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Morphological and Ecological Characterization of *Steinernema feltiae* (Rhabditida: Steinernematidae) Rioja Strain Isolated from *Bibio hortulanus* (Diptera: Bibionidae) in Spain

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Abstract: A new strain of *Steinernema feltiae* (Rhabditida: Steinernematidae) was isolated in La Rioja (Spain) from larvae of *Bibio hortulanus* (Diptera: Bibionidae). A comparative morphometric analysis of this new strain and four additional *S. feltiae* isolates was performed. Although significant differences in morphometric measurements were observed, PCR-RFLP profiles and sequence analysis of the ITS region of rDNA confirmed the identity of the new strain as A2 RFLP type of *S. feltiae*. A comparative morphometric study among nematodes from three hosts, *Galleria mellonella* (Lepidoptera: Pyralidae), *Spodoptera littoralis* (Lepidoptera: Noctuidae) and *B. hortulanus*, was conducted. Ecological characterization of the Rioja isolate was performed in *G. mellonella* larvae. Larval mortality was 75.3 and 78.12% in penetration and sand column assays, respectively, and the percentage of penetrating infective juveniles was 12.0 and 2.8% in these assays. Larval mortality in the one-on-one bioassay was 4.2 %, and in exposure-time bioassays, it was 50% at 11.25 hours. Relationships between morphometric characteristics and host mortality are discussed for this new strain of entomopathogenic nematode.

Key words: molecular markers, natural host, Spain, *Steinernema feltiae*, virulence.

Entomopathogenic nematodes (EPN) in the Heterorhabditidae and Steinernematidae are lethal parasites of insects that are widely distributed in soils throughout the world (Hominick et al., 1996). Their association with bacteria of the genera *Xenorhabdus* (for steinernematids) and *Photorhabdus* (for heterorhabditids) makes them highly virulent (Boemare et al., 1997; Boemare, 2002). Their ability to actively locate their insect hosts, their high reproductive potential, the possibility to mass produce them, and their harmless impact on vertebrates and plants make these organisms highly suitable for the development of environmentally friendly alternatives in the control of insect pests (Gaugler and Kaya, 1990; Kaya and Gaugler, 1993; Burnell and Stock, 2000; Gaugler, 2002). Because survival and virulence of entomopathogenic nematode species and strains are influenced by environmental conditions, a survey was conducted to identify EPN strains in La Rioja, Spain. A new steinernematid strain of *Steinernema feltiae* (Filipjev) was isolated from a natural host. We present results of the ecological characterization of this new strain and discuss its morphological and molecular characterization.

Material and Methods

EPN isolation and host larvae mortality study: A survey was conducted in spring 2001 in La Rioja (Northern Spain), where 149 last instar larvae of *Bibio hortulanus* L. (Diptera: Bibionidae) were collected from the edge of a potato (*Solanum tuberosum* L.) field. Dead *B. hortulanus* larvae were washed and placed individually in White traps to collect infective juveniles (IJ) (White, 1927). Mortality and establishment of IJ in each larva of *B. hortulanus* were assessed. Live *B. hortulanus* larvae were placed in Petri dishes to obtain adult flies to confirm their identity.

Morphological identification and comparative biometric study: One larva from *B. hortulanus* producing nematode progeny (4,215 IJ) was chosen to ensure that only a single EPN species was isolated (Alatorre-Rosas and Kaya, 1990; de Doucet et al., 1998). Twenty-five infective juveniles (IJ) were recovered for morphometric study and the rest were used for in vivo culture on *Galleria mellonella* L. (Lepidoptera: Pyralidae) and *Spodoptera littoralis* Boisdv. (Lepidoptera: Noctuidae) (Woodring and Kaya, 1988). Infective juveniles were fixed and processed in glycerine following the method described by Steinhorst (1966). Semi-permanent slides were prepared following the procedures described by Gómez et al. (2004). Three insect hosts, *B. hortulanus*, *S. littoralis* and *G. mellonella*, were considered. Twenty-five IJ from each host and twenty-five first generation males (FGM) of each host, *S. littoralis* and *G. mellonella*, were considered for morphometric comparisons. Morphological observations and measurements were performed using a Leica DML microscope equipped with DIC optics. Identification was carried out following the keys of Adams and Nguyen (2002) and Hominick et al. (1997). The new strain was compared with other *S. feltiae* strains: Spanish strains M47 and M65 (isolated from two sites with similar environmental conditions) (García del Pino, 1994), 76 UK isolates (Yoshida, 2003) and one isolate from Pakistan (Tabassum and Shahina, 2004).

Molecular characterization: PCR-RFLP and sequence analysis of the ITS region of rDNA were performed to further confirm the identity of the isolate. DNA was extracted from a single female following procedures

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described by Williams et al. (1992). PCR reactions were carried out with 10 µl of digested females. PCR amplification conditions followed procedures described by Hominick et al. (1997) using the primers 18S and 26S described by Vrain et al. (1992). Restriction digests were performed using the enzymes Alu I, Dra I, Eco RI, Hae III, Hha I, Hinc II, Hinf I, Mnl I and Rsa I, following manufacturer's instructions (New England Biolabs, Beverly, MA). The ITS1-5.8S-ITS2 region was cloned into the pGEM-T vector and sequenced. The sequence was compared and analyzed using BLASTN analysis (Altschul et al., 1997). Alignments of the ITS1-5.8S-ITS2 sequences were generated using Clustal W (Thompson et al., 1994). Sequences from four *Steinernema feltiae* isolates and a closely related species, *S. weiseri*, were considered for comparisons. Accession numbers are: AY230169 (*S. feltiae* A2 RFLP type), AY230170 (*S. feltiae* A1 RFLP Type), AY23078 (*S. feltiae* MY9, Japanese type), AF192981 (*S. feltiae* SN strain) and AY230167 (*Steinernema weiseri*) (Spiridonov et al., 2004).

Ecological characterization: The virulence of the new strain was assessed using a method modified from that proposed by Glazer and Lewis (2000). Penetration, exposure time, one-on-one and sand column assays were performed using last instar *G. mellonella* larvae. Infective juveniles were stored at 10 °C for 2-3 weeks before use. The concentration required in each bioassay was adjusted by volumetric dilutions, except in one-on-one assay where each IJ was picked individually. Assays were performed in 25-well plates (4 cm^2/well) (Sterilin Hounslow, Middlesex, UK) containing a thin layer (0.8 g/well) of sterile sand (passed through a 2-mm mesh sieve) dampened with 150 µl of ultra-pure water. The final volume was identical in all the treatments (IJ and control) to ensure similar conditions. All bioassays were carried out in the dark at 22 ± 1 °C and 55% relative humidity, with three repetitions over a 10 day period.

Penetration bioassay: A suspension of 200 IJ in 100 µl was placed in each well followed by a single last instar *G. mellonella* larva. The control was a 25-well plate with 250 µl of ultra-pure water/well. Larval mortality was recorded daily. Dead larvae were rinsed in tap water to remove nematodes from their surface and placed individually in 5-cm Petri dishes with moist filter paper and incubated for a further two days. Insects were dissected under a stereoscopic microscope and the number of males and females in each larva was counted. The penetration rate was calculated as the percentage of the initial IJ that had invaded the insect host (Glazer and Lewis, 2000).

Exposure time bioassay: A suspension of 400 IJ in 100 µl of ultra-pure water was placed in each well (Glazer and Lewis, 2000) followed by a single *G. mellonella* larva. After 1, 2 and 3 hr, eight larvae were randomly removed. Insects were rinsed and incubated in 5-cm Petri dishes with moist filter paper. The mortality was

TABLE 1. Morphometrics of infective juveniles (IJ) and first generation males (FGM) of *Steinernema feltiae* Rioja strain in *G. mellonella*, *S. littoralis* and *B. hortulanus*. Means, standard deviations and ranges (in parenthesis) are given in µm.

	IJ[b]				FGM[c]		
Character/ratio[a]	*G. mellonella*	*S. littoralis*	*B. hortulanus*	ANOVA (df), p	*G. mellonella*	*S. littoralis*	T test (df), p[d]
L	783 ± 75.3* (660–914)	645 ± 60.4** (537–754)	874 ± 57.4* (683–895)	F = 51.157 (2, 71), p < 0.0001	1220 ± 176.4 (820–1648)	1238 ± 146.7 (970–1583)	n.s.
W	26 ± 2.9* (20–30)	21 ± 2.3** (17–26)	25 ± 1.3* (22–28)	F = 23.386 (2, 70), p < 0.0001	107 ± 20.9 (73–141)	115 ± 20.9 (68–161)	n.s.
ES	118 ± 8.3* (102–138)	105 ± 11.4** (88–129)	116 ± 9.2* (92–127)	F = 14.787 (2, 70), p < 0.0001	138 ± 12.4 (100–152)	141 ± 14.1 (100–168)	n.s.
NR	93 ± 6.5* (80–105)	76 ± 8.8** (61–99)	90 ± 9.4* (71–101)	F = 27.211 (2, 70), p < 0.0001			
EP	64 ± 10.6* (50–89)	51 ± 8.5** (41–76)	54 ± 2.4* (50–59)	F = 16.786 (2, 69), p < 0.0001	86 ± 9.9 (61–97)	89 ± 13.0 (68–114)	n.s.
T	72 ± 5.5* (61–80)	64 ± 6.1** (55–76)	71 ± 3.6* (65–79)	F = 14.202 (2, 68), p < 0.0001	28 ± 3.5 (21–33)	24 ± 3.7 (15–29)	n.s.
SL					68 ± 4.6 (58–80)	68 ± 5.1 (58–76)	t = 2.084 (44), p = 0.043
GL					47 ± 3.7 (41–53)	44 ± 4.9 (35–53)	n.s.
ABW					34 ± 3.0 (28–39)	36 ± 3.7 (29–42)	t = –2.620 (48), p = 0.012
a	30 ± 3.3* (20–37)	31 ± 2.4* (27–35)	34 ± 2.9** (28–36)	F = 10.851 (2, 71), p < 0.0001	12 ± 1.86 (8–17)	11 ± 1.7 (9–15)	n.s.
b	6.6 ± 0.51* (5.9–7.6)	6.3 ± 0.94*** (4.8–8.3)	7.0 ± 0.30** (6.3–7.6)	F = 6.589 (2, 69), p < 0.0001	9.3 ± 1.47 (7.0–12.6)	8.8 ± 0.86 (7.2–10.2)	n.s.
c	11 ± 0.7* (10–13)	10 ± 0.8** (8–12)	12 ± 0.8*** (10–13)	F = 24.857 (2, 67), p < 0.0001	51 ± 7.9 (40–70)	45 ± 7.2 (33–60)	n.s.
D%	54 ± 7.7* (40–69)	49 ± 7.2** (39–64)	46 ± 4.8** (37–60)	F = 8.586 (2, 69), p < 0.0001	62 ± 6.7 (46–70)	62 ± 7.2 (48–74)	n.s.
E%	88 ± 10.4* (75–109)	75 ± 10.9** (58–110)	76 ± 5.4** (57–85)	F = 14.505 (2, 63), p < 0.0001			
GS					0.70 ± 0.058 (0.60–0.83)	0.65 ± 0.059 (0.54–0.75)	t = 2.021 (44), p = 0.049
SW					2.05 ± 0.80 (1.69–2.35)	1.91 ± 0.26 (1.52–2.63)	t = 2.484 (48), p = 0.017
MUC					P	P	

[a] L = body length, W = body width, ES = oesophagus length, NR = ant. nerve ring, EP = ant. exc. pore, T = tail length, SL = spicule length, GL = gubernaculum length, ABW = anal body width. Ratios: a = L/W, b = L/ES, c = L/T, D% = EP/ES × 100, E% = EP/T × 100, GS = GL/SL, SW = SL/ABW, MUC = mucron, P = present.

[b] Differences among insect hosts are provided by ANOVA, with Tukey's test to measure differences within host. Different number of * symbol indicate seen statistical differences.

[c] Differences between two insect hosts are provided by *t*-test.

[d] Results statistically not significant, n.s.

recorded 24-48 hrs post-exposure to nematodes, and the ET_{50} value (nematode exposure time in minutes required to achieve 50% insect mortality) was calculated.

One-on-one bioassay: In this bioassay, only one IJ was applied per well with a micropipette in 50 µl. One *G. mellonella* larva was placed in each well, and the plate was incubated for 72 hr. The percent mortality was determined after 72 hr.

Sand column bioassay: Fifteen plastic tubes (45 mm height × 40 mm diameter) were used for this bioassay. A suspension of 100 IJ in 100 µl was placed into each tube, which was subsequently filled with moist sterile sand. One last instar *G. mellonella* larva was placed on top and the tube closed and inverted, allowing the nematodes to migrate towards the larval host. The larvae were removed from the tube after 24 hrs, placed into Petri dishes and incubated for a further 2–3 days. Percent of penetrating IJ (development in male and female) and larval mortality were recorded.

Statistics: Significant differences of morphometric data of *S. feltiae* Rioja IJ from different insect hosts were analyzed by ANOVA. Tukey's test was performed to assess differences between hosts. *Galleria mellonella* and *S. littoralis* FGM were analyzed in a *t*-test. Morphological comparison between all raw data from the Rioja strain and the mean of measurements recorded from the literature of Catalonia, UK and Pakistan strains was carried out using the One-Sample *t*-test. Similarity between IJ and FGM measurements from the Rioja strain recovered from *B. hortulanus*, *G. mellonella* and *S. littoralis* and the other selected reference strains was calculated as a percentage of IJ and FGM measurements not statistically different from reference strains. Crosstabs were performed for statistical differences in percentage of morphological similarity among strains within the same host. Larval mortality was corrected using Abbott's formula (1925). Differences in penetration rate of Rioja males and females were assessed by the Man-Whitney test. Probit analysis was used to calculate ET_{50} values.

TABLE 2. Morphometric comparison of morphometric traits of infective juveniles Rioja strain in the insect hosts and those provided by the bibliography.

Character/ ratio[a]	Insect host[b]	M47 Spain (García del Pino, 1994)		M65 Spain (García del Pino, 1994)		Site 76 UK (Yosida, 2003)		Pakistan (Tabassum and Shahina, 2004)	
		One-sample t-test, df	p[c]	One-sample t-test, df	p[c]	One-sample t-test, df	p[c]	One-sample t-test, df	p[c]
L	*G. m*	t = −3.313, 24	0.003		n.s.	t = −6.015, 24	<0.001	t = −4.754, 24	<0.001
	S. l	t = −15.264, 23	<0.001	t = −11.896, 23	<0.001	t = −18.567, 23	<0.001	t = −17.025, 23	<0.001
	B. h		n.s.	t = 2.861, 24	0.009	t = −4.282, 24	<0.001	t = −2.627, 24	0.015
W	*G. m*	t = −6.140, 23	<0.001	t = −4.754, 24	<0.001	t = −4.162, 23	<0.001	t = −7.459, 23	<0.001
	S. l	t = −16.997, 23	<0.001	t = −15.298, 23	<0.001	t = −14.449, 23	<0.001	= −18.696, 23	<0.001
	B. h	t = −16.395, 24	<0.001	t = −12.663, 24	<0.001	t = −12.663, 24	<0.001	t = −20.127, 24	<0.001
ES	*G. m*	t = −9.453, 23	<0.001		n.s.	t = −7.605, 23	<0.001	t = −5.873, 23	<0.001
	S. l	t = −13.402, 24	<0.001	t = −7.800, 24	<0.001	t = −12.002, 24	<0.001	t = −10.689, 24	<0.001
	B. h	t = −10.002, 23	<0.001	t = −3.067, 23	0.005	t = −8.402, 23	<0.001	t = −6.802, 23	<0.001
NR	*G. m*	t = −2.326, 23	0.029		n.s.			t = 5.269, 23	<0.001
	S. l	t = −11.136, 24	<0.001	t = −8.131, 24	<0.001	Not Available		t = −5.465, 24	<0.001
	B. h	t = −3.362, 23	0.003		n.s.				n.s.
EP	*G. m*		n.s.	t = −3.519, 23	0.002		n.s.		n.s.
	S. l	t = −5.547, 23	<0.001	t = −2.941, 23	0.007	t = −6.995, 23	<0.001	t = −5.836, 23	<0.001
	B. h	t = −14.911, 23	<0.001	t = −4.482, 23	<0.001	t = −19.082, 23	<0.001	t = −14.911, 23	<0.001
T	*G. m*	t = −3.412, 22	0.002	t = −2.186, 22	0.040	t = −11.821, 22	<0.001	t = −4.814, 22	<0.001
	S. l	t = −8.818, 22	<0.001	t = −7.710, 22	<0.001	t = −16.417, 22	<0.001	t = −10.085, 22	<0.001
	B. h	t = −5.846, 24	<0.001	t = −4.453, 24	<0.001	t = −19.786, 24	<0.001	t = −8.634, 24	<0.001
a	*G. m*	t = 2.826, 24	0.009	t = 3.731, 24	0.001	t = −2.452, 24	0.022	t = 3.580, 24	0.002
	S. l	t = 4.217, 23	<0.001	t = 5.434, 23	<0.001	t = −2.879, 23	0.008	t = 5.231, 23	<0.001
	B. h	t = 10.246, 24	<0.001	t = 12.493, 24	<0.001	t = 3.504, 24	0.002	t = 12.493, 24	<0.001
b	*G. m*	t = 4.445, 22	<0.001		n.s.		n.s.		n.s.
	S. l		n.s.		n.s.		n.s.		n.s.
	B. h	t = 12.928, 23	<0.001	t = 8.086, 23	<0.001	t = 6.471, 23	<0.001	t = 8.086, 23	<0.001
c	*G. m*		n.s.		n.s.	t = 4.679, 22	<0.001		n.s.
	S. l	t = −6.372, 22	<0.001	t = −4.115, 22	<0.001		n.s.	t = −5.933, 22	<0.001
	B. h	t = 3.787, 23	0.001	t = 3.787, 23	0.001	t = 10.072, 23	<0.001	t = 3.787, 23	0.001
D%	*G. m*	t = 5.572, 22	<0.001	t = 4.772, 22	<0.001	t = 3.532, 22	0.002	t = 4.772, 22	<0.001
	S. l	t = 2.784, 23	0.011		n.s.		n.s.		n.s.
	B. h		n.s.		n.s.	t = −2.140, 24	0.043		n.s.
E%	*G. m*	t = 3.425, 20	0.003	t = 5.488, 20	<0.001	t = 6.278, 20	<0.001	t = 4.084, 20	0.001
	S. l	t = −2.119, 19	0.048		n.s.		n.s.		n.s.
	B. h	t = −5.070, 24	<0.001		n.s.		n.s.	t = −3.227, 24	0.004

[a] L = body length, W = body width, ES = oesophagus length, NR = ant. nerve ring, EP = ant. exc. pore, T = tail length. Ratios; a = L/W, b = L/ES, c = L/T, D% = EP/ES × 100, E% = EP/T × 100.

[b] Insect hosts code: *G. m, G. mellonella, S. l, S. littoralis, B. h, B. hortulanus.*

[c] Results statistically not significant, n.s.

Statistical analyses were performed by SPSS 12.0 for Windows (SPSS Inc. Chicago, IL); $P \leq 0.05$ was considered significant.

Results

Morphological identification and biometric comparative studies: Morphometric values obtained from IJ from the natural host *B. hortulanus* and the IJ and FGM from *G. mellonella* and *S. littoralis* lie with the ranges established for *S. feltiae* (Hominick et al., 1997; Adams and Nguyen, 2002). This new *S. feltiae* strain is named "Rioja." Variation in all morphometric traits of IJ recovered from different hosts was observed (Table 1). However, only GL, ABW, GS, and SW from FGMs were significantly different among hosts (Table 1). Statistical differences between IJ and males of the Rioja strain and other isolates from different hosts are shown in Tables 2 and 3, respectively. The similarity percentage in the biometric comparative study of Rioja IJ and FGM from *G. mellonella*, *S. littoralis* and *B. hortulanus* with selected strains are shown in Table 4. Variation in percent similarity was observed, although differences were not significant. Infective juveniles from natural host *B. hortulanus* showed 27.3% similarity with the M65 strain. Infective juveniles from *G. mellonella* showed 45.5% similarity with the M65 strain, and their FGM showed a 33.3 % degree of similarity with the Pakistan strain. Infective juveniles and FGM recovered from *S. littoralis* showed a 40% degree of similarity with 76 UK and Pakistan strains, respectively.

Molecular characterization: A restriction profile of the rDNA ITS region from the Rioja strain of *S. feltiae* is shown in Fig. 1. The restriction patterns obtained with Alu I, Eco RI, Hae III, Hha I, Hinc II and Dra I are in agreement with those previously seen in *S. feltiae* (Hominick et al., 1997; Reid et al., 1997; Reid and Hominick, 1998; Nguyen, 2003; Yoshida, 2003). Patterns obtained using Rsa I and Hinf I were similar to those of *S. feltiae* A2 RFLP type (Yoshida, 2003), identifying the Rioja strain as *S. feltiae* A2 RFLP type. The alignment of the ITS1-5.8S-ITS2 region and the partial 18S sequence is shown in Fig. 2. The BLASTN analysis showed a 99% similarity with *S. feltiae* A2 RFLP type.

Ecological characterization: The Rioja strain caused 20% less mortality of larvae than the UK strain (Table

TABLE 3. Morphometric comparison of first generation males' morphometric characteristics of *S. feltiae* Rioja strain from different insect hosts and those provided by the bibliography.

		Site 76 UK (Yosida, 2003)		Pakistan (Tabassum and Shahina, 2004)	
Character/ratio[a]	Insect host[b]	One-sample t-test, df	p[c]	One-sample t-test, df	p[c]
L	*G. m*	t = −4.936, 24	<0.001		n.s.
	S. l	t = −5.304, 24	<0.001		n.s.
W	*G. m*	t = 2.122, 23	0.045	t = 4.231, 23	<0.001
	S. l	t = 3.993, 24	0.001	t = 6.136, 24	<0.001
ES	*G. m*		n.s.		n.s.
	S. l		n.s.		n.s.
EP	*G. m*	t = −10.716, 22	<0.001		n.s.
	S. l	t = −7.138, 22	<0.001		n.s.
T	*G. m*	t = −14.734, 22	<0.001	t = −15.639, 22	<0.001
	S. l	t = −9.714, 23	<0.001	t = −10.683, 23	<0.001
SL	*G. m*	t = −4.342, 24	<0.001	t = −4.665, 24	<0.001
	S. l	t = −4.158, 24	<0.001	t = −4.450, 24	<0.001
GL	*G. m*	t = −6.327, 24	<0.001	t = −8.263, 24	<0.001
	S. l	t = −7.732, 24	<0.001	t = −9.765, 24	<0.001
ABW	*G. m*	t = −12.627, 23	<0.001	t = −11.815, 24	<0.001
	S. l	t = −7.347, 24	<0.001	t = −6.673, 24	<0.001
a	*G. m*	t = −6.917, 23	<0.001	t = −4.545, 23	<0.001
	S. l	t = −9.949, 24	<0.001	t = −7.229, 24	<0.001
b	*G. m*	t = −2.091, 21	0.049	t = 2.375, 21	0.027
	S. l	t = −6.161, 24	<0.001		n.s.
c	*G. m*	t = 6.292, 20	<0.001	t = 10.011, 20	<0.001
	S. l	t = 3.370, 23	0.003	t = 7.722, 23	<0.001
D%	*G. m*	t = −11.065, 22	<0.001		n.s.
	S. l	t = −9.694, 22	<0.001		n.s.
GS	*G. m*	t = −2.139, 24	0.043	t = −2.139, 24	0.043
	S. l	t = −3.829, 24	0.001	t = −3.829, 24	0.001
SW	*G. m*	t = 8.304, 23	<0.001	t = 9.664, 23	<0.001
	S. l	t = 3.147, 24	0.004	t = 4.116, 24	<0.001
Mucron	*G. m*	Present/Present		Present/Present	
	S. l	Present/Present		Present/Present	

[a] L = body length, W = body width, ES = oesophagus length, EP = ant. exc. pore, T = tail length, SL = spicule length, GL = gubernaculum length, ABW = anal body width. Ratios: a = L/W, b = L/ES, c = L/T, D% = EP/ES × 100, GS = GL/SL, SW = SL/ABW.

[b] Insect hosts code: *G. m, G. mellonella, S. l, S. littoralis, B. h., B. hortulanus*

[c] Results statistically not significant, n.s.

TABLE 4. Percent similarity[a] between infective juveniles (IJ) and first generation males (FGM) of Rioja strain cultured in *G. mellonella*, *S. littoralis* and *B. hortulanus* and the bibliographic reports.

	M47 Spain (García del Pino, 1994)	M65 Spain (García del Pino, 1994)	Site 76 UK (Yoshida, 2003)		Pakistan (Tabassum and Shahina, 2004)	
Insect host	IJ	IJ	IJ	FGM	IJ	FGM
Galleria mellonella	18.2	45.5	20.0	13.3	27.3	33.3
Spodoptera littoralis	9.1	17.3	40.0	13.3	27.3	40.0
Bibio hortulanus	18.2	27.3	10.0	–	18.2	–

[a] Percent similarity was calcuated from tables 2 and 3 as percent of IJ and FGM biometric measurements not statistically different from reference strains.
– data not available.

5). Insect mortality occurred within 2-4 days after nematode exposure. Infective juvenile establishment of Rioja (12.0%) also is lower than for the UK strain. Significant differences were observed between the penetration of male and female Rioja IJ (*Mann-Whitney* $U = 320.00$, $df = 65$, $P = 0.004$). Although only 100 IJ were inoculated in the sand column assay, similar results were recorded for larval mortality and numbers of days for mortality to occur when compared with penetration assays. However, OBS III host mortality was greater than the range of host mortalities caused by Rioja. Total penetration rate in sand column assay was 2.8%, and significant differences were observed between males and females (*Mann-Whitney* $U = 46.00$, $df = 43$, $P < 0.001$). Larval mortality in a one-on-one assay was below the range of the SN strain, and the ET_{50}, in exposure time assay, was fifteen times higher than the UK strain.

DISCUSSION

Steinernema feltiae was previously isolated from soil samples in Spain (García del Pino, 1994; García del Pino and Palomo, 1996, 1997) but not from *B. hortulanus*. *Steinernema feltiae* has been isolated from bibionid larvae in Denmark (Bovien, 1937; Poinar and Lindhart, 1971), where these flies are also natural hosts for *Steinernema affine* (Bovien) and *Steinernema intermedium* (Poinar) (Bovien, 1937; Mráček and Sturhan, 2000). These steinernematid species seem to be natural control agents of bibionid flies, and epizootics have been reported (Bovien, 1937; Mráček and Sturhan, 2000). Larval mortality observed in the population collected in the La Rioja region was within the limits of the 23–68% range observed for *Bibio* spp., which showed a moderate level of host parasitism by EPN (Peters, 1996). A survey to determinate the infection levels in the bibionid populations in this region could provide accurate information about the relationship between the *S. feltiae* Rioja strain and its natural host.

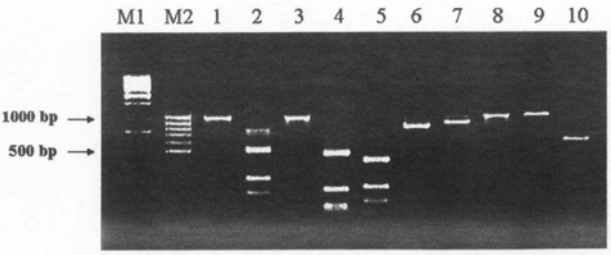

FIG. 1. RFLP analysis of *Steinernema feltiae* Rioja strain, digested with nine restriction enzymes in a 2% (w/v) TBE buffered agarose gel. M1 marker 1 Kb ladder (Band sizes 10,000-500 base pairs) and M2 marker 100 bp ladder (Band sizes 1000-80 base pairs). Lane 1: nondigested amplified product, Lanes 2-10: RFLPs yielded by restriction enzymes: 2, Alu I, 3, EcoR I, 4, Hinf I, 5, RsaI, 6, Hae III, 7, Hha I, 8, Hinc II, 9, Dra I and 10, Mnl I.

In this study, we compared the morphometric traits data of the Rioja strain of *S. feltiae* cultured on three insect hosts: *G. mellonella*, *S. littoralis* and *B. hortulanus*. According to Hominick et al. (1997) and Stock et al. (2000), the most suitable morphometric characters to identify *Steinernema* species are the measurements of third-stage infective juveniles and the first generation males. Infective juveniles from *S. littoralis* were significantly shorter than those recovered from *G. mellonella* and *B. hortulanus*, although total body length of first generation males from *G. mellonella* and *S. littoralis* were similar. Hazir et al. (2001) also observed shorter IJ in "Sinop strain" of *S. feltiae* when reared in *Spodoptera exigua* Hb. (Lepidoptera: Noctuidae). Shishiniova et al. (1998) found morphometrical differences between FGM *Steinernema carpocapsae* (Weisser) "BVC 01 strain" cultured in *G. mellonella* and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). Nevertheless, these authors observed that the differences between isolates from different geographic locations were larger than differences resulting from culturing the same strain on *G. mellonella* or *T. molitor*. Stock et al. (2000) and Poinar (1992) also showed that the geographical origin and habitat can influence morphometric data, so even *G. mellonella* morphometric values can change respective to those provided in the bibliography based on abiotic factors and rearing conditions.

It has been suggested that the morphometric differences are due to intraspecific variability (Poinar, 1992; Stock et al., 2000), and molecular data are useful (Stock and Reid, 2004). Three *S. feltiae* RFLP types had been described: A1 and A2 from strains from the UK and continental Europe and one from Japan (Hominick et al., 1997; Reid et al., 1997; Yoshida, 2003). Molecular analysis of the Rioja strain ITS region showed that this isolate corresponds to A2 RFLP type of *S. feltiae*. Yoshida

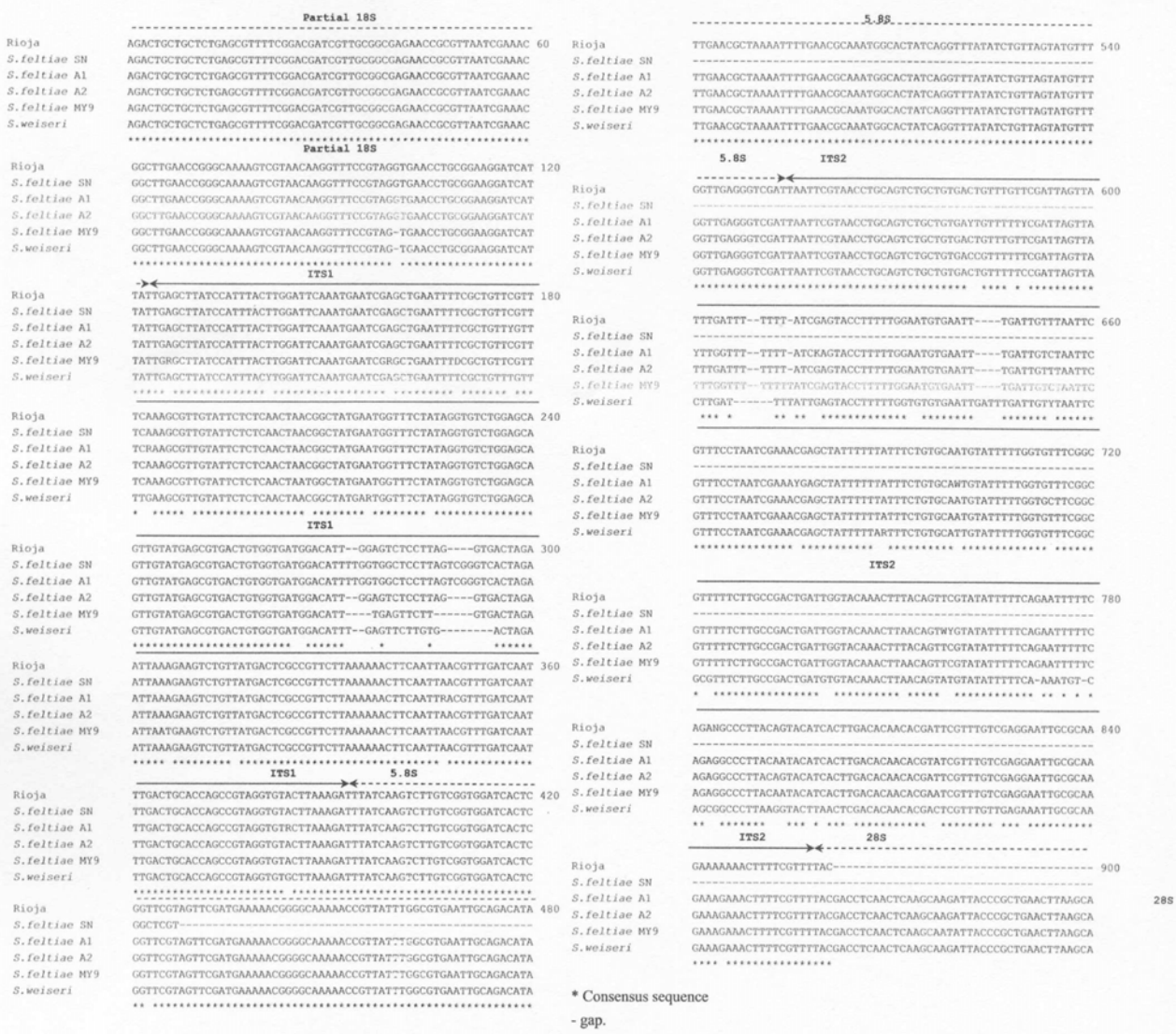

FIG. 2. Sequence alignment of rDNA of 18S (partial)-ITS1-5.8S-ITS2 from *S. feltiae* Rioja strain with other closely related *Steinernema* spp. Continuous line shows ITS regions and dotted lines the ribosomal region.

(2003) described 76 UK strain as A1 RFLP type; this fact could explain the low degree of morphometrical similarity with the Rioja strain. Rioja most strongly resembles the M65 Catalonia and Pakistan strains. Although RFLP types of these reference strains are not yet available, because of their morphometrical similarity it is possible that they belong to the A2 RFLP type. Morphometric variation was observed among all studied isolates. Rioja, Catalonia, UK and Pakistan strains showed intraspecific morphological and morphometric variation. Additional studies on the morphometric and molecular variability of strains from different sites and habitats could provide more accurate and valuable information about geographical and ecological requirements for EPN.

Insect mortality with the Rioja strain ranged between 69.9 to 80.9% and 69.2 to 87% in penetration and sand column assays, respectively. Infective juveniles' penetration in this isolate ranged from 3 to 25% in penetration assay and from 0 to 6% in sand column assay. Similar results were obtained by Tarasco (1997) with eight native *S. feltiae* strains from Italy. Converse and Millar (1999) also observed that the percentage of larval mortality in *G. mellonella* in one-on-one assays differs between species and strains, suggesting that this assay could provide a unique virulence profile to characterize each isolate. In this study, all *S. feltiae* strains tested showed a percent mortality greater than 45%, but no statistical difference among strains was observed. The Rioja strain produced 4.2% mortality in *G. mellonella* larvae. The ET_{50} of the Rioja strain was also 15 times higher than that of the UK strain (Glazer and Lewis, 2000). These outside range values obtained could be due to the foraging behavior of the Rioja isolate. Campbell et al. (2003) described the two broad foraging strategies of the free-living infective juveniles of entomopathogenic nematodes with a continuum between both: cruise (widely foraging) and ambush (sit-and-

TABLE 5. Insect mortality, time to death, penetration rate and ET_{50} in different bioassays with *Steinernema feltiae* strains.

Bioassay n° IJ strain/insect	Penetration		Sand column		One-on-one		Exposure time	
	200 RIOJA	200 UK[c]	100 RIOJA	OBS III[c]	1 RIOJA	SN[c]	400 RIOJA	UK[c]
Insect mortality (%)	75.3 ± 8.05 (69.6–80.9)	95 (90–100)	78.12 ± 12.57 (69.2–87.0)	100	4.2 ± 0.25 (4.0–4.5)	35 (30–70)		
Days to die	2.7 ± 0.67 (2–4)		2.6 ± 0.49 (2–3)					
P (total)[b]	12.0 ± 6.3 (3–25)	48.4 (27.8–63.5)	2.8 ± 1.53 (0–6)					
P (males)[b]	4.7 ± 3.46 (1–13)		0.4 ± 0.59 (0–2)					
P (females)[b]	7.4 ± 4.03 (1–17)		2.4 ± 1.59 (1–6)					
ET_{50} (min)[a]							675, 9	45 (35–70)

[a] ET_{50} value: exposure time in minutes that is required by the nematode to acheive 50% insect mortality.
[b] P: penetration rate: percentage of the initial nematodes that invades an insect host.
[c] Data from Glazer and Lewis (2000).

wait). Cruiser foragers are more effective in finding sedentary and cryptic hosts, and ambushers can find mobile hosts and tend to be shorter (Campbell and Kaya, 2002). *S. feltiae* have an intermediate foraging strategy and raise their bodies off the substrate more frequently than other non-nictating species (Campbell and Gaugler, 1997). The Rioja strain is shorter than other described *S. feltiae* isolates, which could affect their forage behavior, making this strain more ambusher-like than intermediate. Behavioral assays could determine the foraging strategy of this new strain and, combined with basic virulence bioassays, establish its suitability in biological control programs.

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