Identification and intraspecific variability of *Steinernema feltiae* strains from Cemoro Lawang village in Eastern Java, Indonesia

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Summary. Four strains of *Steinernema feltiae* from Eastern Java, Indonesia were characterised based on morphometric, morphological and molecular data. In addition, their virulence against last instar *Tenebrio molitor* and heat tolerance was tested. Infective juvenile have a mean body length ranging from 749 to 792 μm. The maximum sequence difference among the four strains was 7 bp (8.8%) in the ITS and 2 bp (0.3%) in D2D3 regions of the rDNA. All the strains are not reproductively isolated and can reproduce with European strain *S. feltiae* Owiplant. The lowest LC_{50} was observed for strain SCM (373) and the highest for *S. feltiae* strain Owiplant (458) infective juveniles. All the four strains showed relatively better mean heat tolerance when compared with *S. feltiae* Owiplant both in adapted and non-adapted heat tolerance experiments.

Key words: Cross hybridisation, D2D3, ITS, morphometrics, phylogeny, taxonomy, *Tenebrio molitor*, virulence.

Entomopathogenic nematodes (EPN) together with their associated enteric bacteria *Xenorhabdus* and *Photorhabdus*, respectively, are lethal pathogens of several economically important insect pests (Grewal *et al.*, 2005). Many surveys have been conducted all over the world in search of species of EPN (e.g. Hominick, 2002; Grewal *et al.*, 2005). EPN exhibit great variation in their infectivity and survival. Collecting indigenous nematodes can possibly provide isolates more suitable for the control of local pests because of their adaptation to local climatic conditions and population regulators (Stock *et al.*, 2008). The huge natural genetic diversity in EPN populations around the world can also be exploited for breeding programmes to improve beneficial traits of EPN for biological control. However, one of the first and most important needs for use in biocontrol or for domestication of EPN is their accurate identification. New methods using sequence analysis of Internal Transcribed Spacer (ITS) and the D2D3 expansion regions of rDNA have proven to be useful for identification and molecular analysis of nematodes (Spiridonov *et al.*, 2004; Nadler *et al.*, 2006). The objectives of this study were to identify intraspecific variability of the strains using molecular, morphometric, morphological and cross hybridisation methods and to compare their virulence and heat tolerance with a European isolate of *S. feltiae*.

MATERIAL AND METHODS

Nematodes. *Steinernema feltiae* strains SCM, SNGD, SNC and Ssp60 were obtained from Cemoro Lawang village, Eastern Java, Indonesia at 2,329 m above sea level using the *Galleria* baiting technique (Bedding & Akhurst, 1975). *Steinernema feltiae* strain Owiplant from Poland, kindly provided by

Dr.Marek Tomalak, Poznan, Poland, was used to compare virulence and heat tolerance with the newly identified *Steinernema feltiae* strains.

Morphological and morphometric characterisation. Ten *Galleria mellonella* larvae were exposed in a Petri dish (100 × 15 mm) lined with two moistened filter papers to 2000 infective juveniles (IJ) (Nguyen, 2007). First and second generation adult nematodes were obtained by dissecting infected insects in Ringer's solution 2-4 days and 5-7 days, respectively, after the host had died. Infective juveniles were obtained when they emerged from the cadavers after 7-10 days using a White trap (White, 1927).

Specimens of different stages were killed and fixed in TAF (Courtney *et al.*, 1955). Nematodes fixed in TAF were processed to glycerin by the Seinhorst method (Seinhorst, 1959). Morphometric analysis of the nematode specimens were made for 20 individual males, 20 females and 20 IJ using light microscopy and image analysing software (Cell^D, Olympus soft imaging solutions GmbH, Germany). The species were identified by comparing data with species descriptions and keys by Nguyen (2007) and Nguyen *et al.* (2007). For morphometric data analysis measurements of IJ and first generation males were used.

Molecular characterisation and phylogeny. DNA was extracted from three pooled live IJ using the method reported by Spiridonov *et al.* (2004). The ITS regions of the ribosomal DNA (rDNA) was amplified by PCR. The PCR reaction mixture contained 5 µl of 10× PCR reaction buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 1 µM forward and reverse primer, 0.4 µl (2 U μl^{-1}) Taq Polymerase (Invitrogen, Merelbeke, Belgium), 5 µl crude DNA-extract and ddH_2O up to a volume of 50 µl. The primers TW81 (5´-GTT TCC GTA GGT GAA CCT GC-3´) as forward and AB28 (5´-ATA TGC TTA AGT TCA GCG GGT-3´) as reverse primer (Joyce *et al.*, 1994) were used. The D2D3 region was amplified by PCR using the same reaction mixture but with the primers being replaced by D2A (5´-ACA AGT ACC GTG AGG GAA AGT TG-3´) as forward and D3B (5´-TCG GAA GGA ACC AGC TAC TA-3´) as reverse primers (De Ley *et al.*, 1999).

The PCR product was cloned in a pGEM®-T Vector and JM109 High Efficiency Competent Cells according to the manufacturer's instructions (Part number TM042, Promega, USA). Plasmid DNA was purified based on the method described in the Pure Yield Plasmid Miniprep System (Promega). Quantification of both the PCR-product and the plasmid DNA was done by putting 2 µl of the product on a UV-spectrophotometer (Nanodrop-100).

An approximate 20 µl purified plasmid DNA with PCR-insert (100 ng μl^{-1}) was sent to a sequencing service (Macrogen, Seoul, South-Korea) for sequencing. Sequence data of the four strains SCM, SNC, SNGD and Ssp60 were edited using BioEdit (BIOEDIT version 7.0.9, Invitrogen). The sequences of the four strains together with those available in the GenBank were aligned using the default parameters of Clustal X (Thompson *et al.*, 1997).

Phylogenetic trees and pairwise comparisons were obtained by Maximum Parsimony (MP) using PAUP, 4.0b8 (Swofford, 2002). All data were assumed to be unordered, all characters were treated as equally weighted, gaps as missing data. Maximum parsimony was performed with a heuristic search with 1000 replicates (simple addition sequence, stepwise addition, tree-bisection-reconnection (TBR) branch swapping). For the ITS sequences, *Caenorhabditis elegans* (X03680) was used as out-group taxon (Nguyen *et al.*, 2001). For D2D3, *Panagrellus redivivus* (AF331910) was used as out-group taxon (Stock *et al.*, 2001). Branch support was estimated by bootstrap analysis: One thousand replicates for MP and 10,000 replicates for neighbour joining (NJ) (Nguyen *et al.*, 2001, Nguyen, 2007). Trees were displayed with TreeView1.6.1 (Page, 1996).

Cross hybridisation. Twenty males of one nematode strain were cultured on Nutrient Lipid Agar (Wouts, 1981) plates with 20 pre-mature females of another strain and *vice versa*. In each of the combinations, the symbiotic bacteria were taken from the female partner. Ten *G. mellonella* last instar larvae were inoculated with 100 IJ per *G. mellonella* in a Petri dish (100 × 15 mm) with moist filter paper and kept at 25°C in dark room. At 12-24 h after inoculation, the infected *G. mellonella* was sterilised using 95% ethanol. The cadaver was then opened with a sterile needle and a drop of haemolymph was streaked on to NBTA agar (Akhurst, 1980). After sub-culturing and confirming the absence of contaminants, a single colony was transferred in to BSA liquid medium (Ehlers *et al.*, 1998). The bacterium was incubated for 1-3 days at 25 °C in the dark.

Virulence and heat tolerance tests. Virulence was assessed by determination of the lethal concentrations (LC) of four strains from Indonesia and *S. feltiae* Owiplant. Doses of 0, 50, 100, 200, 400 and 800 IJ in 1 ml water were used to inoculate 40 mealworms of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) in Petri dishes filled with moist sand

and incubated at 25°C according to Peters (2005). Larval mortality was corrected using Abbott's formula (Abbott, 1925). LC_{50} and LC_{90} values were calculated using Probit analysis (Finney, 1971).

To adapt IJ to heat, they were kept at 35°C for 3 h prior to heat stress treatment. Afterwards, IJ were left to recover for 1 h at 25°C and were then exposed to five different temperature gradients ranging from 37°C to 41°C for 2 h. Two ml tap water was added on the five cover-slide chambers one hour ahead of introducing 200 IJ. This experiment was repeated three times with different batches of nematodes. The temperature on the bottom of the chambers was recorded by platinum Pt100 thin layer sensors. After the heat treatment the nematodes were left in the dark at 25°C overnight to recover and counting was done after separating active nematodes from the dormant nematodes using a water trap (Ehlers *et al.*, 2005).

RESULTS

Morphological and morphometric characterisation. IJ had a mean body length ranging from 749-792 µm and 53-54 distances from head to excretory pore (Table 1). The analysis of variance showed no significant difference in morphometric data among the four strains in most of the characters. However, significant difference was observed in body length between Ssp60 and SNC ($P \leq 0.05$; Table 1). In addition, Ssp60 is significantly different from the other strains in body diameter, NR, and ABD and significantly shorter in hyaline length ($P \leq 0.05$; Table 1). Strain SNGD has a significantly longer tail length when compared with the other strains ($P \leq 0.05$; Table 1).

In first generation males the mean body length of the strains ranges from the lowest for SNGD (1,081 µm) to the highest for SCM (1,243 µm) (Table 1). No statistically significant differences between the four strains were recorded in ES, T, SL, spicule width, GL and gubernaculum width , D% and SW% ($P \leq 0.05$; Table 1).

Molecular characterisation and phylogeny. Maximum parsimony and NJ criterion produced the same consensus tree in which the strains SCM, SNC, Ssp60 and *S. feltiae* strain SN (AF121050) form a monophyletic group supported by a bootstrap value of 99 and 78%, respectively (Fig. 1a). SNC, SCM, SNGD, Ssp60 and *S. feltiae* strain SN (AF121050) were distinct from the other *Steinernema* species tested, with a bootstrap value of 100% and 94%, respectively, in MP and NJ criteria (Fig. 1).

In the MP consensus tree, the four strains SNC, SCM, and SNGD were grouped together with *S. feltiae* strain SN with a bootstrapping support of 65%. However, the strain Ssp60 is out of this grouping. *Steinernema monticolum* and *S. ashiuense* are distinct from the other *Steinernema* species and the four strains tested with a bootstrap support of 90% (Fig. 1b).

Pairwise comparison of the sequences of the ITS regions of the rDNA indicates that the four strains are closely related with each other and with *S. feltiae* strain SN. Between *S. feltiae* strain SN and SNGD a 99.2% similarity ratio and a 99.9% with the remaining three strains was found (Table 2). The similarity ratio between the tested species ranged between 81.1% (*S. feltiae* strain SN and *S. ashiuense*) to 96.1% (*S. feltiae* strain SN and *S. litorale*) (Table 2).

Similarly, comparison of the sequences of D2D3 region of rDNA of *S. feltiae* strain Bodega Bay and the four strains showed 99.8% to 100% similarity ratio (Table 2). The similarity ratio of the remaining *Steinernema* species with *S. feltiae* strain Bodega Bay was from 93.2% (between *S. feltiae* and *S. monticolum*) to 99.6% (between *S. feltiae* and *S. silvaticum*). There is no difference in the D2D3 regions of rDNA of *S. feltiae* strain Bodega Bay with SNC and SNGD and only one bp with SCM and Ssp60 (Table 2).

Cross hybridisation. In the cross breeding tests, involving males of each of the four strains and females of *S. feltiae* strain Owiplant, copulation started after a few minutes of introduction on agar plates. Similarly, in reverse crosses between females of each strain with males of *S. feltiae* strain Owiplant copulation was observed. In both crosses between males and females and reverse crosses of each of the strains with *S. feltiae* Owiplant offspring were produced starting from the fourth day after introduction of males and virgin females. Further observation on the offspring obtained from the crosses and reverse crosses was continued every day and the offspring were found to be fertile and produced another generation of nematodes.

Virulence and heat tolerance tests. The lowest LC_{50} was observed with strain SCM (373 IJ) and the highest LC_{50} with *S. feltiae* strain Owiplant (458 IJ) followed by SNC (408 IJ) per 40 mealworms (Table 3). Based on LC_{50} and LC_{90} values there was no significant difference ($P \leq 0.05$) in virulence between the strains studied when compared with *S. feltiae* strain Owiplant.

The four strains showed relatively better heat tolerance than *S. feltiae* strain Owiplant, both in the adapted and non-adapted heat tolerance experiments. Moreover, all the strains including *S. feltiae* Owiplant showed an increased tolerance after

Table 1. Comparison of morphometric characters of infective juveniles and first generation males of *Steinernema feltiae* strains SCM, SNC, SNGD , Ssp60 and those provided by the bibliography. All measurements are in µm (mean ± SD (range)). Means followed by the same letter in rows are not significantly different from each other; according to Tukey's HSD test, P ? 0.05. *After Nguyen et al. (2006). **After Campos-Herrera et al. (2006). – Data not available.

Character	Infective Juveniles						First generation males					
	SCM	SNC	SNGD	Ssp60	S. feltiae (SN)*	S. feltiae (Rioja)**	SCM	SNC	SNGD	Ssp60	S. feltiae (SN)*	S. feltiae (Rioja)**
N	20	20	20	20	20	-	20	20	20	20	20	-
L	759 ± 58 ab (647-832)	749 ± 46a (665-807)	789 ± 43 ab (693-868)	792 ± 58 b (684-900)	879 ± 49 (766-928)	783 ± 75.3 (660-914)	1243 ± 96 b (1109-1363)	1142 ± 122 (801-1293) ab	1081 ± 101 (892-1253) a	1169 ± 102 ab (1034-1405)	1612 ± 88 (1414-1815)	1220 ± 176.4 (820-1648)
a	32 ± 3.1 b (27-38)	33 ± 5.1 b (27-43)	32 ± 4.0 b (26-40)	29 ± 2.1 a (24-33)	30 ± 1.9 (27-34)	30 ± 3.3 (20-37°	12 ± 1.7 b (9-14)	10 ± 1.1 a (7-12)	12 ± 0.6 b (11-14)	11 ± 0.8 a (9-13)	11.5	12 ± 1.7 (8-17)
b	6 ± 0.4 ab (6-7)	6 ± 0.5 a (5-7)	7 ± 0.5 b (6-8)	6 ±1 a (4-7)	6.4 ± 0.3 (5.8-6.8)	6.6 ± 0.5 (5.9-6.7)	8 ± 0.6 b (7-9)	8 ± 0.9 ab (6-10)	7 ± 0.6 a (6-8)	8 ± 0.6 b (7-10)	9.5	9.3 ± 1.5 (7-12.6)
c	11 ± 0.8 ab (9-12)	11 ± 0.7 ab (10-13)	10 ± 0.5 a (10-11)	11 ± 0.7 b (10-13)	10 ± 0.5 (9.4 -11)	11 ± 0.7 (10-13)	46 ± 5.6 b (35-54)	41 ± 6.3 b (28-52)	35 ± 3.5 a (29-41)	43 ± 8.9 b (32-68)	41.3	51 ± 7.9 (40-70)
c'	6 ± 1.2 a (4-8)	6 ± 0.9 b (4-8)	6 ± 0.7 b (4-8)	5 ± 0.9 a (4-7)	4.8 ± 0.2 4.5-5.1)	-	0.6 ± 0.1 a (0.5- 0.8)	0.6 ± 0.1 a (0.4-0.8)	0.7± 0.1 b (0.6-0.9)	0.6 ± 0.1 a (0.4-0.7)	0.8	-
Body Diameter	24 ± 1.8 a (20-26)	23 ± 2.9 a (18-28)	25 ± 3.0 a (19-30)	28 ± 1.1 b (25-29)	29 ± 1.9 (26-32)	26 ± 2.9 (20-30)	106 ± 13.4 b (82-135)	118 ± 17.1 b (82-146)	88 ± 6.8 a (80-101)	112 ± 11.9 b (84-130)	140 ± 10 (121-162)	107 ± 20.9 (73-141)
EP	53 ± 4.7 a (44-61)	53 ± 2.4 a (49-57)	57 ± 4.9 b (52-73)	54 ± 3.6 ab (49-61)	63 ± 2.3 (58-67)	64 ± 10.6 (50-89)	81 ± 6.7 b (65-88)	77 ± 9.5 ab (57-93)	79 ± 6.8 b (62-92)	71 ± 9.5 a (55-93)	115 ± 3.4 (110-126)	86 ± 9.9 (61-97)
NR	92 ± 8.5 a (76 -105)	94 ± 6.7 ab (84-112)	91 ± 4.4 a (81-100)	101 ± 16.4 b (87-135)	113 ± 5.1 (108-117)	93 ± 6.5 (80-105)	115 ± 5.3 b (104- 122)	113 ± 8.1 b (98- 128)	113 ± 6.1 b (103-125)	105 ± 4.7 a (97- 112)	-	-
ES	123 ± 12 ab (98-142)	132 ± 8.0 bc (119-151)	120 ± 6.5 a (105-133)	133 ± 16.5 c (115-165)	136 ± 3.5 (130-143)	118 ± 8.3 (102-138)	154 ± 5.2 a (144-162)	153 ± 12.3 a (130- 168)	148 ± 7.2 a (134-160)	146 ± 6.2 a (138-165)	170 ± 3.4 (164-180)	138 ± 12.4 (100-152)
T	70 ± 3.4 a (62-76)	69 ± 4.9 a (59-76)	76 ± 4.6 b (67-85)	72 ± 5.6 a (61-80)	86 ± 2.6 (81-89)	72 ± 5.5 (61-80)	27 ± 4.4 a (23-38)	28 ± 3.5 a (22-35)	31 ± 3.1 a (26-39)	28 ± 4.1 a (18-34)	39 ± 1.2 (37-43)	28 ± 3.5 (21-33)
H	44 ± 4.6 b (32-53)	44 ± 4.7 b (35-53)	46 ± 4.8 b (36-59)	39 ± 3.0 a (34-46)	38 ± 3.2 (32-43)	-	-	-	-	-	-	-
ABD	13 ± 2.1 bc (9-16)	11 ± 1.8 a (8-16)	12 ± 1.8 ab (10-19)	14 ± 2.2 c (10-17)	18 ± 0.8 (16-19)	-	47 ± 2.1 ab (44-50)	49 ± 3.7 b (43-57)	44 ± 2.9 a (40-51)	47 ± 2.7 ab (41-53)	48 ± 1.7 (43-53)	34 ± 3 (28-39)
Spicule Length (SL)	-	-	-	-	-	54 ± 7.7 (40-69)	69 ± 3.6 a (61-74)	69 ± 3.7 a (61-77)	68 ± 4.2 a (60-76)	67 ± 3.2 a (58-71)	66 ± 1.5 (62-68)	68 ± 4.6 (58-80)
spicule width	-	-	-	-	-	88 ± 10.4 (75-109)	14 ± 1.3 a (12-16)	17 ± 10.3 a (48-10)	13 ± 1.5 a (10-15)	13 ± 1.7 a (10-16)	-	_
Gubernaculum length (GL)	-	-	-	-	-	S. feltiae (Rioja)**	47 ± 3.0 a (41-51) a	48 ± 4.7 a (37-56)	49 ± 4.3 a (39-55)	50 ± 2.8 a (45-55)	52 ± 1.9 (48-56)	47 ± 3.7 (41-53)
Gubernaculum width	-	-	-	-	-	-	7 ± 0.8 a (6-9)	7± 0.9 a (5-8)	7 ± 0.9 a (5-9)	7 ± 0.7 a (5-8)	-	-
D% (EP/ES X 100)	43 ± 3.3 a (37-49)	41 ± 3.2 a (33-46)	47 ± 4.9 b (40-60)	41 ± 6.1 a (31-47)	46 ± 1.4 (44- 50)	783 ± 75.3 (660-914)	52 ± 4.3 a (42-58)	51 ± 9.1 a (34-69)	54 ± 4.2 a (43-62)	49 ± 6.8 a (38-63)	68 ± 3.1 (64-72)	62 ± 6.7 (46-70)
E% (EP/T X 100)	76 ± 6.6 a (64-83)	78 ± 4.0 a (73-86)	75 ± 7.1 a (65- 98)	75 ± 5.6 a (66-87)	74 ± 4 (67-81)	30 ± 3.3 (20-37°	302 ± 43.5 b (222-358)	275 ± 39.2 ab (362-194)	258 ± 27.5 a (190-294)	261 ± 55.9 ab (178-382)	-	-
SW% (SL/ABD X 100)	63 ± 5.0 b (52-73)	63 ± 4.2 b (57-74)	61 ± 4.4 b (50-69)	55 ± 3.4 a (49-62)	44 ± 4 (37-51)	6.6 ± 0.5 (5.9-6.7)	136 ± 38.9 a (123-161)	142 ± 11.5 a (126-162)	153 ± 12.8 a (132-173)	143 ± 11.7 a (110-170)	140 ± 10 (130-150)	205 ± 80 (169-235)
GS% (GL/SL X 100)						11 ± 0.7 (10-13)	67 ± 6.2 a (52-74)	70 ± 6.9 ab (53-82)	72 ± 5.4 ab (60-80)	75 ± 4.1 b (66-83)	80 ± 3 (70-90)	70 ± 5.8 (60-83)

Table 2. Pair-wise distances of ITS and D2D3 regions of rDNA between species in *feltiae*-group and strains SCM, SNC, SNGD and Ssp60. Numbers below diagonal are total character differences and above diagonal mean character differences (adjusted for missing data).

Species		1	2	3	4	5	6	7	8	9	10	11	12	13
ITS region														
1	S. monticolum	–	0.07581	0.15632	0.16480	0.15907	0.18443	0.18579	0.18194	0.15580	0.17720	0.18939	0.14716	0.51043
2	S. ashiuense	68	–	0.19344	0.19451	0.19523	0.20055	0.20192	0.20083	0.18940	0.19337	0.19909	0.18544	0.50785
3	S. kraussei	141	171	–	0.06492	0.04145	0.09159	0.09159	0.09045	0.07423	0.08668	0.08516	0.07193	0.49556
4	S. texanum	147	170	62	–	0.06540	0.10614	0.10486	0.10486	0.08691	0.10128	0.10070	0.08765	0.48686
5	S. oregonense	143	172	40	62	–	0.08132	0.08132	0.08015	0.06562	0.07761	0.06964	0.06112	0.49121
6	SNC	135	146	73	83	64	–	0.00248	0.00248	0.00124	0.00878	0.05089	0.04835	0.47690
7	SCM	136	147	73	82	64	2	–	0.00248	0.00124	0.00878	0.05089	0.04835	0.47441
8	Ssp60	133	146	72	82	63	2	2	–	0.00124	0.00754	0.04952	0.04707	0.47625
9	S. feltiae	141	168	72	83	63	1	1	1	–	0.00753	0.04952	0.03858	0.47331
10	SNGD	129	140	69	79	61	7	7	6	6	–	0.04127	0.04198	0.47601
11	S. weiseri	125	131	62	72	50	37	37	36	36	30	–	0.03320	0.49309
12	S. litorale	132	163	72	83	58	38	38	37	37	33	24	–	0.47944
13	C. elegans	465	453	502	463	475	382	380	381	461	377	357	478	–
D2D3 region														
1	SNC	–	0.00000	0.00162	0.00162	0.02435	0.00370	0.00000	0.01020	0.01533	0.00832	0.04708	0.05236	0.27094
2	SNGD	0	–	0.00162	0.00162	0.02435	0. 00370	0.00000	0.01020	0.01533	0.00832	0.04708	0.05236	0.27094
3	SCM	1	1	–	0.00325	0.02597	0.00556	0.00170	0.01190	0.01704	0.00998	0.04870	0.05410	0.26929
4	Ssp60	1	1	2	–	0.002273	0.00185	0.00170	0.00850	0.01363	0.00666	0.04545	0.05061	0.26929
5	S. texanum	15	15	16	14	–	0.02186	0.02791	0.3023	0.03613	0.02864	0.06840	0.06036	0.28669
6	S. silvaticum	2	2	3	1	12	–	0.00364	0.01093	0.01642	0.00546	0.04372	0.04736	0.28885
7	S. feltiae	0	0	1	1	24	2	–	0.01269	0.01736	0.00577	0.06799	0.04812	0.29466
8	S. oregonense	6	6	7	5	26	6	11	–	0.01042	0.01384	0.06551	0.04695	0.29316
9	S. kraussei	9	9	10	8	31	9	15	9	–	0.01852	0.07054	0.05300	0.29569
10	S. weiseri	5	5	6	4	25	3	5	12	16	–	0.06326	0.04408	0.29558
11	S. monticolum	29	29	30	28	58	24	55	53	57	52	–	0.03019	0.29688
12	S. ashiuense	30	30	31	29	51	26	41	40	45	38	24	–	0.30565
13	P. redivivus	165	165	164	164	252	158	254	253	254	261	247	265	–

Table 3. Lethal concentrations and mean tolerated temperatures and temperature at which 10% of the nematode population survived recorded for strains SCM, SNC, SNGD, Ssp60 and *Steinernema feltiae* strain Owiplant for adapted and non-adapted heat tolerance.

Strain	Lethal Concentration (No. IJ/40 mealworms		Non-adapted		Adapted	
	LC50	LC90	Mean (°C)	Best 10% (°C)	Mean (°C)	Best 10% (°C)
SCM	373 ± 113 a	890 ± 209 a	38.7 ab	40.6 a	39.6 bc	41.3 ab
SNC	408 ± 112 a	978 ± 282 a	38.4 a	40.4 a	39.0 abc	40.6 ab
SNGD	406 ± 58 a	893 ± 201 a	38.9 b	40.9 a	39.7 c	41.9 b
Ssp60	386 ± 188 a	913 ± 447 a	38.7 b	40.7 a	38.9 ab	40.3 ab
S. feltiae Owiplant	458 ± 44 a	979 ± 140 a	38.3 a	40.7 a	38.8 a	39.9 a

Means followed by the same letter in a column are not significantly different from each other; according to Tukey's HSD test, $P \leq 0.05$.

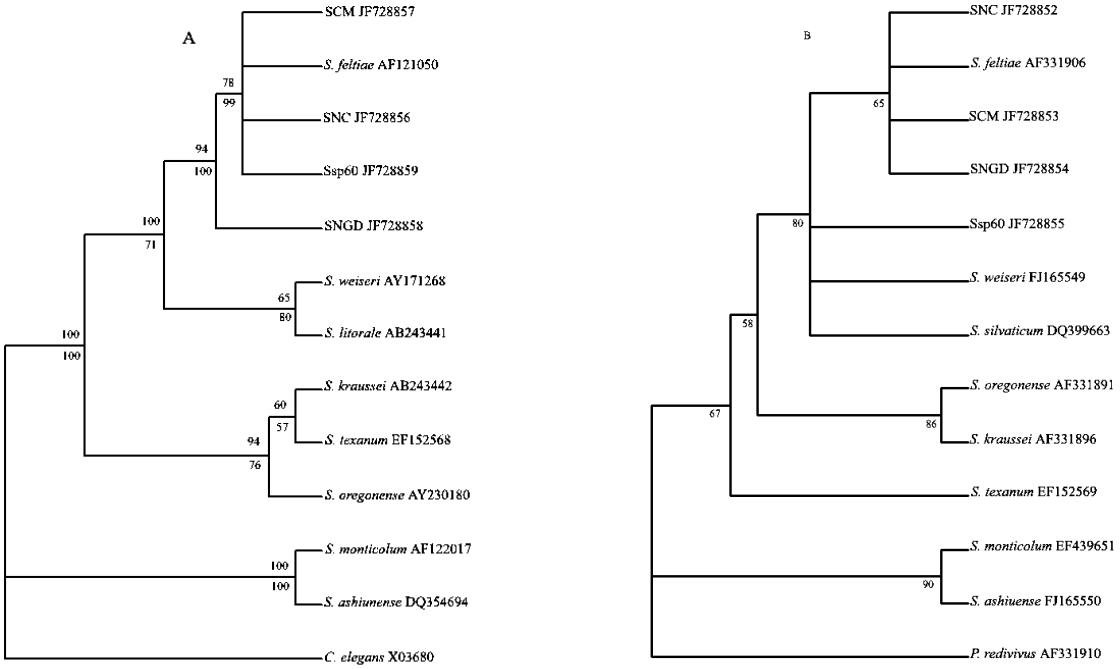

Fig. 1. Phylogenetic relationships of *Steinernema* species from the *feltiae*-group and the strains SCM, SNC, SNGD and Ssp60. A: strict consensus tree of two most parsimonious trees inferred from analysis of ITS rDNA (1212 characters, 194 parsimony informative); B: strict consensus tree of five maximum parsimony trees inferred from analysis of D2D3 LSU rDNA (1095 characters, 56 parsimony informative) regions of rDNA. Bootstrap support over50% is presented at the nodes for MP (below) and NJ (above) analysis.

adaptation to heat. In non-adapted heat tolerance experiment the lowest mean tolerated temperature was recorded for strain *S. feltiae* Owiplant (38.3°C) and the highest for strain SNGD (38.9°C) (Table 3). In both adapted and non-adapted heat tolerance experiments strain SNGD showed better mean tolerated temperatures when compared with the remaining strains and *S. feltiae* Owiplant. Variability among the strains in their heat tolerance was observed. In both adapted and non-adapted heat tolerance experiments, strain SNGD tolerated high mean temperatures of 38.9 and 39.7°C, respectively.

DISCUSSION

Morphological and morphometric characterisation. Morphological characters of all the studied strains have a close resemblance with *S. litorale*. Yoshida (2004) also reported that *S. litorale* resembles the Japanese isolate of *S. feltiae* in many morphological characters. The body length of IJ of the strains ranges from 749 µm for strain SNC to 792 µm for strain Ssp60 and body diameter of 23 µm for SNC to 28 µm for Ssp60. However it is relatively shorter when compared with IJ of *S. feltiae*

strain SN with a mean body length of 879 (766-928) µm and a body diameter of 29 µm (Nguyen *et al*., 2006). Similar to the result of this study, a short mean body length of 783 (660-914) µm and body width of 26 (20-30) µm was reported for IJ of *S. feltiae* strain Rioja by Campos-Herrera *et al.* (2006).

Morphometric trait data of the first generation males showed that all the strains are shorter than that of *S. feltiae* strain SN mentioned by Nguyen *et al* (2006). However, relatively reliable morphometric data were obtained in IJ and first generation males for all the strains to identify them as *S. feltiae*. Hominick *et al.* (1997) and Stock *et al.* (2000) also mentioned that the most suitable morphometric characters to identify *Steinernema* species are the measurements of third-stage IJ and the first generation males.

Molecular characterisation and phylogeny. The maximum intra-specific difference of the four strains with *S. feltiae* strain SN is 6 bp (0.8%) in the ITS rDNA and 1 bp (0.2%) with *S. feltiae* strain Bodega Bay in the D2D3 expansion segment of LSU rDNA. Similar results were reported by Spiridonov *et al*. (2004), who showed intra-specific sequence variability of ITS rDNA region of 14 S. *feltiae* strains ranging from 0-1.6% for European populations and reaching up to 2.4% between the British (A2) and the Armenian isolates. The intra-specific variability of the ITS rDNA region among the strains in this study is lower (0.2-0.9%) when compared with European populations and between the British and Armenian isolates reported by Spiridonov *et al.* (2004).

The maximum inter-specific difference in the ITS rDNA sequences with *S. feltiae* was observed between *S. feltiae* and *S. ashiuense* (18.9%, 168 bp) and the minimal difference was found between *S. feltiae* and *S. litorale* (3.9%, 37 bp). The phylogenetic analysis of the ITS regions of rDNA gave a better resolution than D2D3 with a poor bootstrap support of 65% to group as sister groups. ITS regions of rDNA are highly variable among *Steinernema* species and provide more information characters and resolution among closely related species compared to SSU or LSU rDNA (Nguyen *et al*., 2001, Stock *et al*., 2001; Spiridonov *et al.*, 2004).

Cross hybridisation. According to Yoshida (2004) females of *S. feltiae* strain can copulate and produce offspring when hybridised with the males of *S. litorale*; however, progeny were not generated in the reverse cross when *S. litorale* females were mated with *S. feltiae* males, supporting the result obtained from morphometric, molecular and morphological data.

Virulence and heat tolerance tests. Different authors reported inter and intra-specific variations in the infectivity of different EPN isolates, which have been attributed to the variation in the ability of IJ to find and/or enter a host (Sims *et al.*, 1992). *Tenebrio molitor*, which is a less susceptible host than *G. mellonella*, was used to detect greater differences in virulence between strains. However, significant difference between the studied strains and S. *feltiae* Owiplant was not found. According to Susurluk *et al.* (2001), although more than 50% of *S. feltiae* IJ were able to tolerate a temperature of 36°C for 2 h, survival for longer than 4 h at a temperature of 36°C was not recorded (Susurluk *et al.*, 2001).

The results indicate that the species *S. feltiae* is also indigenous in Eastern Java and the strains can be crossed with a European strain of *S. feltiae*. The data on heat tolerance indicates that a search for genetic variability among tropical strains of *S. feltiae* may be useful to obtain heat tolerant traits to be used in breeding for heat tolerant strains.

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Temesgen Addis, Mulawarman Mulawarman, Lieven Waeyenberge, Maurice Moens, Nicole Viaene, Ralf-Udo Ehlers. Определение и внутривидовая вариабельность изолятов *Steinernema feltiae* из деревни Семоро Лаванг, Восточная Ява, Индонезия.

Резюме. Дана морфологическая, морфометрическая и молекулярная характеристика четырех изолятов *Steinernema feltiae* с Восточной Явы, Индонезия. Исследована инвазионность этих изолятов для личинок мучного хрущака *Tenebrio molitor,* а также их температурная устойчивость. Исследованные инвазионные личинки имеют среднюю длину тела от 749 до 792 µm. Максимальное различие в длине последовательности между изолятами составляла 7 п.н. (8.8%) для ITS-участка и 2 п.н. (0.3%) для D2D3-участка rDNA. Все изоляты скрещивались между собой, а также с европейским изолятом *S. feltiae* Owiplant. Наименьшее значение LC_{50} при заражении мучных хрущаков отмечалось для изолята SCM (373), а наивысшее – для изолята *S. feltiae* Owiplant (458). В экспериментах с адаптацией и без адаптации все четыре изолята показали лучшие средние показатели устойчивости к повышенной температуре по сравнению *S. feltiae* Owiplant.