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Hirschmanniella kwazuna sp. n. from South Africa with notes on a new record of *H. spinicaudata* (Schuurmans Stekhoven, 1944) Luc & Goodey, 1964 (Nematoda: Pratylenchidae) and on the molecular phylogeny of *Hirschmanniella* Luc & Goodey, 1964

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Summary – A new species of the genus *Hirschmanniella*, *H. kwazuna* sp. n., is described from unidentified grass growing in undisturbed veldt from South Africa. *Hirschmanniella kwazuna* sp. n. is characterised by having a very irregular heat-relaxed body posture, body 1522-2049 μm long, lip region low and rounded with four or five lip annuli, stylet 18-22.5 μm long, lateral field areolated along entire body, spermatheca filled with sperm, tail with 62-81 ventral annuli narrowing to a tip bearing a ventral mucro, angular crystal-like inclusions within body cavity in most of the specimens and phasmid situated 12-24 annuli or 15-26 μm anterior to tail tip. Males, like females, have crystal-like inclusions with the tail curved strongly dorsad in most specimens. Juveniles are similar to females. Molecular sequence analysis using the D2-D3 expansion segments of 28S, partial 18S and ITS rRNA sequences distinguished *H. kwazuna* sp. n. from *H. loofi* and other species of the genus. *Hirschmanniella spinicaudata* is reported from South Africa for the first time and described. Phylogenetic analyses based on analysis of the D2-D3, 18S and ITS rRNA genes are given for eight, ten and five valid and unidentified *Hirschmanniella* species, respectively.

Keywords – 18S rRNA, 28S rRNA, description, ITS rRNA, morphology, morphometrics, new species, PCR-RFLP, SEM, taxonomy.

During a study of the KwaZulu-Natal Midlands, plant-parasitic nematodes from wetlands were collected to identify those species most likely to be associated with different wetland conditions. In a few of the sites, nematodes belonging to the genus *Hirschmanniella* Luc & Goodey, 1964 were found. Most of the specimens belonged to a new species while a few were identified as *H. spinicaudata* (Schuurmans Stekhoven, 1944) Luc & Goodey, 1964. Only a few unidentified juveniles of *Hirschmanniella* have previously been reported in South Africa (Marais & Swart, 1998; Marais *et al*., 2004) and one unidentified female was collected in a sugarcane field in 1994 (M. Marais, pers. comm.).

Hirschmanniella species are well adapted to an aquatic environment and parasitise paddy rice and a number of aquatic plants. The genus presently contains 35 valid species (Siddiqi, 2000; Tandingan De Ley *et al.*, 2007). It was originally proposed by Luc and Goodey (1962) for a group of *Radopholus* Thorne, 1949 species with several distinguishing characters: hemispherical and not offset lip region, elongated pharyngeal glands overlapping the intestine ventrally, mucronate tail tip and long body. Fotedar

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and Handoo (1978) erected a new subfamily Hirschmanniellinae within Pratylenchidae for the genera *Hirschmanniella*, *Pratylenchoides* Winslow, 1958, *Zygotylenchus* Siddiqi, 1963 and *Apratylenchoides* Sher, 1973. Siddiqi (2000) considered only *Hirschmanniella* within this subfamily. Using morphological datasets, the phylogenetic relationships of *Hirschmanniella* with other genera were discussed by Luc (1987) and Ryss (1988) who suggested that *Hirschmanniella* might be related to *Pratylenchus* Filipjev, 1936 and *Zygotylenchus* based on the structure of the lip region and pharynx. Molecular phylogenies reconstructed using D2-D3 expansion segments of 28S rRNA gene sequences (Subbotin *et al.*, 2006; Tandingan De Ley *et al*., 2007) and 18S rRNA gene sequences (Holterman *et al.*, 2006; Tandingan De Ley *et al*., 2007) supported a close relationship of *Hirschmanniella* with *Pratylenchus* and *Meloidogyne* Göldi, 1887. However, relationships between these genera still remain poorly resolved. Ribosomal RNA genes have been used for molecular characterisation of *Hirschmanniella* spp. by several research teams. Chen *et al.* (2006) provided ITS-rRNA sequences for *H. oryzae* (van Breda de Haan, 1902) Luc & Goodey, 1964 and *H. mucronata* (Das, 1960) Luc & Goodey, 1964 whilst Tandingan De Ley *et al.* (2007) molecularly characterised four species of *Hirschmanniella* from the USA and presented molecular phylogeny of the genus using four D2-D3 and seven 18S rRNA sequences. In this paper, we provide molecular characterisation of the new species from South Africa together with some unidentified *Hirschmanniella* sp. from Europe using three rRNA gene fragments and reconstructed molecular phylogenetic relationships between species of the genus based on rRNA gene sequences.

Materials and methods

Nematode populations

Soil samples from wetland sites were collected using a bucket auger and the specimens were extracted from the soil using the elutriation technique of Seinhorst (1962). Specimens of *H. loofi* Sher, 1968 and unidentified *Hirschmanniella* spp. were kindly provided for molecular study by Drs W. Bert (Belgium) and D. Sturhan (Germany), respectively. Several *Hirschmanniella* species sequences (Chen *et al.*, 2006; Holterman *et al.*, 2006; Subbotin *et al.*, 2006; Tandingan De Ley *et al*., 2007) were obtained from the GenBank database.

Light microscope and SEM study

Nematodes were killed in FPG (formalin-propionic acid) (Netscher & Seinhorst, 1969) and mounted in pure glycerin using the slow method described by Hooper and Evans (1993). For scanning electron microscopy part of the material was transferred to TAF (triethanolamine formalin), then dehydrated in increasing concentrations of amyl acetate in pure alcohol with a final step in pure amyl acetate. Following conventional critical point drying and gold/palladium coating (15 nm), specimens were viewed with a Philips XL30 DX41 stereoscan electron microscope at 10 kV.

DNA extraction, PCR, cloning, sequencing and PCR-RFLP

For DNA extraction, several specimens from each sample were put into a drop of water on a glass slide and cut under a binocular microscope. Nematode fragments were transferred into Eppendorf tubes containing 16 μl ddH_2O, 2 μl 10× PCR buffer and 2 μl Proteinase K (600 μg ml^{-1}) (Promega, Madison, WI, USA). The tubes were incubated at 65°C (1 h) and then at 95°C (15 min). Detailed protocols for PCR, cloning and automated sequencing are as described by Tanha Maafi *et al*. (2003). The following primers sets were used for amplification and sequencing of ribosomal RNA gene fragments: *i*) forward D2A (5′-CAAGTACCGTGAGGGAAAGTTG-3′) and reverse D3B (5′-TCGGAAGGAACCAGCTACTA-3′) for the D2-D3 of 28S rRNA gene (Nunn, 1992); *ii*) forward TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and reverse AB28 (5′-ATATGCTTAAGTTCAGCGG GT-3′) for the ITS1-5.8S-ITS2 (Curran *et al*., 1994); and *iii*) forward G18SU (5′-GCTTGTCTCAAAGATTAAG CC-3′) (Dorris & Blaxter, 2000) and reverse R18Tyl1 (5′-GGTCCAAGAATTTCACCTCTC-3′) for partial 18S rRNA gene (Chizhov *et al*., 2006). Two clones for each gene fragment were sequenced. The newly obtained sequences were submitted to the GenBank database under the following numbers: EU620460-EU620463 for partial 18S rRNA gene, EU620464-EU620469 for the D2-D3 of 28S gene, and EU620470-EU620475 for the ITS rRNA.

The PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen, Crawley, UK). Three μl of purified product of the ITS of the new *Hirschmanniella* species was digested by one of following restriction enzymes: *Bsh*1236I, *Hin*fI, *Pst*I or *Rsa*I in the buffer stipulated by the manufacturer. The digested DNA was run on a 1% agarose and 0.7% Synergel®

TAE-buffered gel, stained with ethidium bromide, visualised using UV transilluminator and photographed. The exact lengths of each restriction fragment from the PCR products were obtained by a virtual digestion of the sequences using WebCutter 2.0 (available online at www.firstmarket.com/cutter/cut2.html).

Molecular phylogenetic analysis

Sequences for each rRNA gene fragment were aligned separately with outgroup taxa (Holterman *et al.*, 2006; Subbotin *et al.*, 2006) using ClustalX 1.83 with default parameters for gap opening and gap extension penalties. Sequence alignments were analysed using maximum parsimony (MP) and maximum likelihood (ML) with PAUP* 4b10 (Swofford, 2003). We used heuristic search setting with ten replicates of random taxon addition, tree bisection-reconnection branch swapping to seek the most parsimonious trees. Gaps were treated as missing data. Robustness of the clades was assessed using bootstrap analysis yielding a bootstrap percentage (BP) for each node estimated from 1000 replicates. The models for nucleotide substitutions for ML analysis were selected individually for each ribosomal gene using the program MrModeltest 2.2 (Nylander, 2002) with the Akaike Information Criterion (AIC) in conjunction with PAUP*. Bootstrap (BS) analysis for ML was done using 100 pseudo-replicates with tree searches in each replication performed using one random-sequence-addition without branch swapping.

Descriptions

Hirschmanniella kwazuna* **sp. n.**
(Figs 1-5)

Measurements

See Table 1.

Female

Body slender. All heat relaxed specimens with body forms ranging from slightly arcuate ventrally (rare) to highly irregular in most specimens. Lip region low, rounded, not set off, with four or five annuli, indistinct in some specimens. SEM photographs showing no longitudinal lines on lip annuli. Labial disc oblong, slightly raised above labial plate laterally and fused with labial plate dorsally and ventrally. Amphidial openings forming two slits on lateral sides of labial disc. Cephalic framework moderately sclerotised, extending posteriorly for two or three annuli from basal plate. Stylet short, well developed, knobs rounded posteriorly, varying from sloping to hollow anteriorly with outer tips well directed anteriorly in some specimens. Anterior and posterior cephalids situated 3-4 and 12-16 annuli posterior to base of lip region. Hemizonid seen in only a few individuals, two or three annuli long, located from one annulus anterior to one annulus posterior to excretory pore. Hemizonion not seen in any specimens. Excretory pore position highly variable, ranging from just anterior to median bulb to opposite anterior part of pharyngeal lobe, anterior to junction of pharyngeal lumen with intestine. Pharyngeal glands slender, overlapping on ventral side. Beginning of lateral field near lip very indistinct, but apparently starting *ca* a body diam. posterior to stylet knobs, comprising three bands (*i.e.*, four lines) areolated along entire length of body, more distinct in anterior and posterior region under light microscope whereas SEM photographs showing all bands areolated over entire length of body with anastomoses and broken striae in middle band. Spermatheca round to oblong, large, filled with large, rounded sperm cells; anterior spermatheca frequently smaller than posterior spermatheca. Small, round to oval vaginal glands present. Tail with 62-81 ventral annuli, tapering to a narrow terminus, more rounded dorsally with a ventral mucro with a knob-like tip. Intestine not, or only slightly, overlapping rectum. Vacuoles observed more clearly in some specimens. In most specimens, crystal-like inclusions were noticed, in some specimens more in anterior region of body, in some more anterior to anus and in some seen throughout entire length of body. Crystals are of different shapes and sizes but mostly angular. Phasmids situated 12-24 annuli anterior to tail tip but not exactly opposite each other.

Male

Similar to female, including crystal-like inclusions. Spicules well developed, arcuate ventrad. Gubernaculum simple with variously hooked or curved distal end, not protruding. Tail with 68-82 ventral annuli, strongly curved dorsad in most specimens with a ventral mucro. Phasmids situated 17-32 annuli anterior to tail tip.

Juveniles (? J3 and ? J4)

Similar to female in all aspects including presence of crystal-like inclusions. Tail with a ventral mucro as seen

* The species name is derived from the Province of KwaZulu-Natal, the area in which the specimens were found.

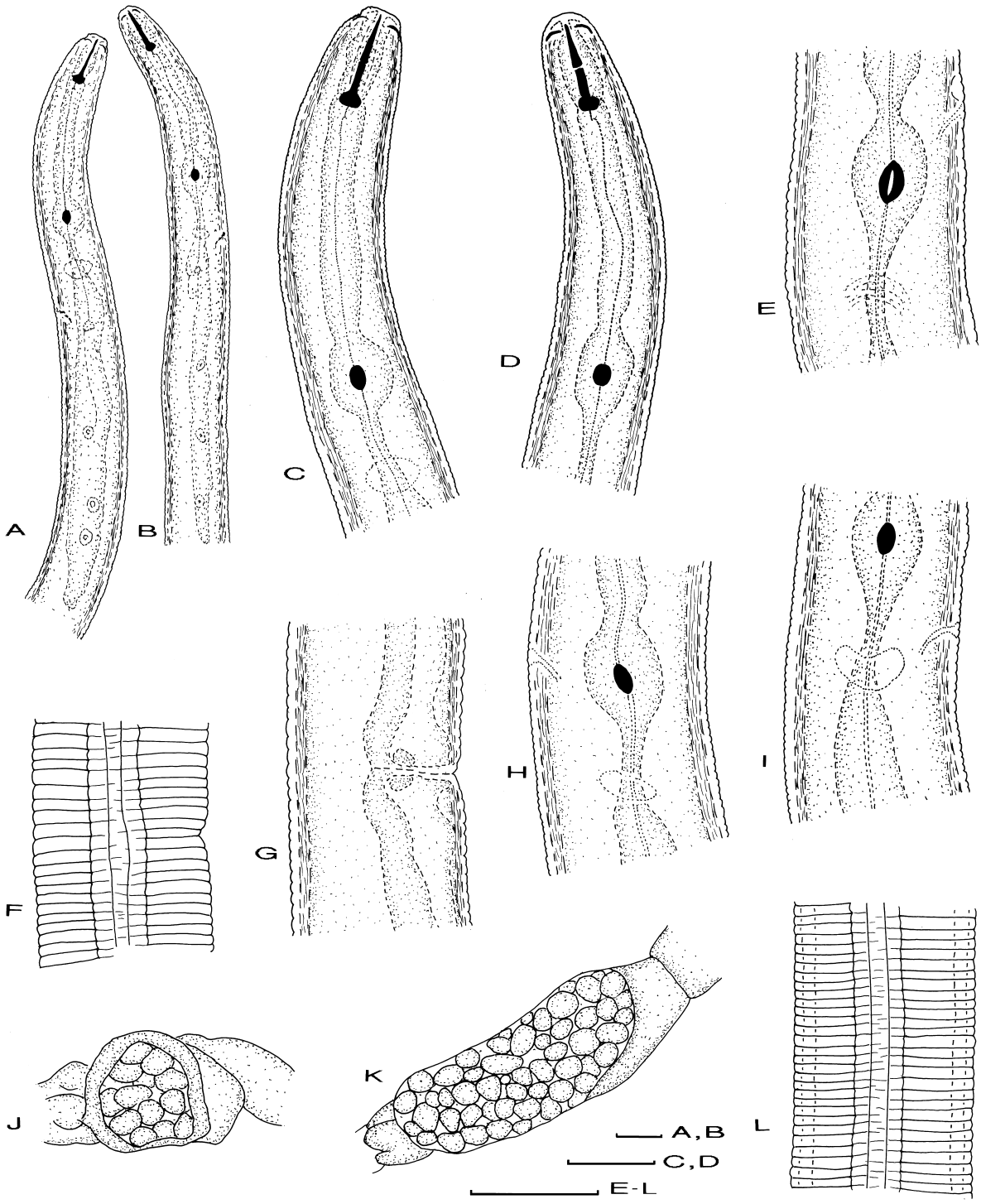

Fig. 1. Hirschmanniella kwazuna *sp. n. A, C: Holotype female anterior region; B, D: Male anterior region; E: Female median bulb region showing anterior position of excretory pore; F: Holotype female lateral field at mid-body; G: Holotype female vulva at mid-body; H, I: Male median bulb region showing position of excretory pore; J: Female anterior spermatheca; K: Female posterior spermatheca; L: Male lateral field at mid-body. (Scale bars = 20 μm.)*

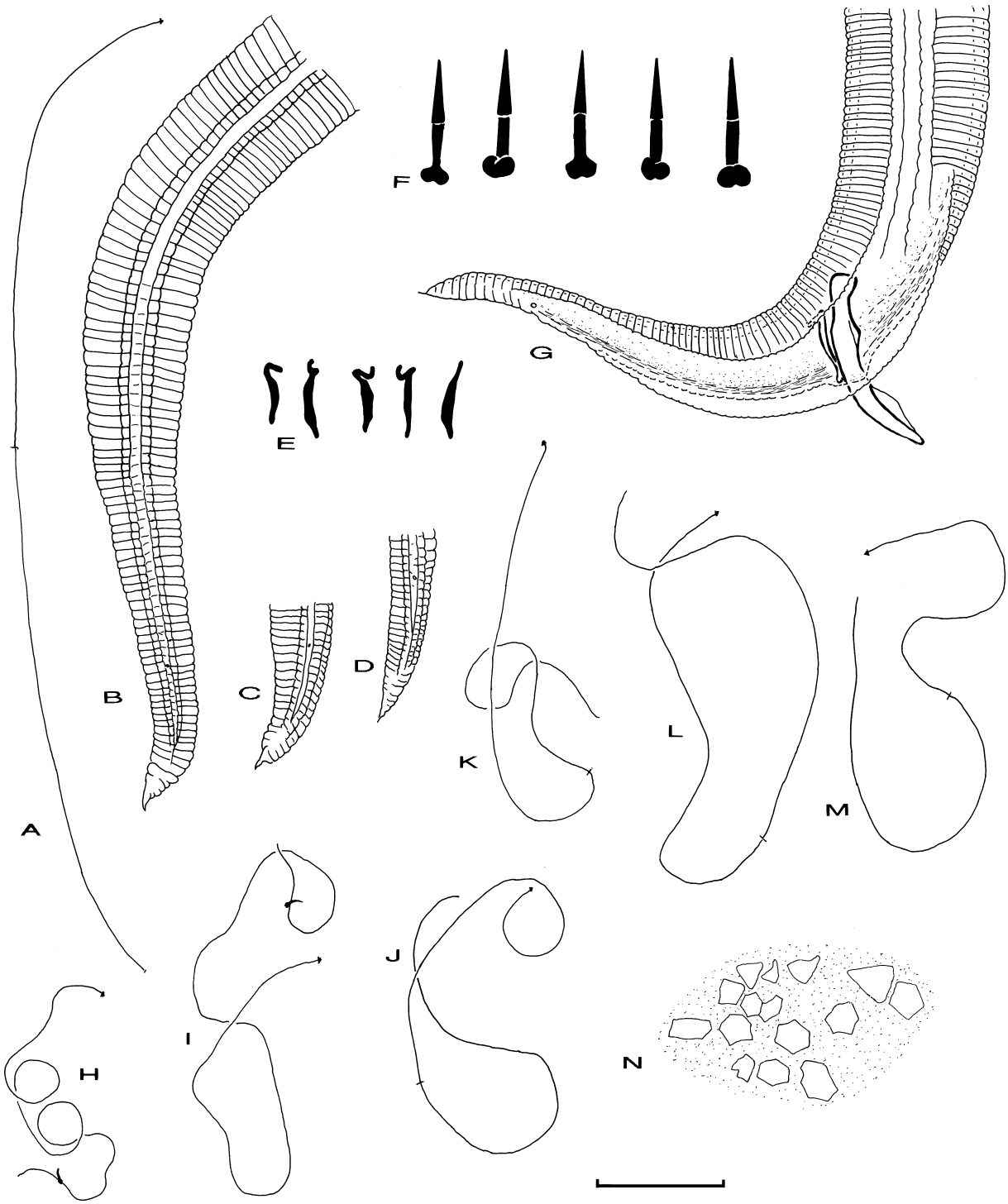

Fig. 2. Hirschmanniella kwazuna *sp. n. A, J-M: Body posture of heat-relaxed females; B: Holotype female, posterior region; C, D: Variation in female tail tip; E: Variation in gubernaculum distal end; F: Variation in female stylet knobs; G: Male posterior region; H, I: Body posture of heat-relaxed males; N: Variation in form and size of crystal-like inclusions in body. (Scale bar: A, H-M = 200 μm; B-G, N = 20 μm.)*

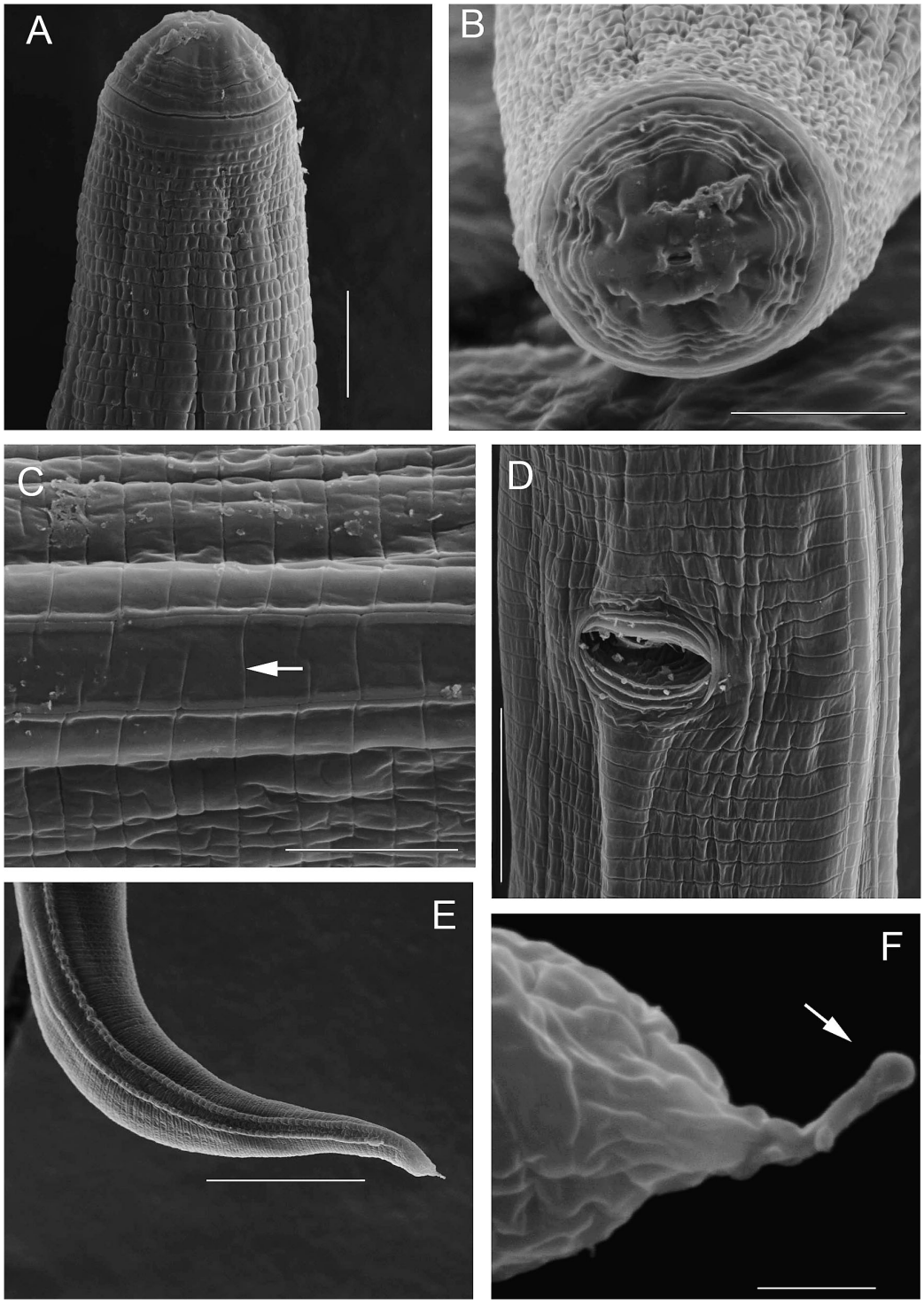

Fig. 3. Hirschmanniella kwazuna *sp. n. Female, SEM. A: Anterior region, lateral view; B: Lip region,* en face *view; C: Lateral field at mid-body with areolation (arrow); D: Vulva, ventral view; E: Tail region; F: Mucro on tail tip (arrow). (Scale bars: A-C = 5 μm; D = 10 μm; E = 20 μm; F = 1 μm.)*

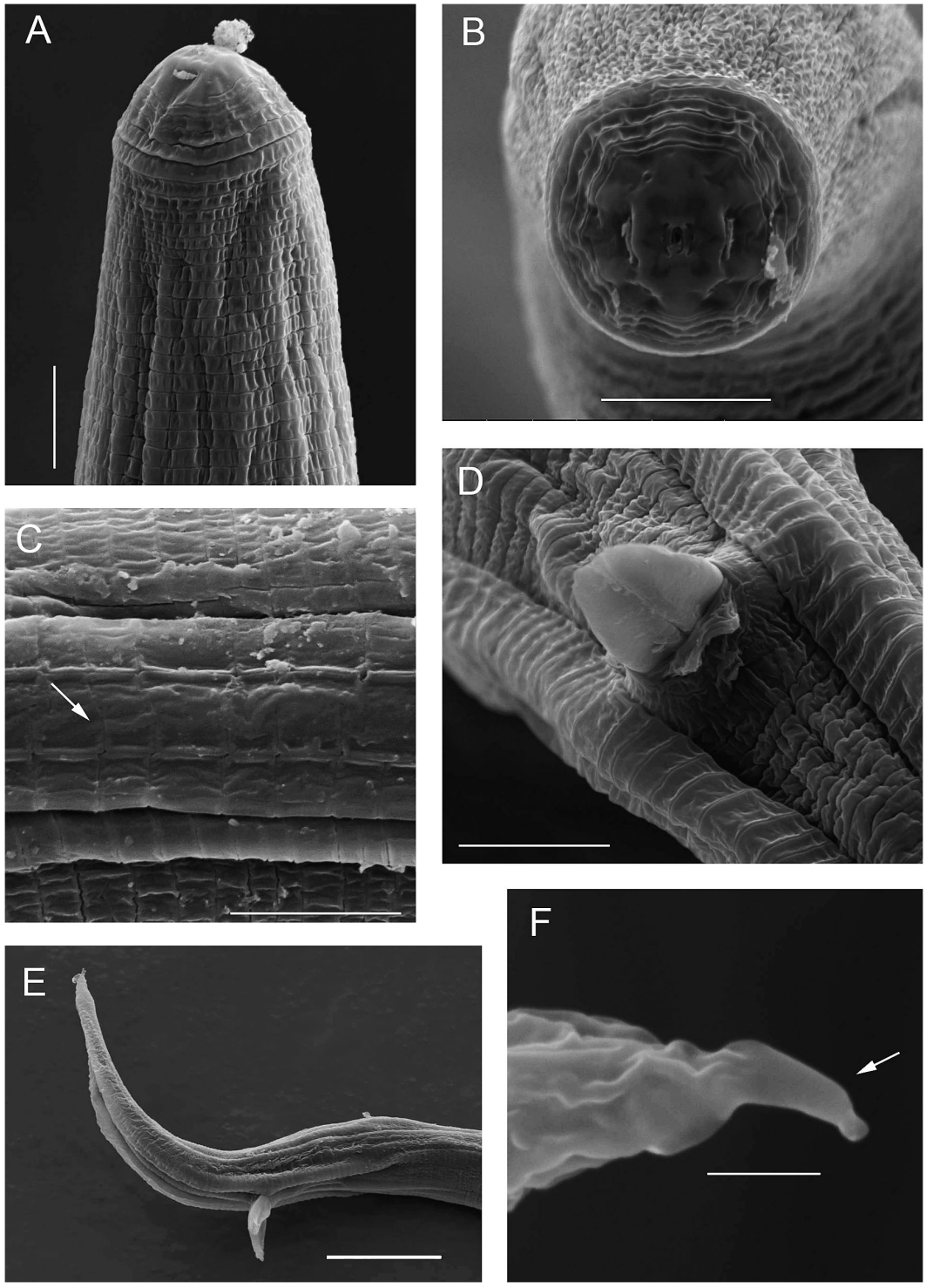

Fig. 4. Hirschmanniella kwazuna *sp. n. Male, SEM. A: Anterior region, lateral view; B: Lip region,* en face *view; C: Lateral field at mid-body with areolation (arrow); D: Cloaca with spicules; E: Tail region; F: Mucro on tail tip (arrow). (Scale bars: A-D = 5 μm; E = 20 μm; F = 1 μm.)*

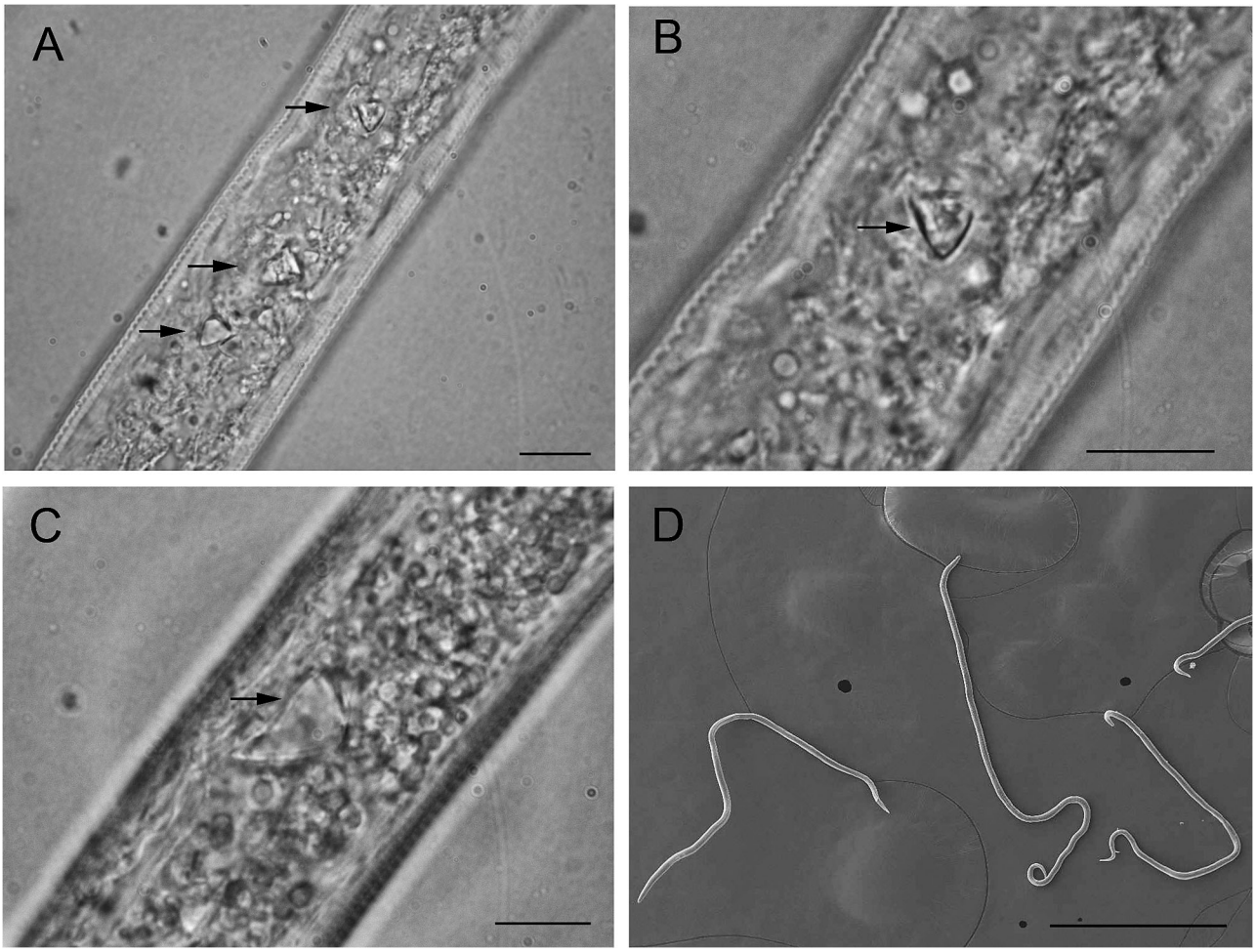

Fig. 5. Hirschmanniella kwazuna *sp. n. A-C: Crystal-like inclusions (arrows); D: Habitus. (Scale bars: A-C = 10 μm; D = 500 μm.)*

in adult males and females but not very distinct in some specimens.

Type habitat and locality

Numerous females, males and juveniles collected on 4 April 2006 by S. Schroeder from unidentified grass in undisturbed veldt in a non-impacted, permanently waterlogged wetland (29°12′15.7″S, 29°50′54.6″E) at an altitude of 1446 m a.s.l. on Watershed farm, a protected crane-breeding site near the town of Mooirivier, KwaZulu-Natal Province, South Africa.

Other localities

One female collected by S. Schroeder at Howard farm, near Nottingham Road, KwaZulu-Natal Province, South Africa, from grass from undisturbed veldt in a non-impacted grassland in a permanently and seasonally waterlogged wetland (29°22′09″S, 29°56′21″E) at an altitude of 1446 m a.s.l. One female collected by V.W. Spaull in 1994 from a sugarcane field at the La Mercy Research Station in the coastal belt near the town of Verulam, KwaZulu-Natal Province (29°37′S, 31°08′E), South Africa, at an altitude of 50 m a.s.l.

Type material

Holotype female and male and numerous females, males and juveniles deposited in the National Collection of Nematodes, ARC-PPRI, Pretoria. One slide T-5478p

Table 1. *Morphometrics of* Hirschmanniella kwazuna *sp. n. All measurements in μm and in the form: mean ± s.d. (range).*

Character	Female		Male	?J4	?J3	Female (*ex* sugarcane)
	Holotype	Paratypes	Paratypes	Paratypes	Paratypes	
n	–	20	22	9	3	1
L	1803	1805 ± 211.7 (1522-2049)	1548 ± 133.3 (1314-1960)	1169 ± 80 (1028-1276)	608-862	1910
a	64.5	64.8 ± 4.3 (56.2-72.2)	63.3 ± 6.1 (54-78)	53 ± 4.4 (45.3-58.2)	37.9-46	61.1
b	4.8	5.2 ± 0.9 (3.7-7.8)	5.5 ± 0.9 (4.3-7.1)	4.5 ± 0.7 (3.8-5.4)	3.4-4.3	–
b′	13	13.3 ± 1.6 (9.7-16.1)	12.7 ± 1.1 (11.4-14.8)	9.8 ± 0.8 (8.3-10.8)	7-8.6	–
c	17.6	20.6 ± 2.6 (17.6-26.4)	22.5 ± 2.9 (16.7-25.2)	18.5 ± 1.2 (15.9-19.6)	15.3-19.5	16.5
c′	5.1	4.5 ± 0.6 (2.9-5.2)	4.1 ± 0.5 (3-4.9)	3.6 ± 0.3 (3.4-4)	2.9-3.3	–
O	18.6	17.5 ± 2.2 (14-21.6)	17.8 ± 2.4 (13.5-21.6)	19.2 ± 3.8 (14.3-25)	14.5-19.5	17.6
DGO	4	3.5 ± 0.5 (3-4)	3.75 ± 0.5 (2.5-4)	3.5 ± 0.6 (2.5-4.5)	2-3	3.5
V	53	52 ± 3.1 (46-59)	–	156 ± 40.3 (109-223)*	22-36*	50.5
G_1	24	26 ± 3.1 (22-34)	–	–	–	17.5
G_2	24	24 ± 3 (18.5-29)	–	–	–	–
M	49.3	48.9 ± 1.9 (45.5-52.5)	47.9 ± 2.1 (44-51)	47.3 ± 1.5 (45.8-51.1)	46.4	49
T	–	–	593 ± 79.4 (514-776)	–	–	–
Ant. ovary length	433	442.5 ± 58.4 (337-558)	–	–	–	338
Post. ovary length	438	424 ± 63.1 (318-547)	–	–	–	–
Stylet length	22	20.5 ± 1.1 (18-22.5)	20 ± 1 (18.5-22)	18 ± 0.5 (17-19)	14-15	18.5
Metenchium	11	10 ± 0.7 (8.5-11.5)	9.5 ± 0.8 (8-11)	8.5 ± 0.4 (8-9)	7	9
Telenchium	11	10.5 ± 0.6 (10-11.5)	10.5 ± 0.4 (10-11)	9.5 ± 0.4 (9-10)	8	9.5
Stylet knob height	2	3 ± 0.5 (2-3.5)	3 ± 0.5 (2-4)	2.25 ± 0.3 (1.5-2.5)	2	3
Stylet knob width	5	4.5 ± 0.4 (4-5)	4.5 ± 0.6 (4-6)	4 ± 0.3 (3.5-4.5)	3-4	4.5
Pharynx	374	337 ± 67.7 (233-437)	296 ± 48.7 (220-373)	263 ± 43.7 (203-330)	177-202	–
Pharyngeal gland overlap	235	215 ± 59.2 (141-297)	165 ± 37.5 (180-246)	143 ± 43.8 (82-200)	101	–
Excretory pore from anterior end	127	116 ± 21.6 (69-151)	102 ± 16.4 (66-121)	88.5 ± 12.4 (60-101.5)	65.5-87	–
Annuli to excretory pore	–	94 ± 8 (88-114)	94 ± 6.6 (86-104)	–	–	88

Table 1. *(Continued).*

Character	Female		Male	?J4	?J3	Female (*ex* sugarcane)
	Holotype	Paratypes	Paratypes	Paratypes	Paratypes	
Anterior to pharyngo/int. junction	139	138 ± 15.4 (110-172)	122 ± 9.9 (100-138)	–	–	–
Excretory pore to pharyngo/int. junction	12	23 ± 13.4 (5-59.5)	23 ± 16.4 (0-65.5)	–	–	–
Mid-body diam.	28	28 ± 2.5 (23.5-33)	24.5 ± 2.5 (21-30.5)	22 ± 2.2 (18.5-24.5)	15.5-19	31
Median bulb length	17.5	17.5 ± 1.5 (15-20.5)	16 ± 1.1 (14-17.5)	16 ± 1.3 (14-18.5)	13-14	–
Median bulb diam.	18	13.5 ± 1.9 (10.5-18)	12.75 ± 1.5 (10-15.5)	11.5 ± 0.7 (10-12.5)	9.5-11	–
Valve length	4	4.5 ± 0.5 (4-5.5)	4 ± 0.6 (3-5.5)	4 ± 0.2 (3.5-4.5)	3-3.5	–
Valve width	3	3.5 ± 0.4 (2.5-4)	3 ± 0.5 (2.5-4)	3 ± 0.3 (2-3)	2.5-3	–
Lip region diam.	11	11.5 ± 0.7 (10-12.5)	11 ± 0.7 (10-12.5)	10 ± 0.9 (9-11)	9-9.5	11
Lip region height	4	4 ± 0.5 (3.5-5)	4 ± 0.6 (3-5)	4 ± 0.4 (3-4.5)	3-4	4
Annulus width	2	1.75 ± 0.3 (1.5-2)	1.5 ± 0.2 (1.5-2)	1.5 ± 0.3 (1-2)	1-1.5	2
Lateral field width	7.5	8.5 ± 1.5 (6.5-12)	8 ± 1.4 (6-11.5)	6 ± 1 (4.5-7.5)	3-5.5	6.5
Ant. spermatheca length	20	28.5 ± 5.7 (20-40)	–	–	–	–
Ant. spermatheca diam.	19	20 ± 2.1 (17-24)	–	–	–	–
Post. spermatheca length	48	38.5 ± 15.7 (26-78)	–	–	–	–
Post. spermatheca diam.	16	18 ± 3.6 (11.5-25.5)	–	–	–	–
Spicule length	–	–	31 ± 1.8 (28-34.5)	–	–	–
Gubernaculum length	–	–	12 ± 1.8 (9.5-16)	–	–	–
Tail annuli	–	72 ± 6.5 (62-81)	75 ± 5.9 (68-82)	–	–	78
Tail length	102	89 ± 13.6 (68-107)	73 ± 10.8 (52-93)	63 ± 4.3 (59-68.5)	36.5-40	116
Annuli from phasmid to tail tip	21	19 ± 3.6 (12-24)	–	19 ± 2.5 (15-22)	–	24
Phasmid to tail tip	–	19.5 ± 3.5 (15-26)	21 ± 3.8 (17-32)	–	–	38.5

* Genital primordium length.

with one paratype male and three female paratypes deposited in the US Department of Agriculture Nematode Collection (USDANC), Beltsville, MD, USA.

DIAGNOSIS AND RELATIONSHIPS

Females of *Hirschmanniella kwazuna* sp. n. are characterised by having a very irregular heat-relaxed body pos-

ture, body 1522-2049 μm long, a low, rounded lip region with four or five lip annuli, stylet 18-22.5 μm long, excretory pore position highly variable ranging from just anterior to median bulb to opposite anterior part of pharyngeal lobe, anterior to the junction of pharyngeal lumen with intestine, lateral field areolated along entire body, spermatheca filled with sperm, tail with 62-81 ventral annuli, narrowing to tip with a ventral mucro, angular crystal-like inclusions in body cavity and the phasmid situated 12-24 annuli or 15-26 μm anterior to tail tip. Males similar to females, including the crystal-like inclusions, and with a dorsally curved tail with ventral mucro.

In having an annulated tail tip with a ventral mucro, a completely areolated lateral field, no ventral notch on tail and a stylet of 18-22.5 μm, *H. kwazuna* sp. n. is distinct from the 34 species listed by Siddiqi (2000) and from the recently described *H. santarosae* Tandingan De Ley, Mundo-Campo, Yoder & De Ley, 2007. The new species is closest to *H. areolata* Ebsary & Anderson, 1982, *H. belli* Sher, 1968, *H. gracilis* (de Man, 1880) Luc & Goodey, 1964, *H. loofi*, *H. mucronata*, *H. oryzae*, *H. pomponiensis* Abdel-Rahman & Maggenti, 1987 and *H. santarosae*.

In having a very irregular body posture and angular crystal-like inclusions in the body *H. kwazuna* sp. n. differs from all the above-mentioned species. Also, all four lines of the lateral field continue 8-14 annuli posterior to the phasmid while in most of the other species listed above the outer lines proceed for 3-7 annuli posteriad to the phasmid and the inner lines stop anterior to the phasmid (see Sher, 1968), although in *H. santarosae* and *H. pomponiensis* the lines end near the tail tip.

Hirschmanniella kwazuna sp. n. is separated from *H. areolata* by the female having a more irregular body form *vs* ventrally arcuate to C-shaped, stylet shorter at 20.5 (18-22.5) *vs* 24-27 μm, stylet knobs sloping to hollow anteriorly with outer tips sometimes well directed anteriorly *vs* bulbous and flange-like, excretory pore location from anterior end much more variable at 116 (69-151) *vs* 130-145 μm, ventral tail annuli numbering 72 (62-81) *vs* 53 (48-61), phasmid situated closer to tail tip at 19.5 (15-26) *vs* 22-29 μm, male stylet shorter at 20 (18.5-22) *vs* 25-27 μm, excretory pore situated more anterior at 102 (66-121) *vs* 121-147 μm and location also more variable, male tail strongly curved dorsad in most specimens *vs* ventrally arcuate and gubernaculum not with such acute endings as illustrated by Ebsary and Anderson (1982).

Hirschmanniella kwazuna sp. n. is differentiated from *H. belli* by the females having 4-5 lip annuli *vs* usually with three indistinct annuli but occasionally with 3-4, stylet knobs varying from slightly sloping to hollow anteriorly *vs* slightly sloping, excretory pore situated from anterior to median bulb to opposite anterior part of pharyngeal lobe *vs* slightly anterior to level of pharyngo-intestinal valve, spermathecae large and filled *vs* inconspicuous and empty, males numerous *vs* rare, lateral field areolated *vs* not areolated to incompletely areolated, especially in tail region, and by the male tail conspicuously curved dorsad *vs* not.

The new species differs from three populations of *H. gracilis* by the following characters: females generally have a shorter stylet of 20.5 (18-22.5) *vs* 23 (21-24), 22 (21-24) and 21 (20-24) μm, respectively, stylet knobs slightly sloping to hollow anteriorly *vs* rounded; lateral fields completely areolated *vs* usually not areolated, or at most incompletely areolated on tail, tail tip always with mucro *vs* usually with ventral projection, tail annulated almost to tail tip *vs* only small part of tail tip non-annulated. The males of *H. kwazuna* sp. n. differ in having a higher c value of 22.5 (16.7-25.2) *vs* 18 (17-20), 19 (17-21) and 17 (16-18), respectively, generally lower c$'$ value of 4.1 (3-4.9) *vs* 4.9 (4.5-5.1), 4.4 (3.8-5.1) and 4.9 (4.3-6.1), respectively, generally shorter stylet of 20 (18.5-22) *vs* 22 (21-23) and 21 (20-22) μm, respectively, spicules shorter at 31 (28-34.5) *vs* 36 (30-38), 34 (27-37) and 35 (31-37) μm, respectively, and tail strongly curved dorsad *vs* not.

Hirschmanniella kwazuna sp. n. can be separated from two populations of *H. loofi* by slightly higher female c value at 20.6 (17.6-26.4) *vs* 17.3 (15-19) and 17 (14-20), respectively, female stylet very much smaller at 20.5 (18-22.5) *vs* 34-37 μm, higher o value of 17.5 (14-21.6) *vs* 12 (8-15) and 13% (11-15%), respectively, lip region with 4-5 *vs* 5-6 annuli, stylet knobs sloping to hollow anteriorly *vs* pear-shaped with a greater height than width, and lateral field areolated *vs* incompletely areolated on tail. Males have a smaller stylet at 18.5-22 *vs* 31-34 μm, higher o value 17.8 (13.5-21.6) *vs* 12% (10-14%) and shorter spicules at 31 (28-34.5) *vs* mean values of 38-44 μm.

Females of *H. kwazuna* sp. n. can be differentiated from three populations of *H. mucronata* by stylet knobs sloping to hollow anteriorly *vs* sloping, lateral field areolated along entire body *vs* areolated only in posterior region, higher o value of 17.5 (14-21) *vs* 14 (10-16), 11 (9-14) and 9% (6-12%), respectively, and shorter stylet of 20.5 (18-22.5) *vs* 27 (26-29), 26 (24-28) and 27 (24-29) μm,

respectively. Males are slightly shorter at 1548 (1314-1960) *vs* 1760 (1700-1830), 1860 (1550-2003) and 1930 (1850-2500) μm, respectively, and have a slightly higher a value of 63.3 (54-78) *vs* 54 (52-60), 57 (49-62) and 57 (54-60), respectively, shorter stylet of 20 (18.5-22) *vs* 26 (25-27), 25 (24-27) and 26 (24-28) μm, respectively, higher o value of 17.8 (13.5-21.6) *vs* 15 (11-19), 11 (9-14) and 9% (6-11%), respectively, slightly shorter mean spicule length of 31 (28-34.5) *vs* 33 (29-35), 33 (30-36) and 34 (32-36) μm, respectively, and 35-38 μm, according to Das (1960).

Hirschmanniella kwazuna sp. n. females can be distinguished from two populations of *H. oryzae* by a longer body at 1805 (1522-2049) *vs* 1300 (1030-1630) and 1440 (1140-1630) μm, respectively, higher b′ value of 13.3 (9.7-16.1) *vs* 10.7 (8.8-12.1) and 10.2 (7.9-11.7), respectively, higher c value of 20.6 (17.6-26.4) *vs* 17 (15-19) and 18 (16-20), respectively, lip region with 4-5 annuli *vs* 3-4 annuli, stylet knobs sloping to hollow anteriorly *vs* rounded to slightly sloping anteriorly, and lateral field completely areolated *vs* not so, but occasionally incompletely areolated in posterior region. Males differ in being longer at 1548 (1314-1960) *vs* 1170 (1010-1400 μm and have a higher b′ value of 12.7 (11.4-14.8) *vs* 10 (9.1-11.3) and 10.1 (8.3-11.1), respectively, higher c value of 22.5 (16.7-25.2) *vs* 17 (16-18), slightly lower c′ value of 4.1 (3-4.9) *vs* 4.9 (4.1-5.4) and 4.8 (4.4-5.1), respectively, stylet length of 20 (18.5-22) *vs* 17 (15-19) μm, longer gubernaculum at 12 (9.5-16) *vs* 8 (7-9) and 8 (7-10) μm, respectively, longer spicules at 31 (28-34.5) *vs* 23 (18-26) and 23 (20-25) μm, respectively, and tail strongly curved dorsad *vs* not.

Females of *H. kwazuna* sp. n. are separated from those of the original description and a new isolate of *H. pomponiensis* by higher c value of 20.6 (17.6-26.4) *vs* 17 (15-22) and 14 (11-17), respectively, lower c′ value of 4.5 (2.9-5.2) *vs* 5 (4-7) and 7.4 (6-9), respectively, longer anterior ovary at 442 (337-558) *vs* 332 (228-407) μm (new isolate population), longer posterior ovary at 424 (318-547) *vs* 308 (218-367) μm (new isolate population), longer stylet of 20.5 (18-22.5) *vs* 18.8 (18-20) μm (new isolate population), longer pharynx at 337 (233-437) *vs* 309 (243-376) and 308 (273-341) μm, respectively, longer pharyngeal overlap of 215 (141-297) *vs* 165 (144-197) μm (new isolate population), excretory pore location more variable at 116 (69-151) *vs* 152 (146-161) and 155 μm, respectively, from anterior end with number of annuli from anterior end also more variable at 94 (88-114) *vs* 101 (95-109) and 102, respectively, shorter tail at 89 (68-107) *vs* 113 (99-136) and 154 (119-189) μm, respectively, lateral field completely areolated *vs* incompletely areolated, and tail with a ventral mucro *vs* without. Males are slightly shorter at 1548 (1314-1960) *vs* 1883 (1810-1990) and 1873 (1725-2064) μm, respectively, have a lower c′ value of 3-4 *vs* 6.3 (6-7) and 5.1-5.2, respectively, shorter distance from anterior end to pharyngo-intestinal junction of 122 (100-138) *vs* 142 (139-149) and 138 (135-141) μm, respectively, excretory pore located more anterior to pharyngo-intestinal junction at 23 (0-65.5) *vs* 9.7 (0-22) and 3.7 (1-12) μm, respectively, slightly shorter spicules at 31 (28-34.5) *vs* 32 (32-33) and 37.3 (34-40) μm, respectively, shorter tail at 73 (52-93) *vs* 119 (101-133) and 107 (97-118) μm, respectively, and tail conspicuously curved dorsad *vs* not.

From *H. santarosae*, the females of *H. kwazuna* differ by a higher c value of 22.5 (16.7-25.2) *vs* 16.4 (14-19), lower c′ value of 4.1 (3-4.9) *vs* 6.3 (5.4-7.5), distance from anterior end to pharyngo-intestinal junction shorter at 122 (100-138) *vs* 145 (117-163) μm, slightly shorter pharynx at 296 (220-373) *vs* 340 (273-412) μm, excretory pore located closer to anterior end at 102 (66-121) *vs* 134 (99-145) μm, excretory pore location from pharyngo-intestinal junction more variable at 23 (0-65.5) *vs* 12.3 (2-23) μm, gubernaculum longer at 12 (9.5-16) *vs* 8.2 (6-11) μm and with a more irregular distal end, shorter tail at 73 (52-93) *vs* 80 (86-127) μm and tail conspicuously curved dorsad *vs* not.

Molecular characterisation

PCR with TW81 and AB28 primers amplifying ITS-rRNA yielded products of 781, 783, and 731 bp in length for *H. kwazuna* sp. n., *H. loofi*, and *Hirschmanniella* sp. from Germany, respectively. *Hirschmanniella kwazuna* sp. n. differs from closely related species *H. loofi* in 50 nucleotides (6.8%). Lengths of amplicons for D2-D3 of 28S rRNA and part 18S rRNA for studied *Hirschmanniella* spp. were on average *ca* 680 and 800 bp, respectively. Sequences of the D2-D3 expansion segments of 28S rRNA and partial 18S rRNA of *H. kwazuna* sp. n. differ from those of *H. loofi* in 26-29 (3.7-4.0%) and 2-3 (0.3-0.4%) nucleotides, respectively. The specific status of *H. kwazuna* sp. n. is supported by the presence of several unambiguous autapomorphies, *viz*., at least four for D2-D3 and 14 for the ITS rRNA, but not by the 18S rRNA sequence.

RFLP-ITS diagnostic profiles with four enzymes obtained for *H. kwazuna* sp. n. is presented in Figure 6.

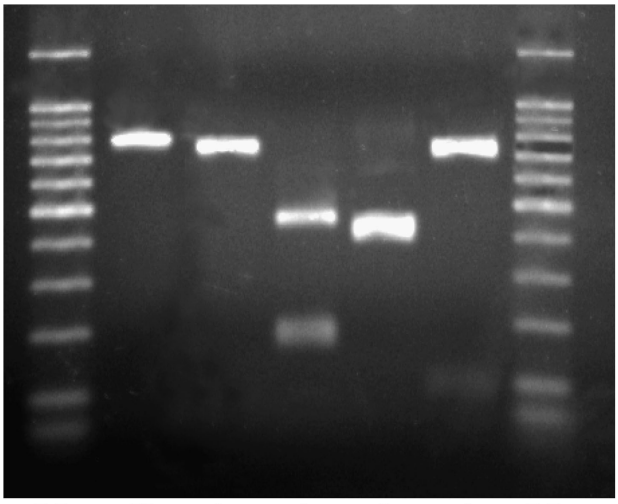

Fig. 6. *Restriction fragments of amplified ITS rRNA digested by four restriction enzymes. M = 100 bp DNA ladder (Promega); U = unrestricted PCR product, 1 =* Bsh*1236I, 2 =* Hin*fI, 3 =* Pst*I, 4 =* Rsa*I.*

RPFLP fragment lengths are: *Bsh*1236I, 718, 63 bp; *Hin*fI, 428, 171, 156, 26 bp; *Pst*I, 397, 384 bp; *Rsa*I, 695, 86 bp.

Hirschmanniella spinicaudata (Schuurmans Stekhoven, 1944) Luc & Goodey, 1964

(Fig. 7)

A few, mainly juvenile, specimens of this species were found during the survey. One poorly preserved female and a male specimen could be studied and their measurements are as follows:

Female

L = 3436 μm; a = 52.2; b′ = 5.6; c = 24.7; c′ = 3.9; o = 8.5; DGO = 4 μm; V = 53.7; stylet length = 47.5 μm; metenchium length = 24.5 μm; telenchium length = 23 μm; stylet knob height = 7.5 μm; stylet knob width = 9 μm; pharynx length = 610 μm; excretory pore from anterior end = 227 μm; mid-body diam. = 66 μm; median bulb = 26.5 × 23 μm; lip region diam. = 21 μm and height = 10 μm; annulus width at mid-body = 3 μm; lateral field width = 17 μm; tail length = 139 μm; Phasmid situated 14 annuli anterior to tail tip; tail with 60 ventral annuli.

Male

L = 2770 μm; a = 62.8; c = 19.9; c′ = 4.6; o = 6.8; DGO = 3 μm; stylet length = 43 μm; metenchium length = 22.5 μm; telenchium length = 20.5 μm; stylet knob height = 3 μm; stylet knob width = 6 μm; mid-body diam. = 44 μm; median bulb = 31.5 × 20.5 μm; lip region diam. = 17 μm and height = 8 μm; annulus width at mid-body = 3 μm; lateral field width = 11.5 μm; tail length = 139 μm; spicule length = 53.5 μm; gubernaculum length = 18.5 μm.

Habitat and locality

Collected from grass from undisturbed veldt in a non-impacted grassland in a permanently and seasonally waterlogged wetland (29°22′09″S, 29°56′21″E) at an altitude of 1446 m on Howard Farm near Nottingham Road, KwaZulu-Natal Province, South Africa.

Phylogenetic relationships within *Hirschmanniella* as inferred from analysis of ribosomal RNA genes

ML and MP analyses generated trees with similar topologies for corresponding gene fragments. ML trees reconstructed using three fragments of rRNA gene (D2-D3 28S, 18S and ITS rRNA) sequences are given in Figures 8 and 9. The monophyly of *Hirschmanniella* was highly supported by 18S rRNA and D2-D3 phylogenetic analysis. In all trees *H. kwazuna* sp. n. and *H. loofi* are sister taxa. The three major moderately or highly supported (BS > 78) clades can be revealed from ML and MP trees: *i*) *H. kwazuna* sp. n. and *H. loofi*; *ii*) *Hirschmanniella* sp. A, B and *H. belli*; and *iii*) *H. pomponiensis*, *H. santarosae*, *H. gracilis*, three unidentified *Hirschmanniella* from The Netherlands and an unidentified *Hirschmanniella* sp. from Germany.

Discussion

DNA barcoding of *Hirschmanniella*

DNA barcoding is becoming a powerful tool for identifying nematodes. It is based on the idea that a particular nucleotide sequence from common genes can serve as a unique identifier for every species. This approach is especially important for diagnostics of nematode groups in which species are distinguished by limited numbers of variable morphological and morphometrical characters.

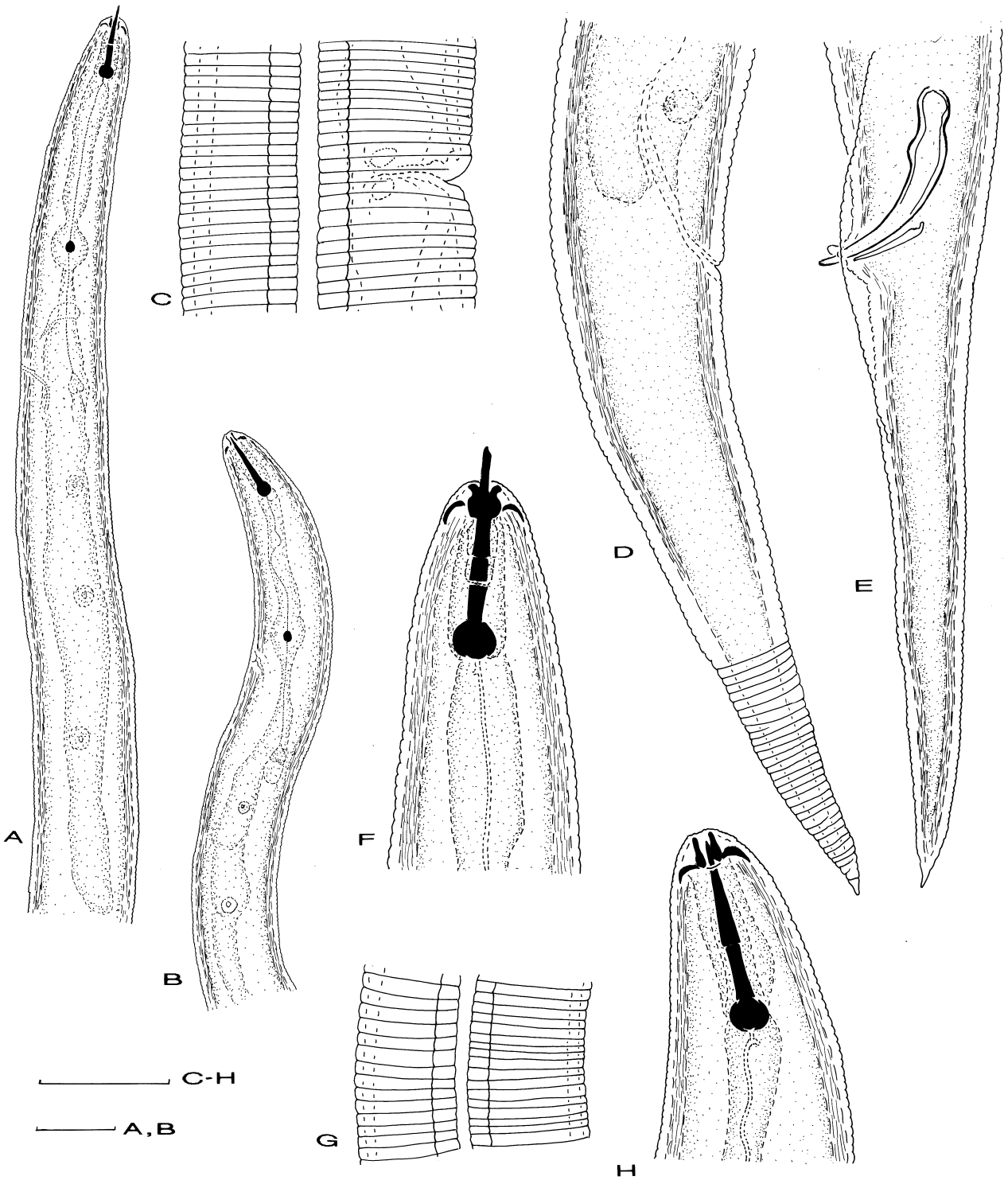

Fig. 7. Hirschmanniella spinicaudata *(Schuurmans Stekhoven, 1944) Luc & Goodey, 1964. A: Female anterior region; B: Male anterior region; C: Female lateral field at mid-body; D: Female posterior region; E: Male posterior region; F: Female lip region; G: Male lateral field at mid-body; H: Male lip region. (Scale bars: A, B = 50 μm; C-H = 30 μm.)*

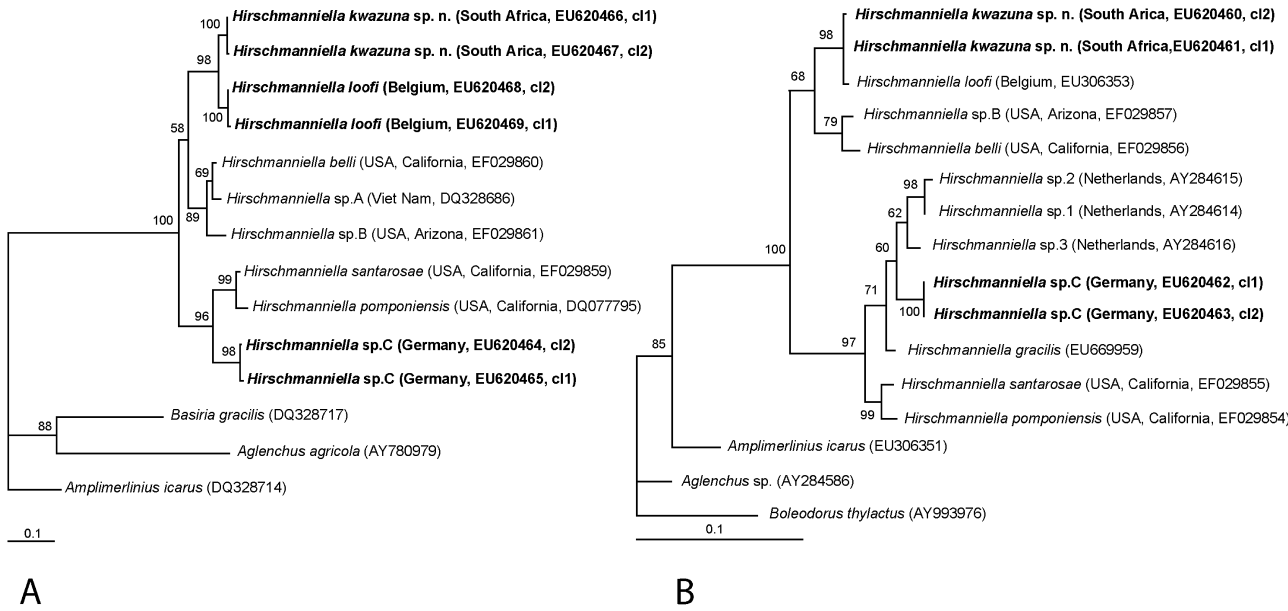

Fig. 8. *Phylogenetic relationships within the genus* Hirschmanniella *as inferred from maximum likelihood analyses of sequences of the D2-D3 expansion segments of 28S rRNA. A: Using GTR + I + G model and part 18S rRNA; B: Using SYM + G model of DNA evolution. Bootstrap values are given on appropriate clade. Newly obtained sequences are indicated in bold.*

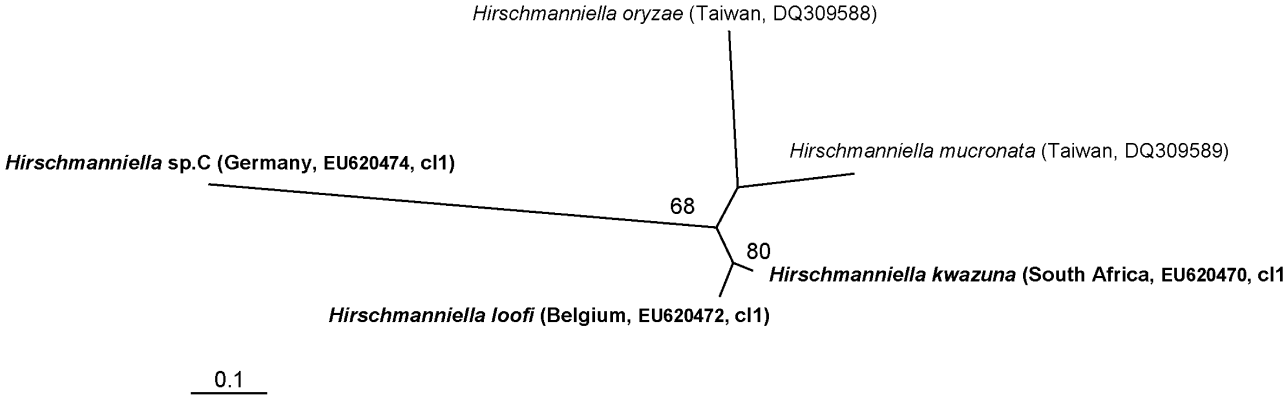

Fig. 9. *Phylogenetic relationships within the genus* Hirschmanniella *as inferred from maximum likelihood analyses of sequences of the ITS rRNA using GTR + I + G model of DNA evolution. Bootstrap values are given on appropriate clades. Newly obtained sequences are indicated in bold.*

The genus *Hirschmanniella* evidently belongs to this category. Ryss (1988) and Tandingan De Ley *et al.* (2007) noted that several reports on identification of common *Hirschmanniella* species in literature might be incorrect and may actually represent cryptic species. Tandingan De Ley *et al.* (2007) and the present study showed that rRNA genes serve as reliable markers to differentiate species of this genus and test species validity. Thus, molecular approach in combination with morphological analysis allows researchers to avoid mistakes in nematode identification.

Application of rRNA markers also allows estimation of species diversity and possible species number for the genus. Species of *Hirschmanniella* have been described from different climatic zones although most occur in tropical and subtropical regions (Ryss, 1988). Although there is a geographical bias in sampling for the present datasets, our preliminary results on comparing known morphos-

pecies in Europe, *i.e.*, *H. behningi* (Micoletzky, 1923) Luc & Goodey, 1964, *H. gracilis*, *H. loofi*, *H. zostericola* (Allgén, 1934) Luc & Goodey, 1964, and species from Europe presently separated using DNA support the possibility that the number of species of *Hirschmanniella* is much greater than is evident from a traditional morphological viewpoint.

Historically the 18S rRNA gene has been proposed as a marker for DNA barcoding of nematodes and this gene is now widely used for such research (see Floyd *et al*., 2002; Powers, 2004). Our present study on the comparison of *H. kwazuna* sp. n. and *H. loofi* showed that this marker has relatively low discriminative power whereas the D2-D3 of 28S and, especially, ITS rRNA fragment clearly differentiated allied species. The results of many studies indicate that sequence divergences at ITS regularly enable the discrimination of closely related species across tylenchids (Powers *et al.*, 1997; Powers, 2004). It is clear that several ribosomal genes, including the D2-D3 of 28S, which can also be considered good markers for phylogenetic reconstruction, the ITS rRNA and protein coding genes should be included in future DNA barcoding projects to facilitate detailed species differentiation.

Phylogeny of *Hirschmanniella* species

Phylogenies of *Hirschmanniella* reconstructed using partial 18S and 28S rRNA gene sequences are generally congruent with those presented by Holterman *et al.* (2006) and Tandingan De Ley *et al.* (2007). The present data included more samples and indicate the presence of three major clades within *Hirschmanniella*. Based on the estimation of rRNA sequence and morphological divergence among pairs of closely related species, the rate of morphological and molecular evolution may vary amongst the different lineages of *Hirschmanniella*. Sequence differences between *H. santarosae* and *H. pomponiensis* were higher (see Tandingan De Ley *et al.*, 2007) than those between *H. kwazuna* sp. n. and *H. loofi*, although the first species pair was less divergent in morphology than the second pair. Molecular datasets from more species are still necessary to reconstruct a comprehensive phylogeny and to reveal correlation trends between molecular and morphological or geographical groupings.

Crystal-like inclusions in *Hirschmanniella*

Several authors have found inclusions in the body of a number of nematodes but they all differ from the angular crystal-like inclusions found in *H. kwazuna* sp. n. Zuckerman *et al*. (1973) found polyhedral virus-like bodies of *ca* 20 nm diam. in the intestinal cells of *Dolichodorus heterocephalus* Cobb, 1914. Nuss (1984) found large numbers of light refracting crystalloid inclusions in the body wall musculature of *Tobrilus gracilis* (Bastian, 1865) Andrássy, 1959 during summertime. In winter the inclusions were rare. Sulphur was found to be the main component of these crystalloid inclusions and was considered as part of a detoxification system for sulphur ions. According to Nuss and Trimkovsky (1984), these crystalloids are the product of a detoxification mechanism necessary for survival in H_2S-rich conditions.

Hirschmanniella belli, *H. oryzae* and *H. anchoryzae* Ebsary & Anderson, 1982 specimens obtained from the USDA Nematode Collection were checked for similar crystals. Only *H. belli* showed one or two crystals in one of the specimens, the other two having none. In one population of *H. oryzae* from Portugal, one individual had a structure which could perhaps be a crystal. Two populations of *H. oryzae* from the Ivory Coast and Burkino Faso from the South African National Collection were also studied, but no crystal-like inclusions were found in any of the specimens.

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