(2) had the second and third lowest number of insect kills respectively. The progeny of 33.D.(2) × 59.F.(2) killed significantly more insects than either of their respective parent isolates (two proportion tests: $z = 2.98$, $P < 0.01$; $z = 2.14$, $P < 0.05$, respectively). By contrast, the IJ produced from crosses involving UK76, 1.E.(1) and 77.J.(1) all killed significantly fewer insects than their parents (two proportion tests: $P = 0.05$ or lower). Of the 937 insects that died,

798 (85%) died within 5 days. The rank order for number of insects killed by each stock isolate or subsequent crosses was significantly different after 5 days than after 10 days (Spearman rank correlation, $P = 0.0562$).

The crosses can be ranked on the basis of each of the three fitness criteria tested above. The sum of the ranks and the mean rank for each cross was calculated on the assumption that the fitness criteria are of equal importance. On this basis, the cross with the lowest mean rank (*i.e.*, ranked first and therefore the best performing isolate) was UK76 × UK76, followed by the lowest ranking Bull Island isolate, 1.E.(1) × 1.E.(1).

Molecular β-tubulin analysis of *Steinernema feltiae* populations

PCR fragments ranged in size from 414-523 bp. Phylogenetic relationships were inferred using the pooled β-tubulin sequences of each isolate. The outgroup taxon sequence (*C. elegans*) was extracted from Genbank. Results of maximum likelihood analysis carried out using Paup* 4.0b are shown in Figure 4; *C. elegans* is separated from all other isolates tested. The two isolates 33.D.(2) and 59.F.(2) are distinct from the other isolates tested with 99% support for this grouping. The remaining ten isolates are separated, again with strong bootstrap support (84%), into three clades and, interestingly, Bull Island isolate 4.G.(1) is grouped with the two outgroups from Counties Kildare and Carlow. Both the commercial isolate, UK76 and Bull Island isolate 2.G.(1), are separated from the other isolates with at least 62% bootstrap support. The separation of *C. elegans* from the *S. feltiae* isolates, and 33.D.(2) and 59.F.(2) from the remaining Bull Island isolates is highlighted in the evolutionary distance between these two isolates and the remaining ten isolates (Fig. 5).

Discussion

Laboratory adaptation has been shown to produce dramatic changes in important biological attributes of EPN, such as storage stability, virulence and reproductive potential (Stuart & Gaugler, 1996; Wang & Grewal, 2002; Bai *et al.*, 2005). Inadvertent selection of traits (Roush, 1990) and the deterioration of the bacterial symbiont (Bil-

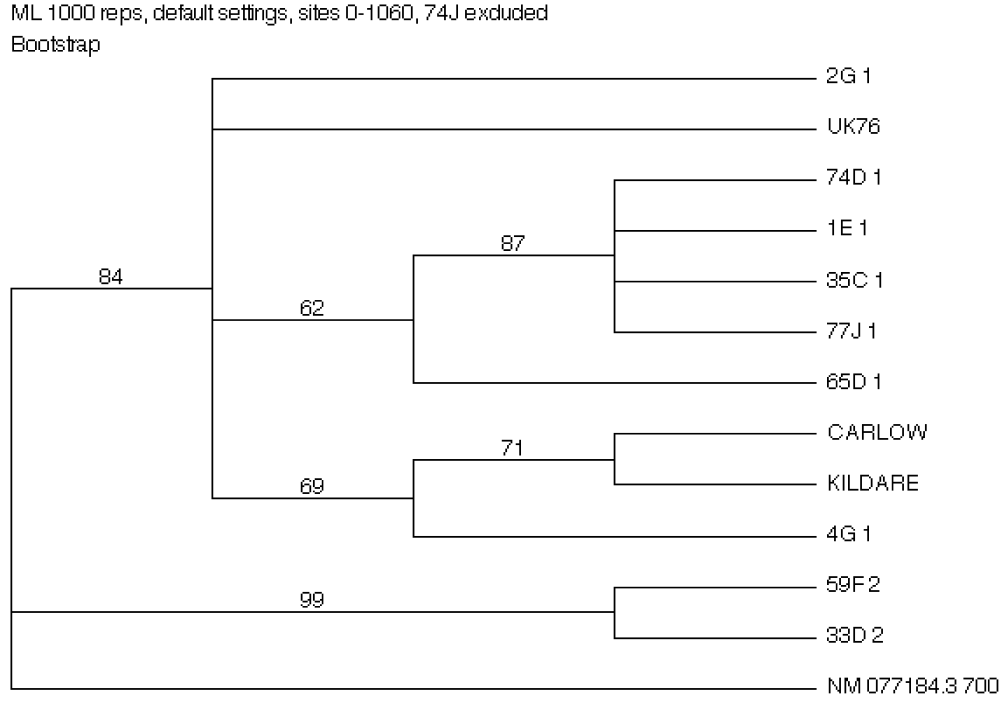

Fig. 4. *Maximum likelihood phylogenetic tree, based on the sequence of a portion of the β-tubulin gene, of 12* Steinernema feltiae *samples: nine from Bull Island, Dublin Bay, one isolate from County Carlow, Ireland, one isolate from County Kildare, and one commercial isolate from the UK (UK76).*

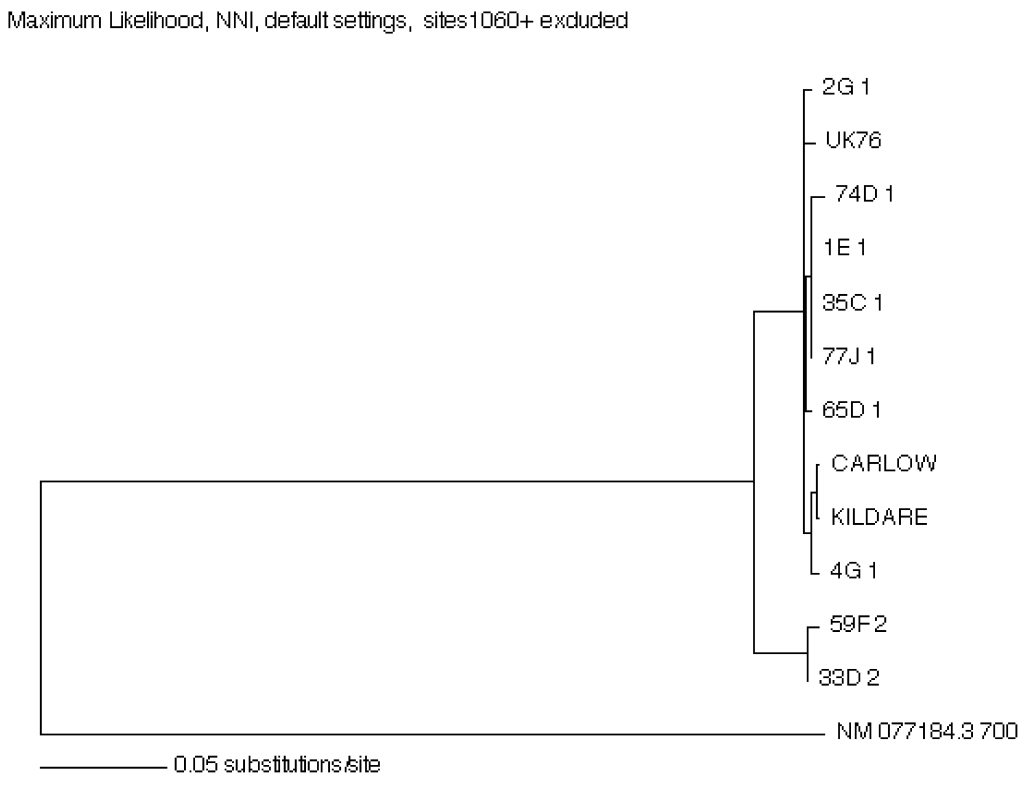

Fig. 5. *Maximum likelihood phylogenetic tree, based on the sequence of a portion of the β-tubulin gene, showing branch lengths representing evolutionary distance between samples. Sample NM 077184.3 700 is the* Caenorhabditis elegans *outgroup.*

grami *et al.*, 2006) when culturing nematodes in the laboratory is also likely to be important. The various isolates of *S. feltiae* used in our study were collected in different years and have subsequently been reared through *G. mellonella* in the laboratory for different time periods. Such culturing and possible inadvertent selection of IJ may have accentuated some of the behavioural and genetic variability observed in this study. Such variability may have arisen due to differing selection pressures acting within varying microhabitats on Bull Island. Somasekhar *et al.* (2002) suggest that high genetic variability among natural populations of *S. carpocapsae* may aid the feasibility of using selection for the genetic improvement of traits such as stress tolerance, virulence and reproductive potential. Attempts at genetic selection of EPN have been made (Segal & Glazer, 2000; Strauch *et al.*, 2004) and the genetic improvement of *S. feltiae* is an attractive proposal as *S. feltiae* is well studied and field tested and is used to control insect pests worldwide (Gaugler *et al.*, 1989; Grewal, 2002). The use of molecular techniques to identify intraspecific differences that may reflect different biological characters might provide foundation populations for selecting positive characteristics (Liu *et al.*, 2000). However, Gaugler *et al.* (1994) warn that, although genetic selection for advantageous traits may be an attractive theory, laboratory-selected strains have rarely been shown to be effective in the field.

Of the *S. feltiae* isolates tested here, 33.D.(2) and 59.F.(2) and their crosses consistently performed poorly in all fitness traits investigated. Although we are not suggesting that any molecular differences shown in this study are the cause of any behavioural traits, it is significant that both 33.D.(2) and 59.F.(2) have been shown to be molecularly distinct from other Bull Island *S. feltiae* with regards to their β-tubulin intron sequence. Reid and Hominck (1992) identified two RFLP types of *S. feltiae*: A1 and A2. It is certainly possible that both 33.D.(2) and 59.F.(2) differ in their RFLP type from the other *S. feltiae* isolates tested here and this needs further investigation. The bacterial preferences of 33.D.(2) and 59.F.(2) may also differ from the other *S. feltiae* isolates investigated here. Boszormenyi *et al.* (2001) showed that two isolates of *H. downesi*

were each unable to utilise the other's symbiotic bacteria, despite these isolates being indistinguishable in both their morphology (Stock *et al.*, 2002) and RFLP patterns (Pamjav *et al.*, 1999). Isolates 33.D.(2) and 59.F.(2) may be similarly limited in their utilisation of the bacteria of other *S. feltiae* isolates, resulting in the restricted out-breeding of these two isolates highlighted in this study. Because of their inability to persist in culture, isolates 33.D.(2) and 59.F.(2) and their symbiotic bacteria, must differ from the other isolates (and respective bacteria) studied in their tolerance of environmental stress and inbreeding and other laboratory-induced factors.

The inability to invade successfully an insect may not necessarily inhibit an individual from reproducing and completing its life cycle. San-Blas and Gowen (2008) report that all of eight species of EPN, including *S. feltiae*, used scavenging as an alternative survival strategy and completed their life cycles in *G. mellonella* cadavers. This, as the authors suggest, could explain the long-term persistence of isolates such as 33.D.(2) and 59.F.(2) in the soil.

Laboratory adaptation can affect biological attributes of EPN (Stuart & Gaugler, 1996; Wang & Grewal, 2002). The cross-breeding of closely related individuals is known to have potential detrimental effects on the subsequent progeny. Phenotypic abnormalities that cause a decline in viability or fertility are often characteristics of inbred individuals (Perrin & Goudet, 2001).

The culturing of the isolates tested here through many rounds of *G. mellonella* over time is likely to have increased inbreeding levels. Progeny of the 33.D.(2) × 59.F.(2) cross failed to invade and kill any host insects except when injected into insects, when not only were the hosts killed, but progeny emerged. It is possible that due to parental inbreeding and inadvertent selection, progeny were incapable of active host invasion and subsequent host mortality and EPN reproduction. However Bai *et al.* (2005) found that inbred lines of *H. bacteriophora* can prevent beneficial trait decline by causing greater mortality to host insects than foundation populations. Bilgrami *et al.* (2006) found that the loss of virulence against *G. mellonella* larvae was the result of bacterial deterioration during subculturing.

Isolates 33.D.(2) and 59.F.(2) were isolated on the second round of baiting of their respective soil samples with *G. mellonella* and have consistently performed poorly in behavioural assays other than presented here (Rolston, unpubl.). It is possible that they and their progeny need more than one exposure to host insects to initiate an infection response, and this option was not provided in these studies. However, it is questionable whether such an infection strategy would be viable in the field where potential host insects may be rare. Local or regional extinction appears to be a common phenomenon in ecological systems, and may affect the richness and population structure of many natural communities (Wright & Coleman, 1993). Wilson *et al.* (2003) found that uniform inundative EPN application resulted in patchier distribution with time as nematodes died, whereas patchy application led to a more even distribution of EPN over time as the nematodes moved from their initial application. Extinction events in the field may not just be as a result of unsuitable insect host numbers, or environmental conditions (Hoy, 1976). In situations where hosts are rare, the chance of mating with a close relative increases. If this continues over several generations, inbreeding may lead to a decline in viability or fecundity that may in turn cause an extinction event. Founder effect is likely to play an important role in EPN genetic diversity (Roush, 1990; Gaugler, 1993). If local insect host numbers are few, numbers of EPN will decrease. A subsequent increase in the number of available insect hosts would lead to a recovery, in terms of numbers, in the local EPN population. However, the EPN population would have passed through an evolutionary bottleneck due to restricted founder numbers and so the new EPN population is likely to contain a lower genotypic diversity compared to the original population. EPN may be an ideal organism for studying inbreeding effects due to their ability to withstand high mortality rates, their short generation time and the large numbers of progeny produced per generation.

The fact that the majority of crosses failed to produce the expected number of emergences suggests that: *i*) all the isolates are somewhat inbred and, therefore, have reduced fitness; *ii*) the IJ were sensitive to the injection procedure and many were unable to survive the induced stress and the host's immune response (although this could also be a result of the first hypothesis) and *iii*) a breeding barrier exists between conspecifics. Nearly all species populations exhibit some degree of genetic differentiation among geographic localities (Ehrlich & Raven, 1969), yet frequently, little variation in a given gene is uncovered when examining individuals in a species. Rosenthal (2001) outlines two hypotheses regarding such lack of variation: *i*) the gene's function is critical and the gene is therefore no longer subject to improvement or change, and chance could only give rise to individuals that would be at a selective disadvantage; *ii*) the lack of variation may

be the result of present or past restrictions in the population size: variation in small or young populations is limited to the variability of the population's founders. The idea of fixed optimal infection strategies has been questioned by Crossan *et al.* (2007). For example, the fastest infecting IJ may not be the fittest as it may respond poorly to other fitness traits. Parasites may adapt their infection behaviour in an attempt to increase their chances of transmission within a given environment. Crossan *et al.* (2007) showed that the infection rate of *S. feltiae* was able to evolve rapidly in response to changes in the rate of host availability. However, there is likely to be a trade off in fitness between being highly infective and surviving in the soil for prolonged periods of host absence. Different intraspecific strains with differing optimum infection strategies may, therefore, exist within a population (Crossan *et al.*, 2007). Dix *et al.* (1992) report reproductive incompatibility resulting from inter-strain crosses of *H. bacteriophora*, but this is seemingly unreported in intraspecific populations of *Steinernema*. Reproductive incompatibility has been well documented in mites and insects, particularly as a result of infection with *Wolbachia* bacteria (*e.g.*, Werren, 1997; Navajas *et al.*, 2000; Perez & Hoy, 2002; Vala *et al.*, 2002). *Wolbachia* bacteria have been found to be widespread in several filarial nematodes (Sironi *et al.*, 1995; Taylor & Hoerauf, 1999; Stevens *et al.*, 2001), but are yet to be found in EPN.

Given the recent geological origin of Bull Island and the small geographical distances between isolates, the molecular and behavioural intraspecific variation of *S. feltiae* is unexpected. The phylogeny inferred from β-tubulin sequences suggests the possibility of two, perhaps three, separate colonisation events of Bull Island. The importation of soil with new genetic lines of EPN is certainly feasible through: *i*) the high level of human traffic that use the island for recreational purposes; *ii*) the migration habits of many of the wildfowl and wading birds that over-winter on the island; and *iii*) animal activity between the mainland and the island *via* the connecting causeway. Comparisons between the Bull Island populations and those isolated from the nearby mainland coastline are yet to be made but may provide important information with regard to the importation of new genetic lines to the island. A shared colonisation origin on Bull Island is the most likely explanation for the genetic similarity of the widely separated 1E(I) and 75J(I) (distance between soil cores 761.64 m); however, it is also possible that sand transported by human activities may have given rise to this disjunct in distribution. In terms of unaided dispersal, the chance of direct gene flow between two populations from either end of the 800 × 100 m sample area of Rolston *et al.* (2005) is low. The greater the geographic distance between populations, the smaller chance of gene flow, which may eventually give rise to genetic isolation by distance (Page & Holmes, 1998). The fact that for some species of nematodes there is no correlation between geographical and genetic distances (Hawdon *et al.*, 2001) suggests that this topic needs further investigation for the isolates tested here, and for EPN in general.

The different microhabitats of the dune system of Bull Island may be a substantial cause of the variation observed between isolates as spatially varying selection can maintain substantial variability, making adaptations to the different habitat conditions possible (Barton, 2000). Information on gene flow between EPN populations is sorely lacking (see Blouin *et al.*, 1999) and is in need of urgent investigation in order to attempt to estimate both the natural diversity of EPN populations and the potential impact of introduced alien or commercially applied nematodes on natural populations in the soil. The EPN populations of Bull Island may be ideal for an extended study of the level of gene flow within each of the two species that exist there due to their seemingly patchy population structures and the variable habitats within the dune system itself (Rolston *et al.*, 2005).

This study has highlighted the significance of intraspecific variation in the population dynamics of EPN. However, it is important to recognise that each Bull Island isolate was possibly established from perhaps no more than two IJ. These successful IJ may not be representative of the population as a whole. Liu *et al.* (2000) importantly state that phylogenetic information is critical to rational implementation and monitoring programmes when EPN are used as biological control agents. When attempting to identify new EPN isolates for the potential control of insect pests, the results presented here agree with Roush (1990), that it is important to consider three genetic problems frequently inflicted by laboratory isolation: *i*) founder effect, where due to small initial sampling sizes, there is little genetic variation in the new population; *ii*) inbreeding depression, which is most apparent in small populations; *iii*) inadvertent selection, where due to laboratory rearing, field-selected adaptations become rare.

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