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Phylogenetic relationships within the genus *Steinernema* (Nematoda: Rhabditida) as inferred from analyses of sequences of the ITS1-5.8S-ITS2 region of rDNA and morphological features

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Summary – Eighty four new and four published ITS1-5.8S-ITS2 sequences of rDNA obtained from different populations of 24 nominal species and 28 isolates of 16 putative *Steinernema* species were analysed using the maximum parsimony method. In most of the phylogenetic trees obtained from different ITS alignments and phylogenetic procedures, the 84 isolates formed five main, highly or moderately supported, clades, *viz* Clade I: '*affine-intermedium*'; Clade II: '*carpocapsae-scapterisci-tami*'; Clade III: '*feltiae-kraussei-oregonense*'; Clade IV: '*bicornutum-ceratophorum-riobrave*'; and Clade V: '*arenarium-glaseri-karii-longicaudum*'. The ITS rDNA data were found to be of little utility in resolving relationships between these clades, but were useful in studying relationships between species within certain clades. The level of intra-specific variability was different between clades with sequence divergence of 2.4-2.8% of ITS rDNA for some species. Analysis of a combined data matrix of both molecular and morphological features was performed with six qualitative and three quantitative features.

Keywords – Entomopathogenic nematodes, ITS, PCR, rDNA, sequence analysis, Steinernematidae.

The genus *Steinernema* Travassos, 1927 is the most intensively studied group of nematodes associated with insects. Although only about 30 species of the genus have been described to date, greater specific diversity is expected (Hominick *et al.*, 1997; Kerry & Hominick, 2002). Nematodes of this genus are found in virtually all terrestrial habitats supporting vegetation (Elawad *et al.*, 1997; Waturu *et al.*, 1997; Phan *et al.*, 2001a, b; Shahina *et al.*, 2001; Hominick, 2002).

Poinar (1993) proposed hypothetical evolutionary links between *Steinernema* and other rhabditids based on morphological data. Relationships of the genus *Steinernema* with alloionematids and strongyloidids were also suggested (Spiridonov & Belostotskaya, 1983). The closeness of *Steinernema* with *Strongyloides* Grassi, 1879 and some other rhabditid genera was recently proved by analyses of 18S rDNA sequences (Blaxter *et al.*, 1998).

Several attempts have been made to compare morphological and molecular approaches for reconstructing the phylogeny of the genus *Steinernema*. Liu and Berry (1996) presented phylogenetic relationships derived from the analysis of both RAPD fragments and 11 morphological features for 14 *Steinernema* species. Reid (1994) and Reid *et al.* (1997) analysed the restriction fragments of ITS1-5.8S-ITS2 PCR products generated by 17 restriction enzymes and proposed a phylogram in which the clades correspond to conditional groups of species such as 'steinernematids with long juveniles' or 'steinernematids with short juveniles'.

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The first attempt to use sequencing data for *Steinernema* phylogenetic analysis was based on analyses of the partial 18S gene of rDNA (Liu *et al.*, 1997). This region, however, was found to be too conservative for constructing a resolved phylogeny of this genus. Sequences of the ITS1 region of rDNA and mitochondrial COII and 16S rDNA genes for seven species of *Steinernema* were analysed by Szalanski *et al.* (2000). The authors obtained trees which were in good concordance with the morphological features of the species and considered these genes to be good candidates for phylogenetic reconstruction of the group. Analyses of sequences of ITS1-5.8S-ITS2 regions obtained from ten steinernematid species revealed several groups with significant bootstrap supports (Nguyen *et al.*, 2001; Nguyen & Duncan, 2002). Comprehensive phylogenetic analyses based on combined morphological and molecular (D2-D3 expansion segment of LSU rDNA) data, including 21 species of *Steinernema*, were made by Stock *et al.* (2001).

All of these studies revealed trends in *Steinernema* phylogeny. Unfortunately, many species and isolates were not included so that their position within the genus was left uncertain. In our study, the ITS1-5.8S-ITS2 sequences of different *Steinernema* isolates maintained at CABI Bioscience, Egham, UK, and CLO, Merelbeke, Belgium were analysed. Several aspects of phylogenetic analyses for the Steinernematidae were set as the main goals for the present work, these being: *i*) to study relationships between the main groups of *Steinernema* using maximum parsimony. It was hypothesised that newly obtained sequences might resolve relationships inside this taxon. In this analysis we included species belonging to different groups (based on gross morphology, *e.g.*, infective juvenile length) in the genus. As alignment ambiguity is a common event during ITS rDNA analysis of divergent sequences, several alignments were generated under different combinations of gap opening and gap extension penalties and then analysed separately; *ii*) to reveal relationships between species and isolates within several main groups. All sequences of isolates belonging to a certain group were included in the alignment and analysed using maximum parsimony; *iii*) to test congruence of morphological and molecular phylogeny of *Steinernema*. The morphological and morphometrical characters used by Liu and Berry (1996) and Stock *et al.* (2001) were re-analysed and a new matrix proposed. The total evidence analysis was applied to obtain a tree from the matrix combining molecular and morphological data; *iv*) to estimate species boundaries in steinernematids based on molecular data. Sequence divergence was estimated firstly between isolates which, according primarily to their morphology, were identified as belonging to the same species, and secondly, isolates which, on the same basis, were identified as belonging to different species. The presence of autapomorphies was estimated for related species. The species delimitation was finally considered on the basis of a combination of molecular (autapomorphies, sequence divergence) and morphological evidence; and *v*) to reveal ecological and biogeographical implications of the contemporary evolutionary hypothesis for *Steinernema*. We expected that the proposed steinernematid phylogeny might demonstrate concordance in features such as habitat preference and geographical distribution patterns.

Materials and methods

Nematode material

The list of studied species and isolates is presented in Table 1. Isolates were obtained either by using *Galleria* baiting of soil samples collected during surveys (Bedding & Akhurst, 1975), or were received from other laboratories. The nematodes were maintained on *Galleria* caterpillars and juvenile suspensions were stored at 4°C.

Morphological and ecological studies, and cross-breeding tests

Juveniles and females were fixed in 6% formalin for both light microscope and SEM studies. For SEM, specimens were processed as described by Kozodoi and Spiridonov (1988). The matrix of morphological features for steinernematids was created on the basis of our own and published observations (Nguyen & Smart, 1996; Artyukhovsky *et al.*, 1997; Hominick *et al.*, 1997; Stock *et al.*, 1998; Pham *et al.*, 2000; Nguyen & Adams, 2003). Cross-breeding experiments were performed to elucidate the specific status of N and Va isolates of *S. feltiae* from Merelbeke, Belgium. Single juveniles from the two populations were injected into *Galleria* caterpillars (Akhurst & Bedding, 1978), which were kept in Parafilm® sealed Petri dishes until the results of the injection (presence or absence of a new generation) became obvious. Information about the habitats of the isolates was obtained during collection of materials for the present study and from the literature (Hominick & Briscoe, 1990; Gwynn & Richardson, 1996; Miduturi *et al.*, 1997; Sturhan, 1999).

DNA EXTRACTION, PCR AMPLIFICATION, CLONING AND SEQUENCING AT CLO

A few (1-20) juveniles were put in an Eppendorf tube containing 8 μl of worm lysis buffer (100 mM KCl, 20 mM Tris-HCl pH 8.3, 3 mM $MgCl_2$, 2 mM DTT and 0.9% Tween 20), 10 μl double distilled water, 2 μl proteinase K (600 μg/ml) and homogenised (Spiridonov & Moens, 1999). PCR amplification was performed as described by Spiridonov and Moens (1999). Two sets of primers were used in the PCR reactions: (1) TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) or (2) 18S (5′-TTGATTACGTCCCTGCCCTTT-3′) and 26S (5′-TTTCACTCGCCGTTACTAAGG-3′) as described by Joyce *et al.* (1994) and Vrain *et al.* (1992), respectively. PCR products were purified using QIAquick PCR or Gel Purification Kit (Qiagen Ltd, Crawley, UK).

PCR products were used for direct sequencing using one of the following primers: AB28, TW81, 18S, 26S, M1 (5′-ACGAGCCGAGTGATCCACCG-3′) or M2 (5′-CTTATCGGTGGATCACTCGG-3′) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems AH Nieuwerkerk a/d Ijissel, The Netherlands). The resulting products were purified using a Centriflex Gel Filtration Cartridge (Edge BioSystems Inc., Gaithersburg, MD, USA).

As initial direct sequencing of several isolates showed ambiguous positions with multiple peaks, the PCR products from these isolates were cloned and re-sequenced. The PCR product was cloned in pGEM-T vector and transformed into JM109 High Efficiency Competent Cells (Promega, Leiden, The Netherlands) according to the instructions of the manufacturer. PCR products from clones were sequenced as described above. Several clones from each isolate were sequenced to confirm polymorphisms observed from the direct sequencing approach.

DNA EXTRACTION, PCR AMPLIFICATION, CLONING AND SEQUENCING AT CABI

Total genomic DNA was isolated as described by Reid and Hominick (1992) or Joyce *et al.* (1994). Primers 18S and 26S, as described by Vrain *et al.* (1992), were used for PCR. PCR amplification was performed as described by Reid *et al*. (1997). All PCR products were cloned and then sequenced as described above.

SEQUENCE ALIGNMENTS AND PHYLOGENETIC ANALYSIS

Alignments of the ITS1-5.8S-ITS2 sequences (with nucleotides belonging to 18S and 28S rDNA removed) were generated using Clustal W (Thompson *et al.*, 1994). Several alignments were generated: *i*) 30 alignments containing 48 *Steinernema* and *Caenorhabditis elegans* (outgroup taxon) sequences with different gap opening and gap extension penalty values for general analysis; *ii*) five alignments for the analysis of steinernematid groups; and *iii*) alignments with 35 sequences for combined morphological and molecular analysis. All studied alignments are available from the first author.

Maximum parsimony (MP) analyses were conducted with PAUP* 4.0b8 (Swofford, 1998). The heuristic search procedure was used with 100 replicates of random taxon addition. Gaps were treated as missing data. Bootstrap (BS) analysis with 100 replicates was conducted to assess the degree of support for each branch on the tree (Felsenstein, 1985) using simple addition sequences with TBR swapping (tree-bisection-reconnection). The *g1* statistic was computed by generating 10 000 random trees using the RANDTREES option in PAUP (Hills & Huelsenbeck, 1992). The partition homogeneity test was run as implemented in PAUP with 100 replicates to determine whether the nematode phylogeny inferred from molecular data was congruent with morphological phylogeny. Trees were displayed with TreeView 1.6.1 (Page, 1996).

Results

SEQUENCE ANALYSES OF THE ITS REGION FOR STEINERNEMATIDS

Sequence characteristics

New complete sequences of the ITS1-5.8S-ITS2 region of the rDNA cistron were obtained for 81 steinernematid isolates and partial sequences (>685 bp) were obtained for a further three species (*S. rarum* – AY 171300; *Steinernema* sp. 13 from Pendelton, Arkansas – AY 171277; and *Steinernema* sp. 13 from Nebraska). Four *Steinernema* sequences deposited in GenBank were also used in the analysis (Table 1). The length of full ITS1-5.8S-ITS2 sequences varied from 712 bp in *Steinernema* sp. 16 to 816 bp in *Steinernema* sp. 7 (E3) from Estonia. The ITS1 region (243-306 bp) was shorter than ITS2 (274-375 bp) except for *Steinernema* sp. 9 from Sri Lanka

Table 1. *Details of* Steinernema *isolates used in the study.*

Species	Location, (strain)	GenBank accession number	Source of material or reference
S. abbasi	Sultanate of Oman	AY230158 (CABI, cl)*	Elawad *et al.* (1997)
S. affine	Merelbeke, Belgium	AY171286 (CLO, di)	S.E. Spiridonov
S. affine	Wageningen, The Netherlands	AY171298 (CLO, di)	S.E. Spiridonov
S. affine	Aberdeen, Scotland, UK	AY171296 (CLO, di)	M. Wilson
S. affine	Berkshire, England, UK	AY230159 (CABI, cl)	A.P. Reid
S. arenarium	Rjazan, Russia	AY230160 (CABI, cl)	A.P. Reid
S. bicornutum	Type locality, Vojvodina, Yugoslavia	AY230163 (CABI, cl)	R.-U. Ehlers
S. bicornutum	Krasnodar, Russia	AY171279 (CLO, cl)	S.E. Spiridonov
S. carpocapsae	USA (All)	AY230164 (CABI, cl)	A.P. Reid
S. carpocapsae	Western Europe (isolate 42)	AY171283 (CLO, cl)	M.A. Ansari
S. carpocapsae	Mozhaisk, Moscow region, Russia	AY171282 (CLO, di)	S.E. Spiridonov
S. ceratophorum	Type locality, China	AY230165 (CABI, cl)	A.P. Reid
S. cubanum	Type locality, Cuba	AY230166 (CABI, cl)	Z. Mráček
S. diaprepesi	Florida, USA	AF122021	Nguyen *et al.* (2001)
S. diaprepesi	Venezuela	AY230187 (CABI, cl)	A.P. Reid
S. feltiae	Type locality, Izhevsk, Russia	AY171246 (CLO, cl)	S.E. Spiridonov
S. feltiae	UK (isolate A1 site 76)	AY230169 (CABI, cl)	A.P. Reid
S. feltiae	UK (isolate A2, site 107)	AY230170 (CABI, cl)	A.P. Reid
S. feltiae	Khosrov National Park, Armenia	AY171256 (CLO, di)	S.E. Spiridonov
S. feltiae	Australia (isolate '*bibionis*')	AY171257 (CLO, di)	R. Akhurst
S. feltiae	Merelbeke, Belgium (isolate N)	AY171254 (CLO, cl)	S.E. Spiridonov
S. feltiae	Merelbeke, Belgium (isolate Va)	AY171274 (CLO, cl)	S.E. Spiridonov
S. feltiae	Belovezha, Poland	AY171266 (CLO, cl)	S.E. Spiridonov
S. feltiae	Czech Republic	AY171249 (CLO, cl)	Z. Mráček
S. feltiae	San Bernardino, Switzerland	AY171247 (CABI, cl)	S.E. Spiridonov
S. feltiae	Tomsk, Russia	AY171273 (CLO, cl)	S.E. Spiridonov
S. feltiae	Artybash, Republic Altai, Russia (isolate 46)	AY171272 (CLO, cl)	S.E. Spiridonov
S. feltiae	St Louis, USA (isolate 88)	AY171275 (CLO, cl)	K. Krasomil-Osterfeld
S. feltiae	Japan (isolate MY9)	AY238178 (CABI, cl)	M. Yoshida
S. glaseri	USA (type)	AY230171 (CABI, di)	A.P. Reid
S. glaseri	Azores, Portugal	AY171288 (CLO, di)	C. Simoes
S. intermedium	Charleston, South Carolina, USA (topotype)	AY230172 (CABI, di)	R.-U. Ehlers
S. intermedium	St Louis, Missouri, USA (isolate 82)	AY171290 (CLO, di)	K. Krasomil-Osterfeld
S. karii	Type locality, Kibingo, Kenya	AY230173 (CABI, cl)	A.P. Reid
S. kraussei	Type locality, Westphalia, Germany	AY230175 (CABI, cl)	Z. Mráček
S. kraussei	Artybash, Republic Altai, Russia (isolate 35)	AY171270 (CLO, di)	S.E. Spiridonov
S. kraussei	Artybash, Republic Altai, Russia (isolate 37)	AY171271 (CLO, di)	S.E. Spiridonov
S. kraussei	UK (isolate Nash)	AY230176 (CLO, cl)	A.P. Reid
S. kraussei	Italy	AY230174 (CLO, cl)	A.P. Reid
S. kraussei	Leignon, Belgium	AY171250 (CLO, cl)	S.E. Spiridonov
S. kraussei	Iceland	AY171248 (CLO, di)	M. Wilson
S. kraussei	Moscow region, Russia	AY171264 (CLO, di)	S.E. Spiridonov
S. kraussei	Kirkhill, Scotland (isolate K5)	AY171253 (CLO, di)	M. Wilson
S. kraussei	S. Gottard, Switzerland (D)	AY171258 (CLO, di)	S.E. Spiridonov
S. kraussei	UK (isolate B2, site 216)	AY230161 (CABI, cl)	A.P. Reid
S. kraussei	S. Gottard, Switzerland (E)	AY171259 (CLO, di)	S.E. Spiridonov
S. kraussei	Velikiye Luki, Pskov region, Russia	AY171251 (CLO, di)	S.E. Spiridonov
S. longicaudum	Type locality, China	AY230177 (CABI, cl)	A.P. Reid

Place of sequencing: CABI, CLO; cl – cloning and sequencing; di – direct sequencing.

Table 1. *(Continued).*

Species	Location, (strain)	GenBank accession number	Source of material or reference
S. monticolum	Type locality, Mt. Chiri, Korea	AF122017	Nguyen *et al*. (2001)
S. neocurtillae	Type locality, La Crosse, Florida, USA	AF122018	Nguyen *et al*. (2001)
S. oregonense	Type locality, Oregon, USA	AY230180 (CABI, cl)	A.P. Reid
S. pakistanense	Type locality, Karachi, Pakistan	AY230181 (CABI, cl)	Shahina *et al*. (2001)
S. rarum	Type locality, Rio Cuarto, Argentina	AY171300 (CLO, cl)	M.E. Doucet
S. riobrave	Type locality, Texas, USA	AY230182 (CABI, cl)	A.P. Reid
S. scapterisci	Type locality, Uruguay	AY230183 (CABI, cl)	K.B. Nguyen
S. siamkayai	Type locality, Lahmsok, Thailand	AF331917	Stock *et al.* (2001)
S. tami	Type locality, Cat Tien, Vietnam	AY171280 (CLO, cl)	S.E. Spiridonov
S. weiseri	UK (isolate D1, site 541 of A. Reid)	AY230167 (CABI, cl)	Mráček *et al.* (2003)
S. weiseri	Germany (isolate F of D. Sturhan)	AY171268 (CLO, cl)	Mráček *et al.* (2003)
S. weiseri	S. Gottard, Switzerland	AY171269 (CLO, di)	S.E. Spiridonov
Steinernema sp. 1	Missouri, USA	AY171276 (CLO, di)	K. Krasomil-Osterfeld
Steinernema sp. 2	Germany (species B)	AY171255 (CLO, cl)	D. Sturhan
Steinernema sp. 2	UK (isolate B3, site 249)	AY230162 (CABI, cl)	A.P. Reid
Steinernema sp. 3	Binh Chau National Park, Vietnam	AY171286 (CLO, di)	S.E. Spiridonov
Steinernema sp. 4	Krasnaya Poliana, Sotchi, Russia	AY171291 (CLO, di)	S.E. Spiridonov
Steinernema sp. 5	UK (species E1 of A. Reid)	AY230168 (CABI, cl)	R. Gwynn
Steinernema sp. 5	Rochefort, Belgium (species E1)	AY171297 (CLO, cl)	S.E. Spiridonov
Steinernema sp. 5	Polva, Estonia (isolate E6)	AY171294 (CLO, di)	S.E. Spiridonov
Steinernema sp. 5	Zvenigorod, Moscow region, Russia	AY171295 (CLO, di)	S.E. Spiridonov
Steinernema sp. 5	Artybash, Republic Altai, Russia (isolate 14)	AY171299 (CLO, di)	S.E. Spiridonov
Steinernema sp. 6	Pennsylvania, USA (isolate 33)	AY171287 (CLO, cl)	K. Krasomil-Osterfeld
Steinernema sp. 7	Polva, Estonia (isolate E3)	AY171292 (CLO, di)	S.E. Spiridonov
Steinernema sp. 7	Switzerland (isolate CH 221)	AY171293 (CLO, di)	J. Grunder
Steinernema sp. 8	North Carolina, USA (isolate NC 513)	AY230179 (CABI, cl)	R.-U. Ehlers
Steinernema sp. 8	St Louis, Missouri, USA (isolate 70)	AY171284 (CLO, di)	K. Krasomil-Osterfeld
Steinernema sp. 9	Sri Lanka (isolate SSL1)	AY230184 (CABI, cl)	Amarasinghe *et al*. (1994)
Steinernema sp. 10	Tichino, Switzerland (isolate CH 213)	AY171285 (CLO, di)	J. Grunder
Steinernema sp. 11	India (isolate 7.2)	AY171281 (CLO, di)	M.A. Ansari
Steinernema sp. 12	Kenya (isolate UH36)	AY230186 (CABI, cl)	A.P. Reid
Steinernema sp. 13	Pendelton, Arkansas, USA	AY171277 (CLO, cl)	K. Krasomil-Osterfeld
Steinernema sp. 13	Nebraska, USA (isolate KKO 133)	AY171278 (CLO, cl)	K. Krasomil-Osterfeld
Steinernema sp. 14	Pennsylvania, USA (isolate 25)	AY171252 (CLO, cl)	K. Krasomil-Osterfeld
Steinernema sp. 15	St Louis, Missouri, USA (isolate 73)	AY171265 (CLO, cl)	K. Krasomil-Osterfeld
Steinernema sp. 16	Nebraska, USA (isolate KKO 122)	AY171260 (CLO, di)	K. Krasomil-Osterfeld
Steinernema sp. 16	Nebraska, USA (isolate KKO 123)	AY171261 (CLO, di)	K. Krasomil-Osterfeld
Steinernema sp. 16	Nebraska, USA (isolate KKO 125)	AY171262 (CLO, di)	K. Krasomil-Osterfeld
Steinernema sp. 16	Nebraska, USA (isolate KKO 126)	AY171267 (CLO, di)	K. Krasomil-Osterfeld
Steinernema sp. 16	Nebraska, USA (isolate KKO 137)	AY171263 (CLO, di)	K. Krasomil-Osterfeld

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                                                       Dde I
                                                         •
S.feltiae (AY171246) Izhevsk, Russia : GACATTTTGGTGGCTCCTTAGTCGGGTCACTAGATCCTA   ┐
S.feltiae (AY171254) Belgium, N      : .......................................   │
S.feltiae  Belgium, NxVa, clone1     : .......................................   │
S.feltiae (AY230169) UK, A1,76       : .......................................   │
S.feltiae (AY171249) Czech Republic  : .......................................   │   a
S.feltiae (AY171275) USA, 88         : .......................................   │
S.feltiae (AY171266) Poland          : .......................................   │
S.feltiae (AY171273) Tomsk, Russia   : .......................................   │
S.feltiae (AY171247) Switzerland     : ..........AT..........A..A.............   ┘
S.feltiae (AY230170) UK, A2,107      : ......GGA..--......----..G.............   ]   b
S.feltiae (AY171272) Altai, Russia   : .......GA..----------..TT..G...........   ┐
S.feltiae (AY230178) Japan, MY9      : .......GA..----------..TT..G...........   │
S.feltiae (AY171257) Australia       : .......GA..----------..TT..G...........   │   c
S.feltiae (AY171274) Belgium, Va     : .......GA..----------..TT..G...........   │
S.feltiae Belgium, NxVa, clone4      : .......GA..----------..TT..G...........   │
S.feltiae (AY171256) Armenia         : .......GA..----------..TT..G...........   ┘
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Fig. 1. *Fragment of the alignment for ITS1 rDNA of 14* Steinernema feltiae *isolates and two ITS-rDNA clones from progeny of cross-breeding. Three types of ITS sequences are indicated with letters: a – without deletions, b – with 6 bp deletion, c – with 10 bp deletion. The* DdeI *restriction site is underlined.*

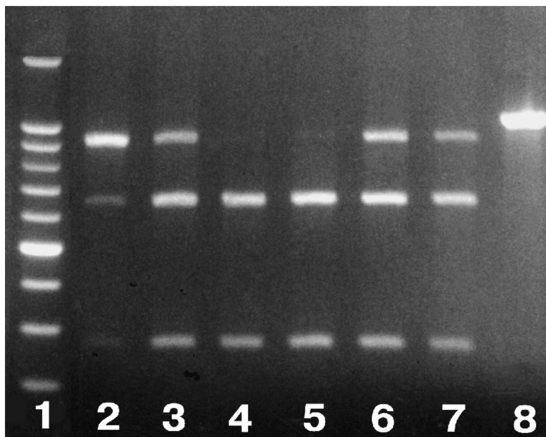

Fig. 2. *Restriction fragments (*DdeI*) of amplified ITS regions of single infective juveniles from two Belgian isolates of* Steinernema feltiae*. Lane 1 – 100 bp DNA marker; Lanes 2 and 3 – two individuals of* S. feltiae *isolate Va; Lanes 4 and 5 – two individuals of* S. feltiae *isolate N; Lanes 6 and 7 – two individuals from the N × Va cross; Lane 8 – unrestricted PCR fragment of* S. feltiae *isolate N.*

(285 bp and 274 bp, respectively). The length of the 5.8S gene sequence was constant for most isolates (156 bp), except for *S. tami* and *Steinernema* sp. 11 (154 bp). Nucleotide base composition was: A – 24.5 (21.2-28.7)%; C – 17.1 (12.5-21.7)%; G – 22.7 (17.4-26.9)%; T – 35.8 (29.9-42.9)%.

Intra-specific and inter-specific variation

The intra-specific variability of the *S. feltiae* ITS sequences ranged from 0-1.6% for European populations and reached 2.4% between the British (A2) and the Armenian isolates. Within clones, three types of ITS1 sequences were distinguished with respect to the presence of indels in positions 150-170 in the *S. feltiae* sequences: (*a*) without deletions, (*b*) with six nucleotide deletions and (*c*) with ten nucleotide deletions (Fig. 1). The third type can be distinguished from the first by the absence of *Dde*I restriction site (Figs 1, 2). Both RFLP and sequences of the ITS region of *S. feltiae* from Belgium revealed that individuals contained ITS sequences of the third (Va, clone 4) and first type (Va, clone 1). Crosses of the Belgian isolate Va containing the two ITS types with another Belgian isolate N having only one ITS type were fertile and the progeny contained both ITS types (Fig. 2).

The sequence differences between *S. kraussei* isolates usually varied between 1-11 bp (up to 1%) but reached 21 bp (2.8%) between the UK (B2) isolate and the Moscow isolate. The sequence divergence of *S. affine* ranged from 0.2-0.6% (2-5 bp). The difference between sequences of *S. carpocapsae* strains from Europe and USA was 3 bp (0.4%). Sequences of the *S. bicornutum* isolate from Krasnodar (Russia) differed from the topotype isolate (Yugoslavia) by 17 bp or 2.2%.

The maximal inter-specific difference in the ITS rDNA sequences was observed between *S. pakistanense* and *S. intermedium* (42.4%, 305 bp), and the minimal difference was found between *Steinernema* sp. 5 and *Steinernema* sp. 7 isolate (2.2-2.5%, 18-19 bp). It should be noted, however, that *Steinernema* sp. 5 and *Steinernema* sp. 7 have yet to be formally described as new species and thus at present our data cannot be used to create molecular criteria for species differentiation.

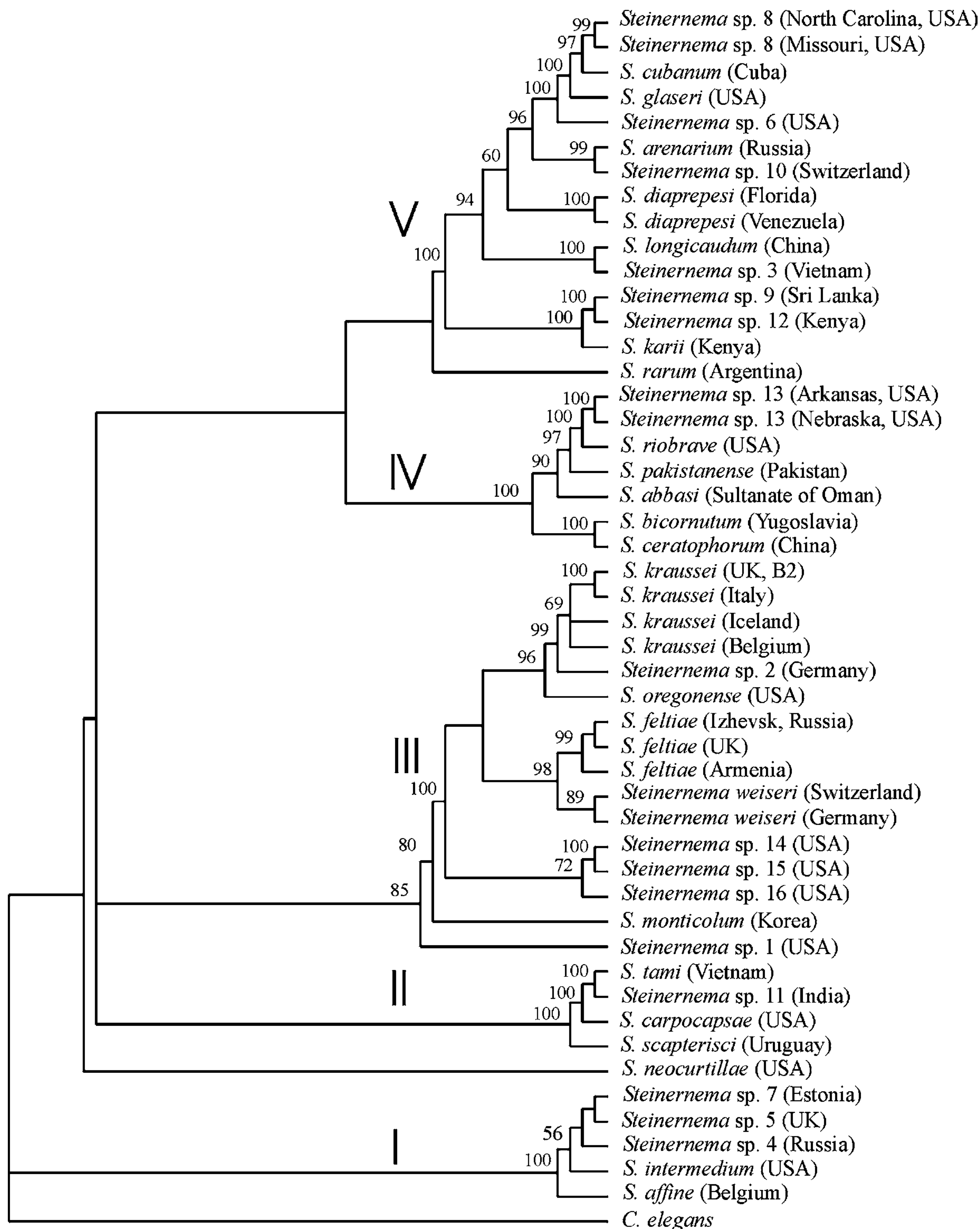

Fig. 3. *Strict consensus of four maximum parsimony trees for the genus* Steinernema *obtained after analysis of alignments generated with default options of Clustal W. Bootstrap numbers (50% and more) are given on appropriate clades.*

Table 2. *Alignment parameters and tree statistics for 48 ITS rDNA sequences of* Steinernema.

N	GOP	GEP	Alignment length	Informative characters	Const. characters	Tree length	Tree number	CI	HI	RI	RC	g1
D	15.0	6.66	921	642	148	3373	4	0.4429	0.5571	0.7395	0.3468	−0.5422
1	1.0	0.5	1496	582	520	2362	5	0.4484	0.5116	0.7466	0.4397	−0.5235
2	2.0	0.5	1514	572	668	2263	16	0.4693	0.5307	0.7509	0.4608	−0.5202
3	4.0	0.5	1332	610	498	2554	36	0.4677	0.5323	0.7460	0.3894	−0.4916
4	7.0	0.5	1146	593	347	2743	2	0.4642	0.5358	0.7466	0.3843	−0.5076
5	15.0	0.5	1069	612	256	3110	9	0.4493	0.5507	0.7338	0.3610	−0.5321
6	30.0	0.5	999	637	205	3493	4	0.4320	0.5680	0.7324	0.3394	−0.5209
7	1.0	1.0	1386	576	543	2226	8	0.4844	0.5156	0.7553	0.4197	−0.5321
8	2.0	1.0	1256	592	453	2369	2	0.4688	0.5312	0.7466	0.3899	−0.5168
9	4.0	1.0	1171	603	254	2769	18	0.4765	0.5244	0.7400	0.4043	−0.5188
10	7.0	1.0	1094	618	298	2790	1	0.4635	0.5365	0.7470	0.3770	−0.5204
11	15.0	1.0	1049	623	245	3120	4	0.4527	0.5473	0.7302	0.3362	−0.5560
12	30.0	1.0	974	639	198	3532	6	0.4334	0.5666	0.7363	0.3625	−0.5152
13	1.0	3.0	1149	615	342	2482	2	0.4706	0.5294	0.7522	0.3891	−0.5284
14	2.0	3.0	1128	607	326	2568	2	0.4633	0.5367	0.7459	0.3802	−0.5105
15	4.0	3.0	1066	612	246	2736	1	0.4607	0.5393	0.7416	0.3738	−0.5138
16	7.0	3.0	1008	615	215	2922	1	0.4593	0.5407	0.7440	0.3713	−0.5444
17	15.0	3.0	943	636	157	3266	2	0.4455	0.5545	0.7371	0.3516	−0.5462
18	30.0	3.0	937	635	165	3581	2	0.4267	0.5733	0.7291	0.3311	−0.5073
19	1.0	6.66	1006	617	234	2709	32	0.4634	0.5366	0.7486	0.3741	−0.5146
20	2.0	6.66	984	619	215	2789	6	0.4562	0.5438	0.7438	0.3646	−0.5075
21	4.0	6.66	962	615	188	2917	4	0.4572	0.5428	0.7431	0.3663	−0.5194
22	7.0	6.66	939	627	175	3091	2	0.4418	0.5582	0.7395	0.3493	−0.5435
23	30.0	6.66	919	640	154	3696	6	0.4138	0.5862	0.7250	0.3176	−0.5519
24	1.0	9.0	963	619	190	2842	6	0.4546	0.5454	0.7450	0.3654	−0.5156
25	2.0	9.0	940	629	178	2933	4	0.4605	0.5395	0.7486	0.3668	−0.5241
26	4.0	9.0	936	622	177	3051	2	0.4535	0.5465	0.7466	0.3609	−0.5220
27	7.0	9.0	927	622	166	3165	6	0.4413	0.5587	0.7414	0.3490	−0.5076
28	15.0	9.0	912	630	153	3395	2	0.4309	0.5691	0.7331	0.3349	−0.5447
29	30.0	9.0	916	652	144	3770	4	0.4181	0.5819	0.7207	0.3181	−0.5404

N – alignment number; GOP – gap open penalty; GEP – gap extension penalty; CI – consistency index; HI – homoplasy index; RI – retention index; RC – rescaled consistency index; g1 – reliability value (after Hillis & Huelsenbeck, 1992).

Molecular phylogeny

Phylogenetic relationships between main Steinernema *groups*

Forty eight steinernematid sequences and one *C. elegans* sequence were aligned with different gap length and gap open penalties to study general relationships between *Steinernema* groups (Table 2). MP analyses of 30 alignments generated trees containing five main clades, *viz* Clade I: '*affine-intermedium*' group (five sequences); Clade II: '*carpocapsae-scapterisci-tami*' group (four sequences); Clade III: '*feltiae-kraussei-oregonense*' group (14 sequences); Clade IV: '*bicornutum-ceratophorum-riobrave*' group (seven sequences); and Clade V: '*arenarium-glaseri-karii-longicaudum*' group (14 sequences). The consensus maximum parsimony tree obtained from one of the alignments (default options) is presented in Figure 3. Strong support for monophyly of Clades I, II and III was evident in all studied alignments. Clade IV was strongly supported in all alignments except two which received moderate support. Monophyly of Clade V was strongly supported in trees obtained from 24 alignments, with moderate or weak support in five trees or one tree, respectively (Table 3). The composition of Clades I-V is presented in detail below.

The positions of *S. monticolum*, *S. neocurtillae*, *S. rarum* and *Steinernema* sp. 1 depended on the alignment (Table 3). *Steinernema monticolum* was a sister for

Table 3. *Positions and bootstrap support of selected clades and species in the MP trees obtained from the general analysis of* Steinernema *sequences.*

Position/alignment	D	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Monophyly of Clade I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Monophyly of Clade II	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Monophyly of Clade III	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Monophyly of Clade IV	S	M	S	S	S	S	S	M	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Monophyly of Clade V	S	W	M	S	S	S	S	S	M	S	S	S	S	S	M	S	S	S	S	M	S	M	S	S	S	S	S	S	S	S
Steinernema spp. 14, 15, 16 + *S. feltiae* and *S. weiseri* clade		M		M	M	M		M		W	W	W		M	M	M	W						W		W		W			
Basal position of *Steinernema* spp. 14, 15, 16 subclade within Clade III	S		M				S		M				S					W	S	W	W	S		S		W		S	S	S
S. oregonense as a sister to *S. kraussei* + *Steinernema* sp. 2	S	M	M	W	S	W	S	S	S	S	M	M	M	M	S	M	M	S	S	M	M	S	M	W	W	W	M	M	S	M
S. monticolum as a sister to Clade III	M	W		W	M	M	M	M		W	M	S	M	W	W	M	W	M	S			M	M		W	W		S	M	
S. monticolum not a sister to Clade III (positioned near other clades)			W						W											W	W			W			W			W
Steinernema sp. 1 as a sister to *S. monticolum* + Clade III	M	W		W	M	M	M	W		W	W	W	M	W	W	W	W	M	M			W	M		M	M		S	S	
Steinernema sp. 1 as a sister to Clade III with *S. monticolum* outside this grouping			W						W											W	W			W			W			W
Basal position of the *S. karii* and *Steinernema* spp. 9, 12 subclade within Clade V	S	W			W	S	S	W		W	M	W	W	W	W	W	S	M	W	W	S	M	S	M	M	W	M	M	W	S
Basal position of *S. glaseri* + *S. cubanum* within Clade V			W	W					W																					
S. pakistanense as a sister to *S. riobrave* + *Steinernema* sp. 13	S	M	W	M	W	M	M	M	W	M	S	M	S	M	M	M	W	M	W	S	M	W	W	M	W	S	M	S	M	S
Basal position of the *S. bicornutum* + *S. ceratophorum* subclade within Clade IV	S			S	S	S	S				S	S	S	S	S		S	S		S		S	S		S		S	S	S	
Basal position of *Steinernema abbasi* within Clade IV		W	W					M	S	S						S			S		S			S		S				S
S. rarum as a sister to Clade V	W	W	W	W	W	M	W		W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
S. neocurtillae as a sister to *S. monticolum* + *Steinernema* sp. 1 + Clade III						W	W				W		W	W	W			W					W	W	W	W		M	W	
S. neocurtillae as a sister to Clade II		W			W				W										W											
S. neocurtillae related to deeper nodes of the *Steinernema* phylogeny tree	W		W	W						W		W				W	W			W	W	W					W			

Bootstrap support (%) for clades: W < 70 (weak); M – 70-90 (moderate); S > 90 (strong).

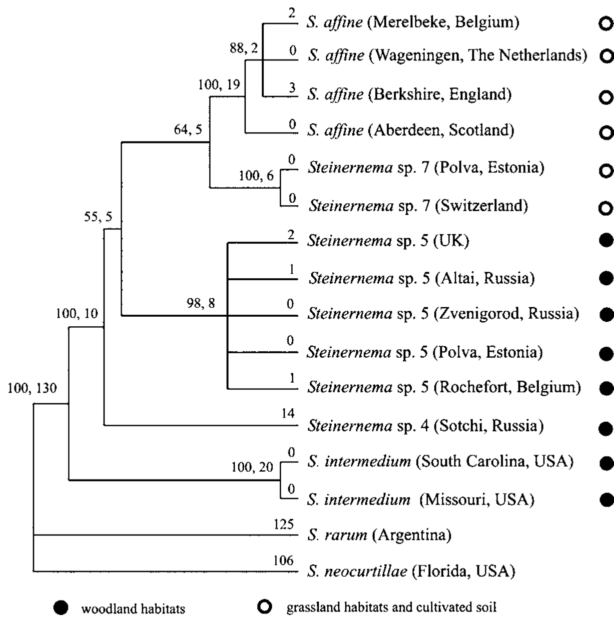

Fig. 4. *Strict consensus of four maximum parsimony trees for Clade I (*'affine-intermedium'*) of the genus* Steinernema. *Bootstrap numbers (50% and more) and nucleotide changes are given on appropriate clades.*

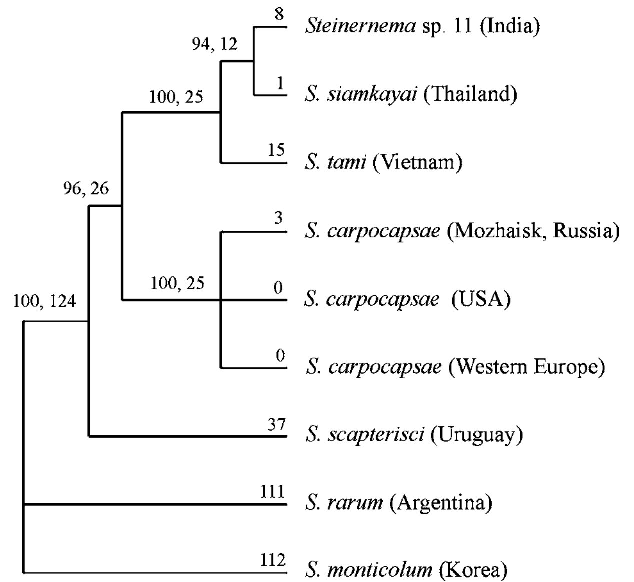

Fig. 5. *Strict consensus of two maximum parsimony trees for Clade II (*'carpocapsae-scapterisci-tami'*) of the genus* Steinernema. *Bootstrap numbers (50% and more) and nucleotide changes are given on appropriate clades.*

Clade III in trees generated from 23 alignments. In three cases, *S. monticolum* was a sister for the group composed of Clade III species and *Steinernema* sp. 1. In most cases, however, the inverse situation was observed when *Steinernema* sp. 1 was a sister for the group formed by the Clade III species together with *S. monticolum*. The position of *S. neocurtillae* was uncertain – in MP trees obtained from 13 alignments, *S. neocurtillae* clustered with the above-mentioned group (*Steinernema* sp. 1 + *S. monticolum* + Clade III), in trees from four alignments it clustered with Clade II, and in the remaining trees it was in a basal or sub-basal position. The bootstrap support for these positions was always weak. The sister taxon status of *S. rarum* for Clade V was evident in the majority of the alignments, but always with low bootstrap support (Table 3).

Relationships within Steinernema *clades*

Clade I – '*affine-intermedium*'. The alignment used for the analyses comprised 16 sequences along with those from *S. rarum* and *S. neocurtillae* (outgroups) and contained 825 bp. MP analysis revealed four equal trees of which the strict consensus tree is presented in Figure 4. Bootstrap support for the basal position of two *S. intermedium* isolates from the USA was very strong. Relationships between *S. affine* and three putative new species (*Steinernema* spp. 4, 5, 7) were not resolved.

Clade II – '*carpocapsae-scapterisci-tami*'. For the analysis of this clade, nine sequences were compared in a 775 bp-alignment, *S. rarum* and *S. monticolum* being selected as the outgroups. MP analysis revealed two equal trees; the strict consensus being presented in Figure 5. *Steinernema scapterisci* was always in the basal position. *Steinernema carpocapsae* was a sister to the clade composed of three isolates originating from tropical or subtropical regions: *S. siamkayai*, *Steinernema* sp. 11 and *S. tami*.

Clade III – '*feltiae-kraussei-oregonense*'. Sequences of 42 isolates were included in the phylogenetic analysis of this clade. *Steinernema monticolum* and *Steinernema* sp. 1 were chosen as the outgroup for the clade as both species usually clustered with this clade in the trees obtained in the general analyses. The length of this alignment was 825 bp. MP analysis revealed 100 maximum parsimony trees of which the strict consensus is presented in Figure 6. The monophyly of the clade was clearly supported as well as its subdivision into the three main subclades: *i*) *S. feltiae* and *Steinernema weiseri*; *ii*) *S. kraussei*, *Steinernema* sp. 2 and *S. oregonense*; and *iii*) *Steinernema* spp. 14, 15 and 16 from the USA.

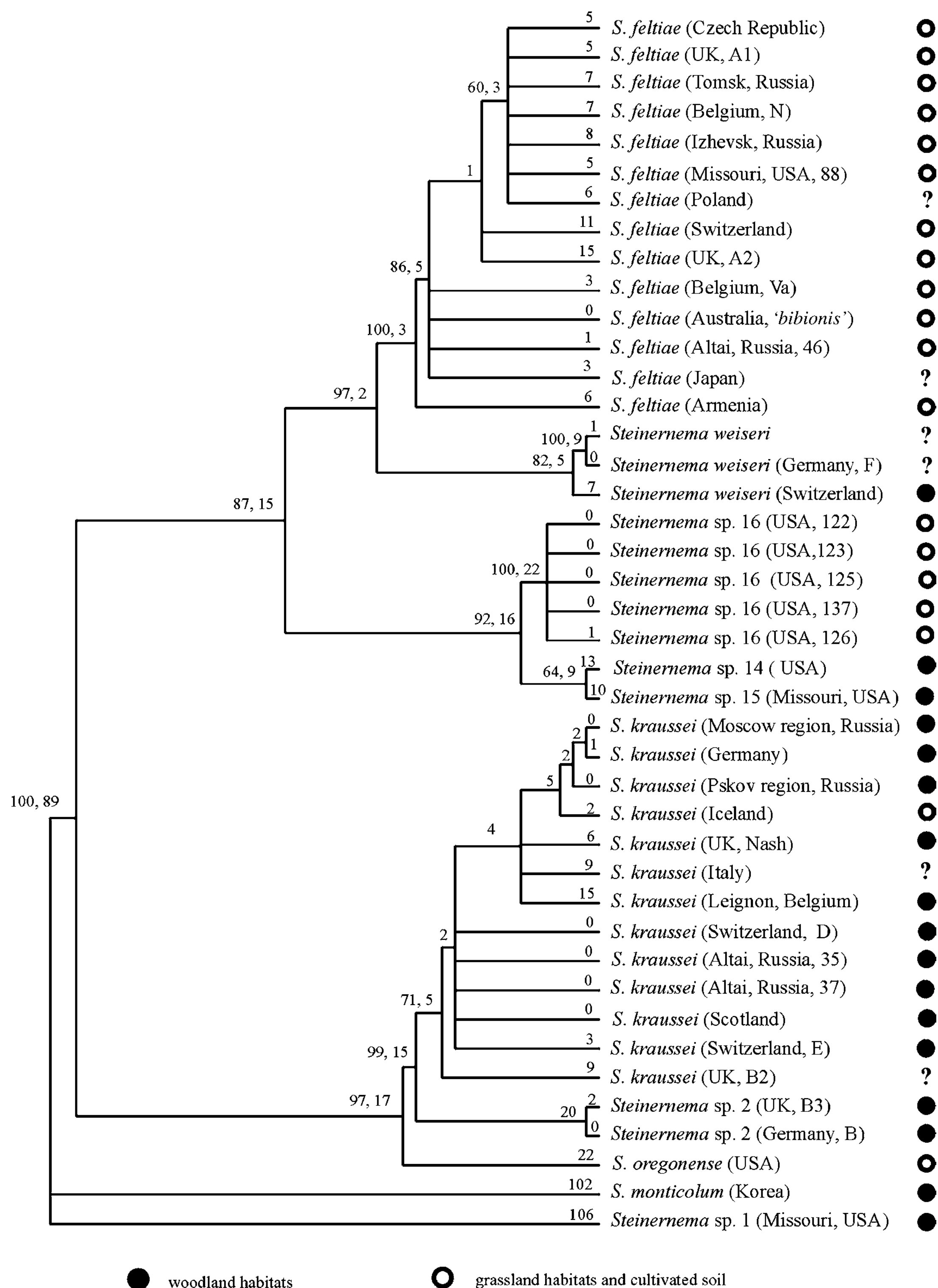

Fig. 6. *Strict consensus of 100 maximum parsimony trees for Clade III ('*feltiae-kraussei-oregonense*') of the genus* Steinernema. *Bootstrap numbers (50% and more) and nucleotide changes are given on appropriate clades.*

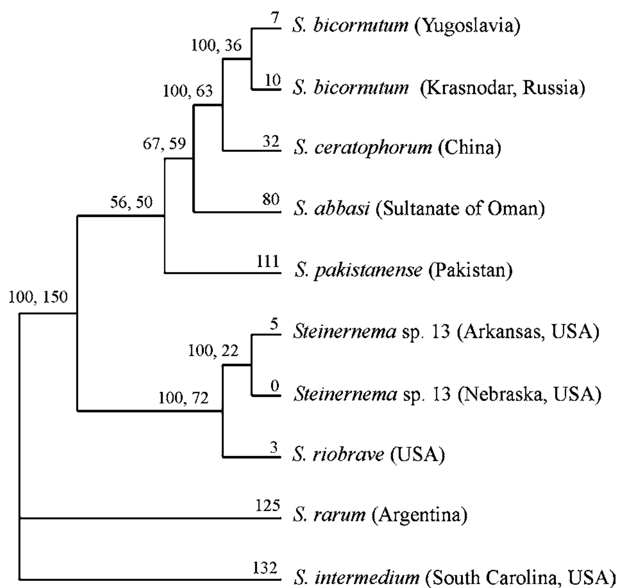

Fig. 7. *Strict consensus of four maximum parsimony trees for Clade IV (*'bicornutum-ceratophorum-riobrave'*) of the genus* Steinernema*. Bootstrap numbers (50% and more) and nucleotide changes are given on appropriate clades.*

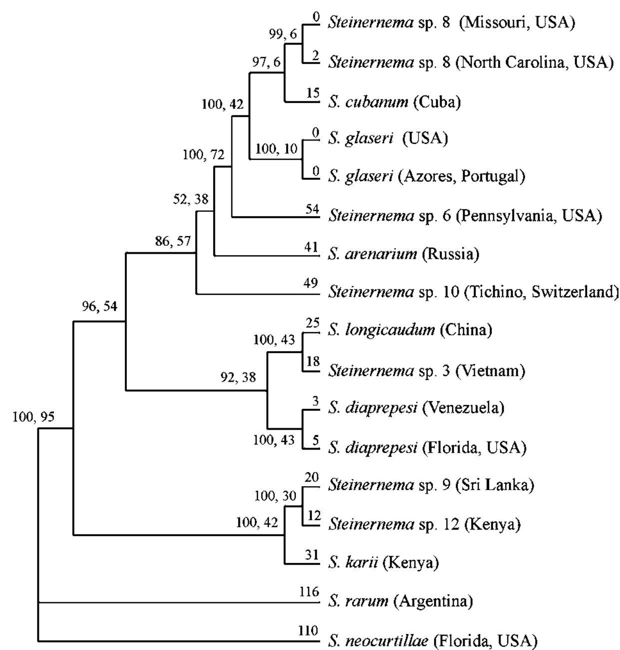

Fig. 8. *Strict consensus of four maximum parsimony trees for Clade V (*'arenarium-glaseri-karii-longicaudum'*) of the genus* Steinernema*. Bootstrap numbers (50% and more) and nucleotide changes are given on appropriate clades.*

Clade IV – '*bicornutum-ceratophorum-riobrave*'. Sequences of eight isolates and two outgroups (*S. rarum* and *S. intermedium*) were used for the analysis. The length of this alignment was 840 bp. The single maximum parsimony tree (Fig. 7) revealed strong bootstrap support for sister relationships of *S. bicornutum* and *S. ceratophorum*, and for *S. riobrave* and *Steinernema* sp. 13.

Clade V – '*arenarium-glaseri-karii-longicaudum*'. Seventeen sequences were aligned for the analysis of this clade (alignment length: 836 bp; outgroups: *S. rarum* and *S. neocurtillae*). A single maximum parsimony tree was obtained (Fig. 8). The bootstrap support for monophyly of the entire clade was strong. The topology of the tree coincided with the one presented in the general analyses, but differed with respect to the relationship between *S. arenarium* and *Steinernema* sp. 10 (Switzerland) and between *S. diaprepesi* and the clade formed by *S. longicaudum* and *Steinernema* sp. 3 (Fig. 8). The sequence of the Azorean population of *S. glaseri* was identical with the topotype isolate from the USA. The 'tropical' clade, comprising *S. karii*, *Steinernema* sp. 9 (Sri Lanka) and *Steinernema* sp. 12 (Kenya), occupied a basal position with strong support.

Phylogenetic relationships between species as inferred from morphological and combined data

Stock *et al.* (2001) included 22 morphological and morphometrical characters with sequence data from the D2-D3 region of the LSU rDNA and concluded that the majority of these were homoplastic. We re-evaluated the characters of their matrix as follows: all highly homoplastic qualitative characters were removed and new states for three qualitative characters were proposed; new ranges (borders between states) were given for the three quantitative features; and three new morphological characters (spermatozoon structure, presence of bacterial vesicle, and coloration of copulatory apparatus) were added.

A light microscopy study revealed lateral horn-like projections on the anterior end of infective juveniles of two isolates of *Steinernema* sp. 13 from USA. These features were not mentioned in the original descriptions of *S. riobrave* (Cabanillas *et al.*, 1994) or *S. abbasi* (Elawad *et al.*, 1997), although their presence was recently reported in both species by Nguyen and Adams (2003). A total of six qualitative and three quantitative features were used to estimate the relationships between the isolates (Table 4).

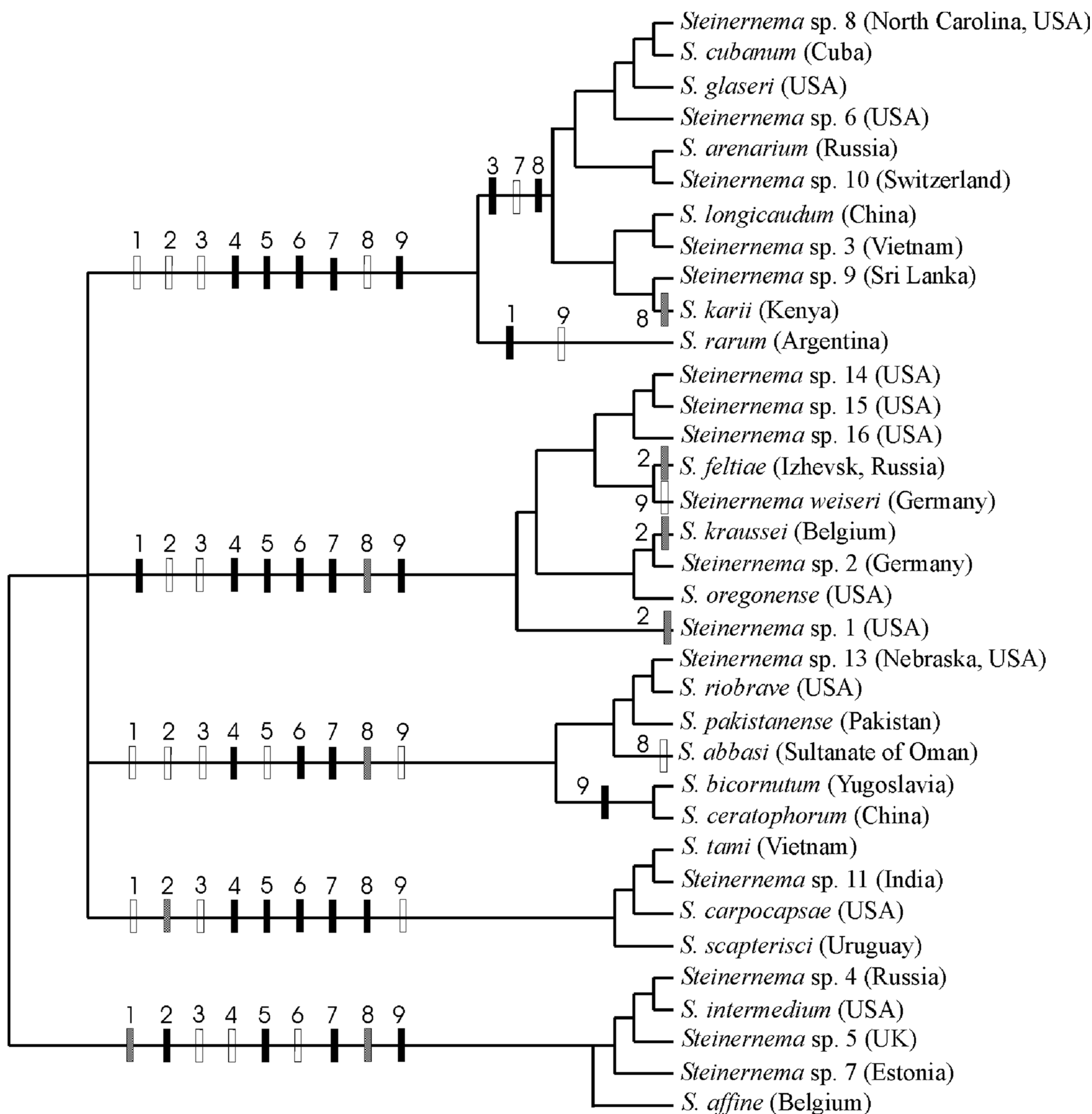

Fig. 9. *Strict consensus of three trees obtained from maximum parsimony analysis of combined morphological and molecular dataset. The morphological character states are indicated on appropriate branches (state '0' – □; state '1' – ■ and state '2' – ▩) and designated with numbers corresponding to Table 4.*

Table 4. *Morphological character states for* Steinernema *species.*

Species/State	1	2	3	4	5	6	7	8	9
Steinernema abbasi	0	0	0	1	0	1	1	0	0
Steinernema affine	2	1	0	0	1	0	1	2	1
Steinernema arenarium	0	0	1	1	1	1	0	1	1
Steinernema bicornutum	0	0	0	1	0	1	1	2	1
Steinernema carpocapsae	0	2	0	1	1	1	1	0	0
Steinernema ceratophorum	0	0	0	1	0	1	1	2	1
Steinernema cubanum	0	0	1	1	1	1	0	1	1
Steinernema feltiae	1	2	0	1	1	1	1	2	1
Steinernema glaseri	0	0	1	1	1	1	0	1	1
Steinernema intermedium	2	1	0	0	1	0	1	2	1
Steinernema karii	0	0	1	1	1	1	0	2	1
Steinernema kraussei	1	2	0	1	1	1	1	2	1
Steinernema longicaudum	0	0	1	1	1	1	0	1	1
Steinernema oregonense	?	?	0	1	1	1	1	2	1
Steinernema pakistanense	?	0	0	1	0	1	1	2	0
Steinernema rarum	1	0	0	1	1	1	1	0	0
Steinernema riobrave	0	0	0	1	0	1	1	2	0
Steinernema scapterisci	0	2	0	1	1	1	1	0	0
Steinernema tami	0	2	0	1	1	1	1	0	0
Steinernema weiseri	1	0	0	1	1	1	1	2	0
Steinernema sp. 1	1	2	0	1	1	1	1	2	1
Steinernema sp. 2	1	0	0	1	1	1	1	2	1
Steinernema sp. 3	0	0	1	1	1	1	0	1	1
Steinernema sp. 4	2	1	0	0	1	0	1	2	1
Steinernema sp. 5	2	1	0	0	1	0	1	2	1
Steinernema sp. 6	0	0	1	1	1	1	0	1	1
Steinernema sp. 7	2	1	0	0	1	0	1	2	1
Steinernema sp. 8	0	0	1	1	1	1	0	1	1
Steinernema sp. 9	0	0	1	1	1	1	0	1	1
Steinernema sp. 10	0	0	1	1	1	1	0	1	1
Steinernema sp. 11	0	2	0	1	1	1	1	0	0
Steinernema sp. 13	0	0	0	1	0	1	1	2	0
Steinernema sp. 14	1	0	0	1	1	1	1	2	1
Steinernema sp. 15	1	0	0	1	1	1	1	2	1
Steinernema sp. 16	1	0	0	1	1	1	1	2	1

[1] Giant amoeboid cells – transformed sperm in female oviducts – (0); Sperm cells in females forming chains, each cell about 5-7 μm – (1); Sperm cells in females forming chains, each cell about 10-12 μm – (2);

[2] Lateral field of infective juveniles with eight or six equal ridges – (0); lateral field of infective juvenile with two central ridges and less prominent marginal or submarginal lines – (1); lateral field of infective juvenile with three or four pronounced ridges in the centre with two prominent marginal and less prominent submarginal ridges – (2).

[3] Bacterial vesicle well defined, with visible walls – (0); no clear walls of bacterial vesicle – (1).

[4] Spicules and gubernaculum nearly transparent or of slight yellowish coloration – (0); spicules and gubernaculum not transparent, well coloured – (1).

[5] Two lateral horns present on both sides of closed mouth in infective juveniles – (0); head end of infective juveniles without lateral horns – (1).

[6] Refractive inclusion present in tail tip of some infective juveniles in population – (0); no refractive inclusion in the juvenile tail tip – (1).

[7] Infective juvenile excretory pore opening at level of posterior half of pharynx (*i.e.*, mean value for D $>$ 55%) – (0); Excretory pore may be situated on either sides of mid-part or at level of anterior half of pharynx – (1).

[8] Average infective juvenile body length not exceeding 600 μm – (0); more than 1000 μm – (1); infective juveniles of intermediate size – 600-1000 μm (2).

[9] Tail of infective juveniles shorter than 61-62 μm – (0); tail of juveniles longer than 61-62 μm – (1).

The partition homogeneity test indicated substantial congruence between morphological and molecular data ($P = 0.6$), and the two datasets were combined. Maximum parsimony analysis generated a single tree (Fig. 9). Inclusion of nine morphological and morphometrical characters did not influence the relationships between the species (data not shown).

Discussion

Molecular and morphological characters in steinernematid species delimitation

Traditionally, species delimitation in steinernematids has been based on morphology and cross-breeding experiments (Hominick *et al.*, 1997). The availability of molecular data presents a new basis for estimating species boundaries in this group. Adams (1998) considered several paradigms for species delimitation in nematology and concluded that the presence of autapomorphy is the main prerequisite for the establishment of a new species. Stock *et al.* (2001) noted the absence of autapomorphies for four species during the analysis of the LSU domain for *Steinernema*. However, these were readily found from the ITS rDNA analysis of some of these species. Our ITS rDNA analysis of a richer set revealed two species (*S. karii* and *S. siamkayai*) lacking autapomorphies in this domain. It can be expected that reported autapomorphies for some well established species may disappear as the number of species studied increases. In this case the search

for autapomorphies as an approach to species delimitation would no longer appear to be a sound procedure.

Alternatively, sequence divergence might be considered as an indication for independent evolutionary history in steinernematids. In our analysis, we revealed different levels of intra-specific sequence divergence in *Steinernema* clades ranging from 0.3-0.6% in Clade I (*S. intermedium*, *S. affine*, *Steinernema* sp. 5) and up to 2.4-2.8% in Clade III (*S. feltiae* and *S. kraussei*). The difference between closely related species was 2.4-2.9% in only two cases and was in excess of 3% for the others.

One of the explanations for different levels of intra-specific sequence divergence is the differences in rate of ITS evolution in steinernematids groups. It is known that higher mutation rates occur in the loops than in stems of rRNA (Dixon & Hillis, 1993) and that differently structured ITS molecules could diverge at different rates. For example, sequence analyses showed that both *S. feltiae* and *S. kraussei*, species with the highest intraspecific divergence in our analysis, have nucleotide positions with high level of substitutions in few loci along the ITS, probably in the loops. Perhaps these species have a similar secondary RNA structure, facilitating the nucleotide changes and thus increasing intra-specific divergence.

Hillis and Dixon (1991) suggested that homogenisation mechanisms tend to lower the level of rDNA polymorphism in a population and the authors hypothesised the existence of 'concerted evolution' as a result of such mechanisms. These mechanisms, however, can be disturbed by intracellular factors, leading to heterogeneity. Heterogeneity was clearly visualised by the multiple peaks during direct sequencing of PCR products of both *S. feltiae* and *S. bicornutum*. High levels of intra-specific sequence divergence were observed in both species. If one assumes that such homogenisation mechanisms would somehow be less active in these steinernematid species, their higher level of sequence divergence could then be explained. However, we did not detect any ITS rDNA heterogeneity for *S. kraussei*, another species with high intraspecific divergence. It is probable that multiple factors may influence the level of intraspecific divergence in steinernematids and we acknowledge that the different levels of intraspecific sequence divergence reported herein may be an artefact caused by the varying number and geographical spread of isolates within different species.

The levels of intra- and inter-specific divergence were analysed together with morphological and ecological data. The results suggested that 27 of the isolates of putative species belonged to 15 new species and that only one isolate, *Steinernema* sp. 11, was probably conspecific with a nominal species, *S. siamkayai*, as a very low level of sequence divergence (1.2%) was observed between the two isolates. Study of the RFLP of this isolate revealed its similarity with an undescribed isolate from Sri Lanka (Hussaini *et al.*, 2001), which was considered conspecific with the recently described *S. asiaticum* (Anis *et al.*, 2002). The proposed differential diagnosis of this species is not decisive and additional studies are needed to elucidate its status and its relationships with *S. siamkayai* and *Steinernema* sp. 11. Our molecular data revealed that some isolates of species that are currently identified, on the basis of their morphological similarity, as being conspecific, can be separated into several species. For example, our analyses suggest that '*S. intermedium*'-like isolates from Europe do not belong to *S. intermedium* (USA, topotype) but to three, as yet undescribed, species (*Steinernema* spp. 4, 5, 7).

PHYLOGENY OF THE GENUS *STEINERNEMA*

Our comprehensive analyses of the phylogeny based on the ITS region provides a new insight into the relationships between groups and species of steinernematids. This study supports the same evolutionary lines previously found by Stock *et al.* (2001) on the basis of the partially sequenced 28S rDNA gene. Five main clades are observed in both analyses. However, relationships between the clades are not clear when using ITS sequences (Nguyen *et al.*, 2001; present study).

Clearly, some of our results differ from previous studies. In most of our trees the basal position of Clade I ('*affine-intermedium*'-group) was evident whereas the analysis of the very conservative 5.8S rDNA by Nguyen *et al.* (2001) 'tenuously but consistently' placed *S. intermedium* in the basal position for the genus. In the phylograms of Stock *et al.* (2001), this basal position is occupied by the group *S. carpocapsae* + *S. scapterisci* + *S. siamkayai* and *S. monticolum*.

A contradiction between our results and published phylograms was also found in the position of *S. monticolum.* In the LSU tree of Stock *et al.* (2001), and in some maximum parsimony trees for ITS by Nguyen *et al.* (2001), this species clustered with *S. carpocapsae* + *S. scapterisci* + *S. siamkayai*. In our phylogenetic trees, *S. monticolum* clustered mainly with Clade II ('*feltiae-kraussei*' group) with weak to strong bootstrap support (Table 3). Based on its morphometric features, such as the infective juvenile body length of 612-821 μm (Stock *et al.*, 1997), this species fits into the group of steinernematids with 'me-

dium sized' juveniles ('*feltiae-kraussei-oregonense*'), but not into the '*carpocapsae-scapterisci-tami*' group with short juveniles.

Another difference between the LSU analysis and our data is the position of two populations identified as *S. longicaudum* by Stock *et al.* (2001). In the LSU tree, the Chinese population of *S.* '*longicaudum*' clustered with *S. cubanum*, whereas the American *S.* '*longicaudum*' population was a sister taxon of *S. karii*. In our analyses, after excluding several species from the alignment (data not shown), the topotype Chinese population of *S. longicaudum* was always related to *S. karii*, whereas *Steinernema* sp. 8 was a sister group for *S. cubanum.* Such a clustering appears to be logical from the biogeographical point of view (Fig. 8). It is possible that the two *S.* '*longicaudum*' isolates studied by Stock *et al.* (2001) may have been mislabelled and that one of the Californian isolates may be identical to our *Steinernema* sp. 8, which was collected from North Carolina and Missouri.

Morphological basis for steinernematid phylogeny

In the analysis by Stock *et al.* (2001), only three characters were not homoplastic, *viz* two autapomorphies in *S. glaseri* and *S. affine* (presence/absence of notch on the spicular lamina and refractive inclusion in tail of infective juveniles, respectively) and one synapomorphy for *S. bicornutum* and *S. ceratophorum* (presence of infective juvenile lateral projection or horn-like structures). In addition, our study showed the highest consistency index (CI = 1.0) for the following morphological features: structure of bacterial vesicles; colour of male copulatory apparatus; and position of the excretory pore. A CI of 0.67 was observed for sperm structure.

Spermatozoon morphology is proposed for the first time in the reconstruction of steinernematid phylogeny. It was previously shown that spermatozoa in *Steinernema* are diverse (Spiridonov *et al.*, 1999). The formation of chains of several steinernematid spermatozoa was reported by Bovien (1937), and a rich diversity of giant amoeboid cells (transformed sperm) was found between *Steinernema* species. Species of Clade I and Clade III are characterised by chain-forming spermatozoa, giant transformed sperm only being reported in representatives of Clades II, IV and V. Features of the sperm may be easily discovered during examination of living females and the entire character set of features related to steinernematid spermatozoa is an attractive addition to the analysis.

The presence of lateral projections or horn-like structures on the cephalic end of the infective juvenile was synapomorphic for Clade IV and the character of slightly yellowish or colourless spicules was synapomorphic for Clade I.

In our matrix, differences in lateral field morphology of infective juveniles were grouped into states similar to, but distinct from, those used by Stock *et al.* (2001). The consistency index was still low (0.4) for this feature, the variability of juvenile lateral field structure in some clades probably being responsible for this. Several clades are characterised by uniform structure of the lateral field: species in Clade I have a lateral field with two ridges in the centre; in Clade II species have four central ridges and pronounced marginal ridges, but weakly developed submarginal ridges; in Clades IV and V species have eight equal ridges (which appear as nine lines with equal spaces between). Different types of lateral field were reported for Clade III: *S. kraussei* and *S. feltiae* are characterised by a lateral field with three or four central ridges plus prominent marginal and weakly developed submarginal ones, whereas some other species of this clade have a lateral field with either eight or six (as in *S. oregonense*) equal ridges (Table 4).

The presence of the bacterial vesicle is a stable characteristic for steinernematid species (Bird & Akhurst, 1983). In our analysis the CI value for this feature equals 1.0. Representatives of Clade I ('*affine-intermedium*' group) have swollen, thin-walled vesicles with a large inner space, and fill the body diameter in mature juveniles. Species of Clade III ('*feltiae-kraussei-oregonense*' group) are characterised by more compact and thick-walled vesicles, which usually do not impact upon the general shape of the intestine. Nevertheless, the latter type of vesicle is not synapomorphic for this clade, as similar, though smaller and usually rounded, bacterial vesicles are common for the species of Clades II and IV. Similar vesicles were observed in *S. rarum*. A true vesicle is probably absent in the species composing Clade V. Further research is needed to validate the use of detailed vesicle morphology in the phylogenetic analysis of *Steinernema*.

Ecological and geographical patterns in *Steinernema* phylogeny

Recently, general patterns of natural steinernematid distribution were discussed by Hominick (2002) who reviewed the available, often contradictory, information. The distribution study of *Steinernema* by Sturhan (1999), which was based on several hundred soil samples col-

lected throughout Germany, revealed that some species have habitat preferences (*e.g.*, *S. affine* is a species characteristic of grasslands and arable soil). The habitat preferences of steinernematids are presumably largely determined by the distribution of their host insects (Peters, 1996) and other factors, including soil type and temperature. As insects correspond in their distribution to plant associations, habitat preference of steinernematids may also be expected. Some cases of congruence between ecological features and the genetic relationships of steinernematids became obvious during mapping of our data. Species of Clade I are divided into several groups each with characteristic habitat preferences (Fig. 4): *S. affine* is a species of open landscapes, *viz* grassland or cultivated soil, whereas *S. intermedium* (USA) and *Steinernema* sp. 5 (Europe) appear to be inhabitants of woodlands (Spiridonov & Moens, 1999). This result contradicts that of Kramer *et al.* (2000), who reported that *S. intermedium* is a common inhabitant of grasslands in Swiss lowlands. The identification of this species, however, was based on morphological characters only, and the conspecific status of this Swiss isolate with the North American *S. intermedium* was not evident. Sequence analysis of one of these Swiss isolates (CH 221) revealed its similarity to *Steinernema* sp. 7 from Estonia. This latter isolate was only found in grasslands of Southern Estonia, whereas *Steinernema* sp. 5 (isolate E6) was common under the canopy of deciduous and mixed forests in the same area.

Habitat preference patterns were also observed in Clade III (Fig. 6). All of our *S. feltiae* isolates originated from grasslands or cultivated soil, though Sturhan (1999) also reported it from woodland. *Steinernema kraussei* isolates in our collection were mainly obtained from woodlands. However, *S. kraussei* was also reported from alpine grasslands in Switzerland (Steiner, 1996) and Scotland (Gwynn & Richardson, 1996), and from alpine meadows in Bulgaria (Shishinova *et al.*, 2000). The Iceland isolate of *S. kraussei* was obtained from a swampy, treeless, area in a volcanic valley. The distribution of *S. kraussei* in woodland habitats is almost a rule in lowland parts of Europe, although this species can also be commonly found outside woodlands at high altitudes and latitudes.

An attempt was recently made to analyse the geographical distribution of steinernematids (Hominick, 2002). Any such estimation based on morphological identification only might be risky because of the difficulties in accurately identifying *Steinernema* species. Kerry and Hominick (2002) indicated that accurate identification is a key factor in understanding of geographical distribution and biodiversity of steinernematids.

Several geographical patterns of steinernematids can be observed from the data presented here. Some species are found throughout the temperate belt of the northern hemisphere. Hence, *S. feltiae* from the USA (isolate 88) is identical in its ITS sequence to the topotype isolate from Europe. However, this must be interpreted with the caveat that the distribution could be the result of introduction events.

Steinernematids quite often form groups of closely related isolates with a specific geographical area of distribution. Separate clades for North American and Eurasian species are obvious in Clades I, III and IV, with some level of independence of subtropical and tropical species from those inhabiting temperate regions in Clades II and V.

Conclusions

The analysed set of sequence data from the ITS rDNA region of steinernematids provided new information concerning the composition of five main clades in the genus *Steinernema* and the phylogenetic relationships inside these main steinernematid evolutionary lineages. ITS rDNA data were confirmed to be of little value for resolving relationships between clades, something previously suggested by Nguyen *et al.* (2001). The level of intraspecific variability differs between clades, reaching 2.5% in *S. feltiae*. These different levels of variability should be taken into account when delimitating new steinernematid species. The morphology of steinernematids adds little data for the reconstruction of their phylogeny, but can support decisions about species independence when the observed differences in ITS rDNA nucleotides are close to the intra-specific level (*e.g.*, *Steinernema weiseri* and *Steinernema* sp. 2 *vs S. feltiae* and *S. kraussei*, respectively). Although original data about habitat preferences of steinernematids are sometimes contradictory – and this phenomenon can be latitude- or altitude-dependent – the data suggest some preferences for specific ecosystems between species of Clades I and III. Biogeographical patterns are apparent in the phylogeny of some clades.

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References

ADAMS, B.J. (1998). Species concept and the evolutionary paradigm in modern nematology. *Journal of Nematology* 30, 1-21.

AKHURST, R.J. & BEDDING, R.A. (1978). A simple cross-breeding technique to facilitate species determination in the genus *Neoaplectana*. *Nematologica* 24, 328-330.

AMARASINGHE, L.D., HOMINICK, W.M., BRISCOE, B.R. & REID, A.P. (1994). Occurrence and distribution of entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae) in Sri Lanka. *Journal of Helminthology* 68, 77-86.

ANIS, M., SHAHINA, F., REID, A.P. & ROWE, J. (2002). *Steinernema asiaticum* sp. n. (Rhabditida: Steinernematidae) from Pakistan. *International Journal of Nematology* 12, 220-231.

ARTYUKHOVSKY, A.K., KOZODOI, E.M., REID, A.P. & SPIRIDONOV, S.E. (1997). Redescription of *Steinernema arenarium* (Artyukhovsky, 1967) topotypes from Central Russia and a proposal for *S. anomalae* (Kozodoi, 1984) as a junior synonym. *Russian Journal of Nematology* 5, 31-37.

BEDDING, R.A. & AKHURST, R.J. (1975). A simple technique for the detection of insect parasitic rhabditid nematodes in soil. *Nematologica* 21, 109-110.

BIRD, A.F. & AKHURST, R.J. (1983). Bacterial vesicle in the nematodes of the genus *Steinernema* (Rhabditida, Steinernematidae). *International Journal of Parasitology* 13, 599-606.

BLAXTER, M.L., DE LEY, P., GAREY, J.R., LIU, L.X., SCHELDEMANN, P., VIERSTRAETE, A., VANFLETTEREN, J.R., MACKEY, L.Y., DORRIS, M., FRISSE, L.M., VIDA, J.T. & THOMAS, W.K. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* 392, 71-75.

BOVIEN, P. (1937). Some types of association between nematodes and insects. *Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening, Kobenhavn* 101, 1-114.

CABANILLAS, H.E., POINAR JR, G.O. & RAULSTON, J.R. (1994). *Steinernema riobravis* n. sp. (Rhabditida: Steinernematidae) from Texas. *Fundamental and Applied Nematology* 17, 123-131.

DIXON, M.T. & HILLIS, D.M. (1993). Ribosomal RNA secondary structure: compensatory mutations and implications for phylogenetic analysis. *Molecular Biology and Evolution* 10, 256-267.

ELAWAD, S., AHMAD, W. & REID, A.P. (1997). *Steinernema abbasi* sp. n. (Nematoda, Steinernematidae), from the Sultanate of Oman. *Fundamental and Applied Nematology* 20, 433-442.

FELSENSTEIN, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783-791.

GWYNN, R.L. & RICHARDSON, P.N. (1996). Incidence of entomopathogenic nematodes in soil samples collected from Scotland, England and Wales. *Fundamental and Applied Nematology* 19, 427-431.

HILLIS, D.M. & DIXON, M.T. (1991). Ribosomal DNA: molecular evolution and phylogenetic inference. *The Quarterly Review of Biology* 66, 411-428.

HILLIS, D.M. & HUELSENBECK, J.P. (1992). Signal, noise, and reliability in molecular phylogenetic analyses. *Journal of Heredity* 83, 189-195.

HOMINICK, W.M. (2002). Biogeography. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 115-144.

HOMINICK, W.M. & BRISCOE, B.R. (1990). Occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in British soils. *Parasitology* 100, 295-302.

HOMINICK, W.M., BRISCOE, B.R., DEL PINO, F.G., JIAN HENG, HUNT, D.J., KOZODOY, E., MRÁČEK, Z., NGUYEN, K.B., REID, A.P., SPIRIDONOV, S.E., STOCK, P., STURHAN, D., WATURU, C. & YOSHIDA, M. (1997). Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. *Journal of Helminthology* 71, 271-298.

HUSSAINI, S.S., ANSARI, M.A., AHMAD, W. & SUBBOTIN, S.A. (2001). Identification of some Indian populations of *Steinernema* species (Nematoda) by RFLP analysis of the ITS region of rDNA. *International Journal of Nematology* 11, 73-76.

JOYCE, S.A., REID, A., DRIVER, F. & CURRAN, J. (1994). Application of polymerase chain reaction (PCR) methods to the identification of entomopathogenic nematodes. In: Burnell, A.M., Ehlers, R.-U. & Masson, J.-P. (Eds). *COST 812 Biotechnology: Genetics of entomopathogenic nematodes-bacterium complexes. Proceedings of symposium and workshop, St Patrick's College, Maynooth, County Kildare, Ireland.* Luxembourg, European Commission, DGXII, pp. 178-187.

KERRY, B.R. & HOMINICK, W.M. (2002). Biological control. In: Lee, D.L. (Ed.). *The biology of nematodes*, London & New York, Taylor & Francis, pp. 483-510.

KOZODOI, E.M. & SPIRIDONOV, S.E. (1988). Cuticular ridges on lateral fields of larvae of *Neoaplectana*. *Folia Parasitologica* 35, 359-362.

KRAMER, I., HIRSCHY, O. & GRUNDER, J.M. (2000). Survey of baited insect parasitic nematodes from Swiss lowland. In: Griffin, C.T., Burnell, A.M., Downes, M.J. & Mulder, R. (Eds). *COST 819; Developments in entomopathogenic nematode/bacterial research.* Luxembourg, European Commission, DGXII, pp. 172-176.

LIU, J. & BERRY, R.E. (1996). Phylogenetic analysis of the genus *Steinernema* by morphological characters and randomly amplified polymorphic DNA fragments. *Fundamental and Applied Nematology* 19, 463-469.

LIU, J., BERRY, R.E. & MOLDENKE, A.F. (1997). Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) inferred from partial 18S rRNA gene sequences. *Journal of Invertebrate Pathology* 69, 246-252.

MIDUTURI, J., WAEYENBERGE, L. & MOENS, M. (1997). Natural distribution of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) in Belgian soils. *Russian Journal of Nematology* 5, 155-165.

MRÁČEK, Z., STURHAN, D. & REID, A. (2003). *Steinernema weiseri* n. sp. (Rhabditida, Steinernematidae), a new entomopathogenic nematode from Europe. *Systematic Parasitology* 56, 37-47.

NGUYEN, K.B. & ADAMS, B.J. (2003). SEM and systematic studies on *Steinernema abbasi* Elawad *et al.*, 1997, and *Steinernema riobrave* Cabanillas *et al.*, 1994. *Zootaxa* 179, 1-10.

NGUYEN, K.B. & DUNCAN, L.W. (2002). *Steinernema diaprepesi* n. sp. (Rhabditida: Steinernematidae), a parasite of the citrus root weevil *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae). *Journal of Nematology* 34, 159-170.

NGUYEN, K.B. & SMART, G.C. JR (1996). Identification of entomopathogenic nematodes in the Steinernematidae and Heterorhabditidae (Nemata: Rhabditida). *Journal of Nematology* 28, 286-300.

NGUYEN, K.B., MARUNIAK, J. & ADAMS, B.J. (2001). Diagnostic and phylogenetic utility of the rDNA internal transcribed spacer sequences of *Steinernema*. *Journal of Nematology* 33, 73-82.

PAGE, R.D.M. (1996). TREEVIEW: an application to view phylogenetic trees on personal computer. *CABIOS* 12, 357-358.

PETERS, A. (1996). The natural host range of *Steinernema* and *Heterorhabditis* spp. and their impact on insect population. *Biocontrol Science and Technology* 6, 389-402.

PHAM, V.L., NGUYEN, B.K., REID, A.P. & SPIRIDONOV, S.E. (2000). *Steinernema tami* sp. n. (Rhabditida, Steinernematidae) from Cat Tien forest, Vietnam. *Russian Journal of Nematology* 8, 33-43.

PHAN, K.L., NGUYEN, N.C. & MOENS, M. (2001a). *Steinernema sangi* sp. n. (Rhabditida, Steinernematidae) from Vietnam. *Russian Journal of Nematology* 9, 1-17.

PHAN, K.L., NGUYEN, N.C. & MOENS, M. (2001b). *Steinernema loci* sp. n. and *Steinernema thanhi* sp. n. (Rhabditida: Steinernematidae) from Vietnam. *Nematology* 3, 503-514.

POINAR JR, G.O. (1993). Origins and phylogenetic relationships of the entomophilic rhabditids *Heterorhabditis* and *Steinernema*. *Fundamental and Applied Nematology* 16, 333-338.

REID, A.P. (1994). Molecular taxonomy of *Steinernema*. In: Burnell, A.M., Ehlers, R.-U. & Masson, J.-P. (Eds). *COST 812 Biotechnology: Genetics of entomopathogenic nematodes-bacterium complexes. Proceedings of symposium and workshop, St. Patrick's College, Maynooth, County Kildare, Ireland.* Luxembourg, European Commission, DG XII, pp. 49-58.

REID, A.P. & HOMINICK, W.M. (1992). Restriction fragment length polymorphisms within the ribosomal DNA repeat unit of British entomopathogenic nematodes (Rhabditida: Steinernematidae). *Parasitology* 105, 317-323.

REID, A.P., HOMINICK, W.M. & BRISCOE, B.R. (1997). Molecular taxonomy and phylogeny of entomopathogenic nematode species (Rhabditida: Steinernematidae) by RFLP analysis of the ITS region of the ribosomal DNA repeat unit. *Systematic Parasitology* 37, 187-193.

SHAHINA, F., ANIS, M., REID, A.P., ROWE, J. & MAQBOOL, M.A. (2001). *Steinernema pakistanense* sp. n. (Rhabditida: Steinernematidae) from Pakistan. *International Journal of Nematology* 11, 124-133.

SHISHINOVA, M., BUDUROVA, L. & GRADINAROV, D. (2000). Entomopathogenic nematodes from Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in Bulgaria. *IOBC Bulletin* 23, 75-78.

SPIRIDONOV, S.E. & BELOSTOTSKAYA, F.N. (1983). [New data about morphology and biology of the nematodes of the genus *Neoaplectana* and their position in the system of rhabditids.] *Parasitologyia* 17, 119-125.

SPIRIDONOV, S.E. & MOENS, M. (1999). Two previously unreported species of steinernematids from woodlands in Belgium. *Russian Journal of Nematology* 7, 39-42.

SPIRIDONOV, S.E., HOMINICK, W.M. & BRISCOE, B.R. (1999). Morphology of amoeboid cells in the uterus of *Steinernema* species (Rhabditida: Steinernematidae). *Russian Journal of Nematology* 7, 49-56.

STEINER, W.A. (1996). Distribution of entomopathogenic nematodes in the Swiss Alps. *Revue Suisse de Zoologie* 103, 439-452.

STOCK, S.P., CHOO, H.Y. & KAYA, H.K. (1997). *Steinernema monticolum* sp. n. (Rhabditida: Steinernematidae), an entomopathogenic nematode from Korea with a key to other species. *Nematologica* 43, 15-29.

STOCK, S.P., SOMSOOK, V. & REID, A.P. (1998). *Steinernema siamkayai* n. sp. (Rhabditida, Steinernematidae) an entomopathogenic nematode from Thailand. *Systematic Parasitology* 41, 105-113.

STOCK, S.P., CAMPBELL, J.F. & NADLER, S.A. (2001). Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology* 87, 877-889.

STURHAN, D. (1999). Prevalence and habitat specificity of entomopathogenic nematodes in Germany. In: Gwynn, R.L., Smith, P.H., Griffin, C., Ehlers, R.-U., Boemare, N. & Mas-

son, J.-P. (Eds). *COST 819, Entomopathogenic nematodes: application and persistence of entomopathogenic nematodes.* Luxembourg, European Commission, DG XII, pp. 123-132.

SWOFFORD, D.L. (1998). PAUP*. *Phylogenetic analysis using parsimony. Version 4*. Sunderland, MA, USA, Sinauer.

SZALANSKI, A.P., TAYLOR, D.B. & MULLIN, P.G. (2000). Assessing nuclear and mitochondrial DNA sequence variation within *Steinernema* (Rhabditida: Steinernematidae). *Journal of Nematology* 32, 229-233.

THOMPSON, J.D., HIGGINS, D.G. & GIBSON, T.J. (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment though sequence weighting, position gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673-4680.

VRAIN, T.C., WAKARCHUK, D.A., LEVESQUE, A.C. & HAMILTON, R.J. (1992). Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563-573.

WATURU, C.N., HUNT, D.J. & REID, A.P. (1997). *Steinernema karii* sp. n. (Nematoda, Steinernematidae), a new entomopathogenic nematode from Kenya. *International Journal of Nematology* 7, 68-75.