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Morphological and molecular studies on *Heterodera sacchari*, *H. goldeni* and *H. leuceilyma* (Nematoda: Heteroderidae)

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Summary – *Heterodera sacchari*, *H. leuceilyma* and *H. goldeni* are closely related members of the *H. sacchari* species complex, which is mainly characterised and distinguished from all other described *Heterodera* species by the presence of finger-like projections of the strongly developed underbridge in the vulval cone of the cysts. Males are rare in all three species and are described here in *H. goldeni* for the first time. Reproduction appears to be parthenogenetic. There are only minor morphological distinctions between the three species, particularly after our present studies have emended their original descriptions from various populations. *Heterodera sacchari* and *H. goldeni* showed differences in the ITS-rRNA gene sequences. *Heterodera sacchari* was described and reliably identified from many tropical African countries, *H. leuceilyma* is known only from Florida, USA, and *H. goldeni* has been identified in Egypt, Israel and Iran. All three species have grasses and other Poaceae as hosts, *H. sacchari* commonly attacking rice and sugarcane, and *H. goldeni* reproducing successfully on sugarcane ratoon seedlings. Morphological data emending the descriptions of *H. sacchari*, *H. goldeni* and *H. leuceilyma* from various populations are presented and discussed along with their host and distribution. Molecular characterisation of *H. sacchari* and *H. goldeni* is provided. An analysis of phylogenetic relationships within species of the *sacchari*-group using ITS-rRNA gene sequences is also presented.

Keywords – cyst nematodes, distribution, Egypt, hosts, Iran, Israel, ITS-rDNA, morphology, morphometrics, phylogeny.

Three *Heterodera* species, *H. sacchari* Luc & Merny, 1963, *H. leuceilyma* Di Edwardo & Perry, 1964 and *H. goldeni* Handoo & Ibrahim, 2002, are currently known, these being distinguished from all other species of the genus by the presence of distinct finger-like projections in the strongly developed underbridge in the vulval cone of the cysts. The sugarcane cyst nematode *H. sacchari* was originally reported from sugarcane, *Saccharum officinale* L., in Congo-Brazzaville (Luc & Merny, 1963) and was later found parasitising roots of rice, *Oryza sativa* L. (Merny, 1970; Babatola, 1983). It is considered to be a potential pest for these crops and is placed on the list of quarantine pests of several countries. *Heterodera leuceilyma* has been described as a pathogen of St Augustinegrass, *Stenotaphrum secundatum* (Walt.) Kuntze, in Florida, USA. The recently described cyst nematode *H. goldeni* was found attacking Qasabagrass *Panicum coloratum* L. in Egypt. Besides the peculiarities in underbridge shape, these three species share other diagnostic characters, like the presence of three incisures in the lateral field of the

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Table 1. Heterodera *species and populations used in the present study.*

Species	Locality	Host	GenBank accession No.	Study	Source
H. sacchari	Ghana, Ashani	*Oryza sativa* (Rice)	EF143604, EF143605	S	D.J. Hunt
H. sacchari	Benin	*Saccharum officinale* (Sugarcane)		M	D. Sturhan
H. sacchari	Côte d'Ivoire	*Saccharum officinale* (Sugarcane)	AF274403	S	Subbotin *et al*. (2001); J. Rowe
H. leuceilyma	USA, Florida, West Palm Beach	*Stenotaphrum secundatum* (St Augustinegrass)		M	Beltsville, MD, USA, USDA Nematode Collection, Type specimens
H. leuceilyma	USA, Florida, Atlantis	*Cynodon dactylon* (Bermudagrass)		M	Beltsville, MD, USA, Nematode Collection
H. leuceilyma	USA, Florida, Atlantis	*Cynodon dactylon* (Bermudagrass)		M	
H. goldeni	Egypt, Alexandria	*Panicum coloratum* (Qasabagrass)	EF143607, EF14608	M, S	Z. Handoo; I.K.A. Ibrahim
H. goldeni	Iran, Mazandaran, Nashtaroud	*Phragmites australis* (Common reed)	AF498383	M, S	Tanha Maafi *et al*. (2003)
H. goldeni	Iran, Gilan, Bandar Anzali	Rhizosphere of *Phragmites australis*	EF143610	S	Z. Tanha Maafi
H. goldeni	Iran, Gilan, Bandar Anzali	Rhizosphere of *Phragmites australis*		M	Z. Tanha Maafi
H. goldeni	Iran, Gilan, Bashman	Rhizosphere of *Juncus acutus* (Dutch rush)	EF143609	M, S	Z. Tanha Maafi
H. goldeni	Israel, Arava	*Pennisetum clandestinum*	EF143606	M, S	M. Mor

M – Morphological and morphometrical study; S – sequencing of the ITS-rDNA.

second-stage juveniles, and all are specialised to Poaceae and related monocotyledon hosts. An underbridge with finger-like projections has also been reported by Mulvey (1972) for *H. oryzae* Luc & Berdon-Brizuela, 1961, but the material studied by him was clearly a mixture containing *H. sacchari* (see Luc & Taylor, 1977).

During nematological surveys conducted by the first author in Iran, an unidentified cyst nematode similar to *H. sacchari* was found on roots of *Phragmites australis* (Cav.) Trin. *ex* Steud. in two northern provinces of the country. Molecular and morphological analyses showed that it appeared to differ from the sugarcane cyst nematode. It was suggested that it may be considered as a new species (Tanha Maafi *et al.*, 2003). A population of a cyst nematode close to *H. sacchari* and *H. leuceilyma* was also found parasitising a wild grass in Israel. Comparative morphological and molecular analyses of these nematode populations revealed that the unidentified cyst nematodes from Iran and Israel belong to *H. goldeni*. In this paper morphological data emending the descriptions of *H. sacchari* and *H. goldeni* are presented; males of *H. goldeni* are described for the first time, diagnostic morphological characters are detailed and a molecular characterisation of *H. sacchari* and *H. goldeni* is provided. An analysis of phylogenetic relationships within species of the *sacchari*-group using the ITS-rRNA gene sequences is also presented. Data emending the original description of *H. leuceilyma* are added. Preliminary results have already been briefly reported (Maafi *et al*., 2005).

Materials and methods

Nematode populations

A list of studied populations is given in Table 1. The cysts were mostly extracted by a combination of a modified Cobb sieving method and the sugar flotation method (Caveness & Jensen, 1955; Dunn, 1969). In addition, males were isolated from roots of *Pennisetum clandestinum.* The plants were grown in a glasshouse at

25°C in 10 dm^3 containers in sandy soil. Males were extracted from soil by centrifugation-flotation methods. Some cysts were dried and kept at room temperature for molecular studies and a number of those were used for morphological and morphometric studies. The vulval cone region was excised, and embedded in a heated drop of glycerin jelly on a cover slip mounted in aluminium slide. Second-stage juveniles and eggs from the same cysts and males were fixed in TAF (2 ml triethanolamine, 7 ml formaldehyde, 91 ml distilled water) and transferred to dehydrated glycerin (De Grisse, 1969). The juveniles were mounted in a small drop of dehydrated glycerin, the cover slip being sealed with a paraffin ring and mounted on an aluminium slide.

Light microscopic and SEM observations

Morphological and morphometric characters were studied by using a camera lucida installed on a light microscope (Reichert and Olympus, BH-2). The light microscopic photographs of cysts, cyst vulval cones and second-stages juveniles were taken with an automatic Olympus camera attached to a compound Olympus BX50 microscope equipped with an interference contrast system. The photographs of cysts were taken with the same camera attached to a dissecting microscope. For photomicrographs of males a Zeiss microscope provided with DIC optics was used. For scanning electron microscope (SEM) observations juveniles preserved in glycerin were first dehydrated by ethanol and dried by CO_2, then placed on stubs and coated with gold. The specimens were observed with a scanning electron microscope model JMS 840 operating at 15 kV.

DNA extraction

For each population several cysts were soaked overnight in double distilled water. One to four cysts were transferred into an Eppendorf tube containing 20 μl double-distilled water and 2 μl 10× PCR buffer and then crushed by a microhomogeniser Vibro Mixer (Zürich, Switzerland). Proteinase K (3 μl of a 600 μg/ml solution) (Promega Benelux, Leiden, The Netherlands) was added and the tubes were frozen at −80°C for at least 10 min and then incubated at 65°C (1 h) and 95°C (10 min) consecutively. After incubation, the tubes were centrifuged for 2 min at *ca* 13 000 *g* and kept at −20°C until use.

PCR

Extracted DNA (2-4 μl) was transferred into an Eppendorf tube containing 2.5 μl 10× *Taq* incubation buffer, 5 μl Q solution, 0.5 μl dNTPs mixture (*Taq* PCR Core Kit, Qiagen, Hilden, Germany), 0.15 μl of each primer (1.0 μg/μl) (synthesised by Life Technologies, Merelbeke, Belgium), 0.2 μl *Taq* polymerase and double-distilled water to a final volume of 25 μl. The forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) were used in PCR (Tanha Maafi *et al.*, 2003). The PCR amplification profile consisted of 4 min at 94°C; 35 cycles of 1 min at 94°C, 1.5 min at 55°C, and 2 min at 72°C, followed by a final step of 10 min at 72°C. Two μl of the PCR product were run on a 1% TAE-buffered agarose gel (100 V, 40 min).

Cloning and sequencing

Purified PCR products were cloned into the pGEM-T vector and transformed into JM109 High Efficiency Competent Cells (Promega Benelux). Several clones were isolated using blue/white selection and submitted to PCR with vector primers. PCR products from clones were sequenced using primers TW81, AB28 or internal reverse primer 5.8SM5 (5′-GGCGCAATGTGCATTCGA-3′) as described by Zheng *et al.* (2000), with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems Benelux, Maarssen, The Netherlands) according to the manufacturer's instructions. The resulting products were purified using a Centriflex Gel Filtration Cartridge (Edge BioSystems, Gaithersburg, MD, USA). Sequences were run on a 377 DNA Sequencer (PE Applied Biosystems, Warrington, UK). The newly obtained sequences have been submitted to GenBank database under the numbers indicated in Table 1.

Sequencing and phylogenetic analyses

DNA sequences of cyst nematodes from the *sacchari*-group were aligned with ClustalX 1.64 using default options (Thompson *et al.*, 1997). *Heterodera hordecalis* Andersson, 1975 and *H. latipons* Franklin, 1969 were designated as outgroup taxa (Subbotin *et al.*, 2001). Sequence alignment was analysed with equally weighted maximum parsimony (MP) method and maximum likelihood (ML) method using PAUP* 4.0b4a (Swofford, 1998). For MP we used heuristic search setting with ten replicates of random taxon addition, tree bisection-reconnection branch

swapping to seek for the most parsimonious trees. Gaps were treated as missing data. To obtain an estimation of the support for each node, a bootstrap analysis (1000 replicates, heuristic search, and simple addition of sequence) was also performed. Best fit model of DNA evolution for ML analysis was obtained using ModelTest program (Posada & Crandall, 1998). Bootstrap analysis for ML was made using 100 pseudo-replicates with tree searches in each replication performed using one random-sequence-addition without branch swapping.

Results and discussion

MORPHOLOGY

Heterodera sacchari

Detailed morphological descriptions and morphometrics of different stages are given by Luc and Merny (1963), Netscher *et al*. (1969), Merny (1970), Luc (1974), Nobbs *et al.* (1992) and Shahina and Maqbool (1995). Morphometrics of cysts and second-stage juveniles are summarised here in Tables 2, 3 and 4, with new data added for juveniles from a population from Benin. Netscher (1969) showed that *H. sacchari* is a triploid parthenogenetic species, but males have been described from different populations (Table 4). Among the measurements compiled in Tables 2, 3 and 4, cyst lengths (means 550-735 μm) and fenestrae in particular show a remarkable wide range of variation and amongst the juveniles mean body lengths (460-592 μm) and mean stylet lengths (21-24.1 μm) are also variable.

Second-stage juveniles from Benin had the following characteristics (besides measurements given in Table 3): Lip region measuring about 9 $\times$ 4 μm; three lip annules (plus perioral disc) with the anterior ones mostly indistinct. Stylet knobs anteriorly concave, stylet base 5 μm diam. Subventral glands mostly filling body cavity. Cuticle annuli 1.7-1.8 μm wide at mid-body; lateral fields generally irregularly areolated. Phasmids distinct, lens-like, two to four annuli behind anus level. Figures by Vovlas *et al*. (1986) and Nobbs *et al*. (1992) showing punctiform phasmids in a more posterior position are considered by us to be incorrect.

Heterodera goldeni

Morphometrics of cysts and second-stage juveniles from the original description of this species by Handoo and Ibrahim (2002) from Egypt and from populations from Iran are given in Tables 5 and 6, measurements of second-stage juveniles and males from Israel in Tables 4 and 6.

Cysts of the Iranian populations closely agree in measurements and other morphological details with the original description of *H. goldeni*. Old cysts often turn black in colour; large bullae are scattered in the vulval cone at level of the prominent underbridge with finger-like projections at the middle, which occasionally extend to the length of

Table 2. *Morphometrics of cysts of* Heterodera sacchari *and* H. leuceilyma*. All measurements in μm and in the form: mean (range).*

Species	*H. sacchari*	*H. sacchari*	*H. sacchari*	*H. sacchari*	*H. sacchari*	*H. leuceilyma*
Country	Congo-Brazzaville	Côte d'Ivoire	Liberia	Côte d'Ivoire	Pakistan	Florida, USA
Host	*Saccharum officinale*	*Oryza sativa*	*Oryza sativa*	*Oryza sativa*	*Saccharum officinale*	*Stenotaphrum secundatum*
Source	(Luc & Merny, 1963; Mulvey, 1972)	(Merny, 1970)	(Vovlas *et al*., 1986)	(Nobbs *et al*., 1992)	(Shahina & Maqbool, 1995)	(Di Edwardo & Perry, 1964; Mulvey, 1972)
Cyst						
n	100	60	16	50	20	10
Length	654 (380-1030)	640 (500-890)	590 (450-1250)	735 (442-983)	550 (485-860)	830 (650-930)
Diam.	445 (280-830)	430 (320-620)	470 (350-774)	456 (279-753)	448 (337-605)	480 (420-570)
Length/diam.	1.5 (1.0-2.2)	1.5 (1.2-2.0)	–	–	1.7 (1.0-2.0)	–
Vulval plate						
n	25		8	10	20	40
Vulval slit length	(50-52)	–	50 (48-52)	52.6 (44-62)	50 (45-55.4)	(50-65)
Fenestral length	(45-55)	–	49 (47-52)	40.1 (29-46)	52 (48.5-60)	(48-50)
Fenestral width	(35-45)	–	31 (30-32)	48.5 (36-68)	38 (33.4-40)	(38-40)
Underbridge length	(100-150)	–	140 (120-150)	129.6 (111-172)	130.4 (120-145.5)	(100-140)

Table 3. *Morphometrics of second-stage juveniles of* Heterodera sacchari *and* H. leuceilyma*. All measurements in µm and in the form: mean (range).*

Species	*H. sacchari*	*H. sacchari*	*H. sacchari*	*H. sacchari*	*H. sacchari*	*H. sacchari*	*H. leuceilyma*
Country	Congo-Brazzaville	Côte d'Ivoire	Benin	Liberia	Côte d'Ivoire	Pakistan	Florida, USA
Host	*Saccharum officinale*	*Oryza sativa*	*Saccharum officinale*	*Oryza sativa*	*Oryza sativa*	*Saccharum officinale*	*Stenotaphrum secundatum*
Source	(Luc & Merny, 1963)	(Merny, 1970)	(original)	(Vovlas *et al*., 1986)	(Nobbs *et al*., 1992)	(Shahina & Maqbool, 1995)	(Di Edwardo & Perry, 1964)
n	25	60	15	60	20	20	10
L	480 (420-530) [n = 100]	500 (420-530)	525 (495-560)	460 (360-510)	592 (569-609)	475 (394-520)	550 (520-580)
a	26 (24-28)	29 (22-33)	–	26 (24-28)	–	24.5 (23-26)	28.3 (26.3-31.5)
b	3.0 (2.3-3.6)	2.2 (1.8-2.5)	–	2.5 (2.2-2.7)	–	3.4 (2.5-4.0)	4.4 (4.0-4.9)
c	8.8 (8.3-9.5) [n = 11]	8.8 (7.3-9.7)	–	7.6 (7.1-8.5)	–	7.8 (7.5-9.0)	8.6 (7.9-9.3)
c′	–	–	–	–	–	–	–
Stylet length	22 (21-24)	22.5 (21-26)	23.3 (22.5-24.5)	21 (19-23)	24.1 (21-26)	21 (20-24)	26 (23-28)
DGO	–	–	–	5-6	–	–	5
Body diam. at mid-body	18.5 (17-19)	17.5 (16-20)	–	–	19.4 (18-20)	–	–
Body diam. at anus	–	–	–	–	13.4 (11-15)	–	–
Tail length	49-60	–	62 (56-66)	60 (55-64)	61.5 (49-69)	50.5 (45.5-60.2)	–
Length of hyaline part of tail	26 (20-30)	30 (27-35)	31 (28-36)	28 (26-30)	31.9 (28-39)	26 (24.2-32.5)	38 (31-41)
Hyaline part/stylet length	0.9-1.5	1.3 (1.0-1.5)	–	1.3 (1.1-1.5)	–	–	*ca* 1.5

Table 4. *Morphometrics of male* Heterodera sacchari*,* H. leuceilyma *and* H. goldeni*. All measurements in µm and either in the form: mean (range) or range.*

Species	*H. sacchari*	*H. sacchari*	*H. sacchari*	*H. leuceilyma*	*H. goldeni*
Country	Congo-Brazzaville	Ivory Coast	Pakistan	Florida, USA	Israel
Host	*Saccharum officinale*	*Oryza sativa*	*Saccharum officinale*	*Stenotaphrum secundatum*	*Pennisetum clandestinum*
Source	(Netscher *et al*., 1969)	(Merny, 1970)	(Shahina & Maqbool, 1995)	(Di Edwardo & Perry, 1964)	(original)
n	20	1	7	10	15
L	1040-1510	1035	954 (800-1210)	1230 (950-1450)	1290 (1060-1400)
a	44-57	40	50 (45.2-56.2)	34.8 (25.0-41.9)	–
b	3-7	5	5.0 (4.5-5.6)	6.7 (5.0-8.6)	–
c	–	–	–	–	–
c′	–	–	–	–	–
Stylet length	24-30	26	26 (25.5-28)	35 (32-40)	28 (26-29)
T	42-67	32	–	61 (54.9-67.6)	–
Spicule length	29-38	30	34 (30.5-35.2)	35.5 (30-37)	34 (30-38)
Gubernaculum length	8	7	–	–	9 (8-10.5)

Table 5. *Morphometrics of cysts of* Heterodera goldeni. *All measurements in μm and either in the form: mean ± s.d. (range) or mean (range).*

Country	Egypt	Iran Mazandaran province, Nashtaroud	Iran Gilan province, Bandar Anzali	Iran Gilan province, Bashman
Host	*Panicum coloratum*	*Phragmites australis*	Rhizosphere of *Phragmites australis*	Rhizosphere of *Juncus acutus*
Source	(Handoo & Ibrahim, 2002)	(original)	(original)	(original)
Cyst				
n	20	19	8	30
Length	756 ± 204 (510-1150)*	714 ± 178 (480-1100)**	766 ± 99 (600-900)**	707 ± 3 (500-900)**
Diam.	466 ± 196 (257-995)	456 ± 156 (300-900)	481 ± 73 (350-580)	437 ± 47 (340-540)
Length/diam.	1.6 ± 0.2 (1.3-2.1)	1.6 ± 0.33 (1.29-1.90)	1.60 ± 0.1 (1.47-1.71)	1.62 ± 0.11 (1.38-1.86)
Vulval plate				
n	20	15	10	8
Vulval slit length	42 (35-50)	49 ± 6.3 (40-65)	44 ± 3.4 (38-48)	48 ± 7.3 (40-60)
Fenestral length	–	60 ± 8.4 (47-75)	61 ± 4.6 (54-68)	60 ± 5.2 (51-65)
Fenestral width	52 (33-65)	45 ± 6.4 (38-63)	42.3 ± 2.8 (37-46)	45 ± 4.7 (40-53)
Underbridge length	117 ± 28.3 (102-150)	130 ± 23.3 (98-170)	117 ± 5.30 (110-125)	115 ± 12.3 (100-130)
Width of middle part of underbridge	–	48 ± 7.5 (36-60)	52.3 ± 8.3 (45-70)	55.5 ± 7.6 (45-66)

*including neck; ** excluding neck.

the fenestra (Fig. 1). Cysts from Israel had semifenestra 48 μm high and 46 μm wide, vulval slit 47-55 μm wide and the underbridge *ca* 120 μm long, few peripheral bullae present and cyst wall with distinct punctation.

Second-stage juveniles of the Iranian populations have a slightly shorter body but longer stylet than specimens from Egypt (see Table 6). The body is curved ventrally after fixation, the lip region dome-shaped, low, with two or three annules and a labial disc, slightly offset from the rest of the body (Figs 2, 3). Stylet strong, with robust and anteriorly deeply concave knobs. Dorsal pharyngeal gland orifice distinct, median bulb oval to rounded, pharyngeal glands overlap ventrally, junction with intestine distinct. Hemizonid two annules wide, cuticle in this region slightly swollen, excretory pore just posterior to hemizonid. Lateral field with three incisures starting 10-12 annules posterior to lip region and ending at second half of tail with outer incisures crenate and areolated in some parts of tail region. Phasmids rather large, two or three annules posterior to anus level (Figs 2, 3), tail long and tapering to a finely rounded terminus. SEM photographs of the lip region in *en face* view identical to those presented by Handoo and Ibrahim (2002) (Fig. 3).

Second-stage juveniles from Israel also have a relatively short body and a somewhat longer stylet (Table 6). They agree in other morphological characteristics with the description above. The lateral field is irregularly areolated, the subventral pharyngeal glands often do not fill the body cavity, the stylet knobs are slightly concave anteriorly, the phasmids are lens-like and situated at two or three annules posterior to the anus.

Males are not known from the Egyptian and Iranian populations, but were found in the Israel population reared in the glasshouse. Measurements are given in Table 4. Lip region dome-shaped with three to four (exceptionally five) annules plus labial disc. Stylet base rounded, 5-6 μm in diam., with anteriorly flat or slightly concave knobs. Excretory pore 5-6 annules posterior to hemizonid. Cuticle annules 2.0-2.5 μm wide in mid-body region; lateral field with three longitudinal lines, but often only inner line developed, areolation present. Gonads mostly well developed and containing sperm. Long penial tube present, spicules with notched tip, gubernaculum 8-10.5 μm long, phasmids absent (Fig. 4).

Heterodera leuceilyma

Di Edwardo and Perry (1964) gave a description of this species based on cysts, females, males and second-

Table 6. *Morphometrics of second-stage juveniles of* Heterodera goldeni. *All measurements in μm and either in the form: mean ± s.d. (range) or mean (range).*

Species	*H. goldeni*	*H. goldeni*	*H. goldeni*	*H. goldeni*	*H. goldeni*
Country	Egypt	Iran	Iran	Iran	Israel
	Alexandria	Mazandaran province, Nashtaroud	Gilan province, Bandar Anzali	Gilan province, Bashman	Arava
Host	*Panicum coloratum*	*Phragmites australis*	Rhizosphere of *Phragmites australis*	Rhizosphere of *Juncus acutus*	*Pennisetum clandestinum*
Source	(Handoo & Ibrahim, 2002)	(original)	(original)	(original)	(original)
n	38	10	14	20	15
L	546 ± 41.6 (450-612)	532 ± 22.4 (495-572)	513 ± 20 (503-530)	512 ± 21 (472-548)	520 (465-570)
a	31 ± 2.1 (28-36)	29.8 ± 1.0 (28.8-31.8)	26.7 ± 1.6 (23.2-28.23)	28.6 ± 1.4 (26.1-30.3)	–
b	2.5 ± 0.2 (2.3-3.4)	–	4.3 ± 0.3 (3.8-5.1)	4.5 ± 0.5 (3.9-6.5)	–
c	8.3 ± 0.4 (7.7-9.3)	8.5 ± 0.7 (7.6-9.9)	8.6 ± 0.5 (7.9-9.3)	8.2 ± 0.6 (7.1-9.5)	8.6 (8.0-9.6)
c′	–	4.6 ± 0.5 (3.9-5.1)	4.3 ± 0.3 (3.7-4.8)		–
Stylet length	22.6 ± 0.4 (22-23.5)	24 ± 0.7 (23-25)	23.7 ± 0.5 (22.8-24.3)	24.2 ± 0.7 (22.8-25.2)	24 (23-25)
Lip region height	–	4.8 ± 0.4 (4-5)	4 ± 0.2 (3.8-4.3)	3.9 ± 0.2 (3.6-4.3)	–
Lip region diam.	–	9.2 ± 0.4 (9-10)	9 ± 0.3 (8.6-9.5)	9 ± 0.5 (8.6-10)	–
DGO	4.0 ± 0.9 (2.5-6.0)	6.2 ± 0.4 (6-7)	6 ± 0.7 (5.2-6.6)	6.6 ± 0.7 (5.2-7.2)	–
Distance from anterior end to excretory pore	–	105 ± 5.4 (98-113)	97 ± 5.3 (85-103)	103 ± 3.4 (95-110)	–
Body diam. at mid-body	18.6 ± 2.1 (16-20)	18 ± 0.8 (17-19)	18.9 ± 0.9 (18-20.4)	17.9 ± 0.4 (17-19)	–
Body diam. at anus	–	13.8 ± 0.6 (13-15)	13.6 ± 0.7 (12.3-15.23)	13.6 ± 0.6 (12-14.3)	-
Length of hyaline part of tail	38.4 ± 2.9 (33-43)	36.7 ± 2.5 (34-42)	34 ± 2.6 (28.6-38.5)	34.3 ± 3 (28.5-40)	34 (26-39)
Tail length	65.4 ± 3.8 (60-75)	63 ± 5.4 (54-70)	59 ± 4 (51-64)	63 ± 4.8 (56-70)	60 (51-66)
Hyaline part/stylet length	–	1.5 ± 0.1 (1.4-1.75)	1.4 ± 0.1 (1.22-1.58)	1.4 ± 0.1 (1.2-1.6)	–
Length/median bulb distance	–	7.6 ± 0.6 (6.90-9.15)	7.4 ± 0.6 (6.5-8.8)	7.4 ± 04 (6.3-8.1)	–

stage juveniles from St Augustinegrass in Florida, whilst Mulvey (1972) added cyst cone measurements (see Tables 2, 3, 4).

Cysts are mainly characterised by a massive underbridge with dorso-ventral finger-like projections, bullae present, cuticle with zigzag pattern and extensive punctation internally, colour changing with age from yellow to brown and glossy black.

Males are sporadic in occurrence according to the original description. Based on our study of six paratype males the following emended description is given: Lip-region dome-shaped, 12.5-13 μm diam. and 6.5-7.7 μm high, with four (exceptionally five) annules plus labial disc. Stylet 28-30 μm long (*vs* 32-40 μm according to original description). Cuticle with distinct annuli, annuli 2.5 μm wide in mid-body region; lateral field with three longitudinal lines, irregularly crossed by annules; exceptionally only one or two lines developed, but inner line generally present; occasionally two indistinct additional lines present between main lines. Spicules 33-44 μm long, notched at tip, gubernaculum 9-10 μm long. Penial tube well developed, phasmids absent (*vs* present according to original description).

Second-stage juveniles (emended description based on recent examination of six paratypes and six additional second-stage juveniles from type locality and type host): Lip region *ca* 10 μm diam. and 4 μm high, with three annules plus perioral disc. Stylet 22.5-25 μm long (n = 5), stylet base *ca* 6 μm diam., knobs rounded posteriorly with slightly concave anterior face. Pharyngeal glands more or less filling body cavity; hemizonid extending over two body annules, excretory pore immediately posterior. Cuticle annules 1.8 μm wide in mid-body region; lateral field

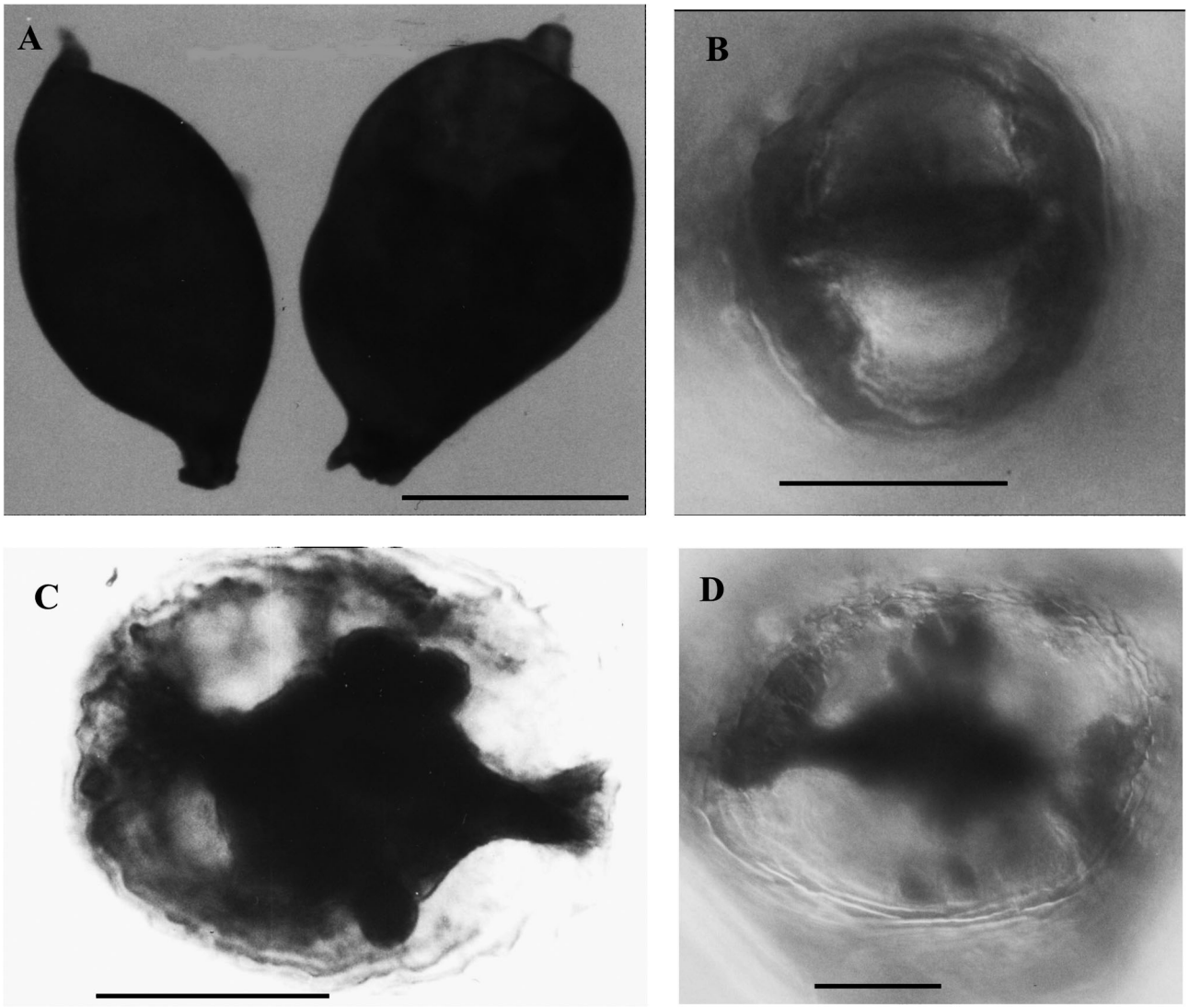

Fig. 1. Heterodera goldeni *(Iranian population). LM photos of cysts. A: Cysts; B: Vulval plate; C, D: Underbridge. (Scale bars: A = 200 μm; B-D = 40 μm.)*

with three lines and only occasional areolation. Tail length = 67 (59-74) μm (n = 8), length of hyaline tail portion = 36.5 (31-40) μm (n = 10). Phasmids located two to three annules posterior to anus, lens-like (*vs* pore-like according to original description). Second-stage juveniles originating from bermudagrass at Atlantis, Florida and reared on bermudagrass in a glasshouse at Beltsville were similar in morphological characters to those from the type locality. They also showed lens-like phasmids located closely posterior to the anus and stylet knobs with concave anterior faces.

Differential diagnoses

The *H. sacchari* species complex, comprising the species *H. sacchari*, *H. leuceilyma* and *H. goldeni*, is mainly characterised and distinguished from all other described *Heterodera* species by the presence of finger-like projections of the strongly developed underbridge in the vulval cone. The cysts are lemon-shaped and ambifenestrate with the vulval slit longer than the fenestra width; bullae are present and the cyst wall shows a distinct punctation. The lateral field in males and second-stage juveniles has three incisures, phasmids are lens-

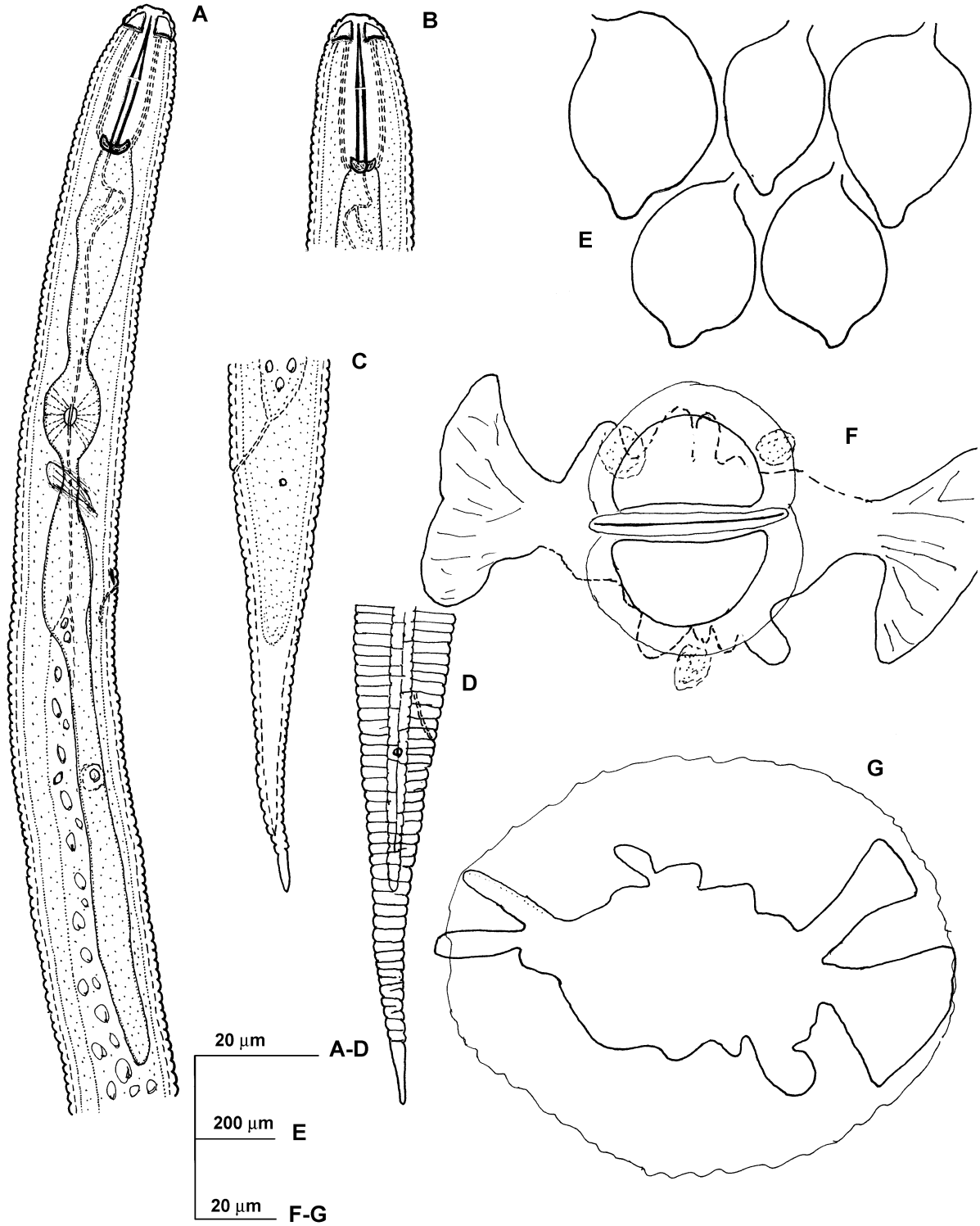

Fig. 2. Heterodera goldeni *(Iranian population). A-D: Second-stage juvenile. A: Anterior end; B: Head; C, D: Tail (C: Gilan population, D: Mazandaran population); E: Cysts; F: View of vulval-fenestral region showing fenestration, bullae and underbridge; G: Shape of underbridge.*

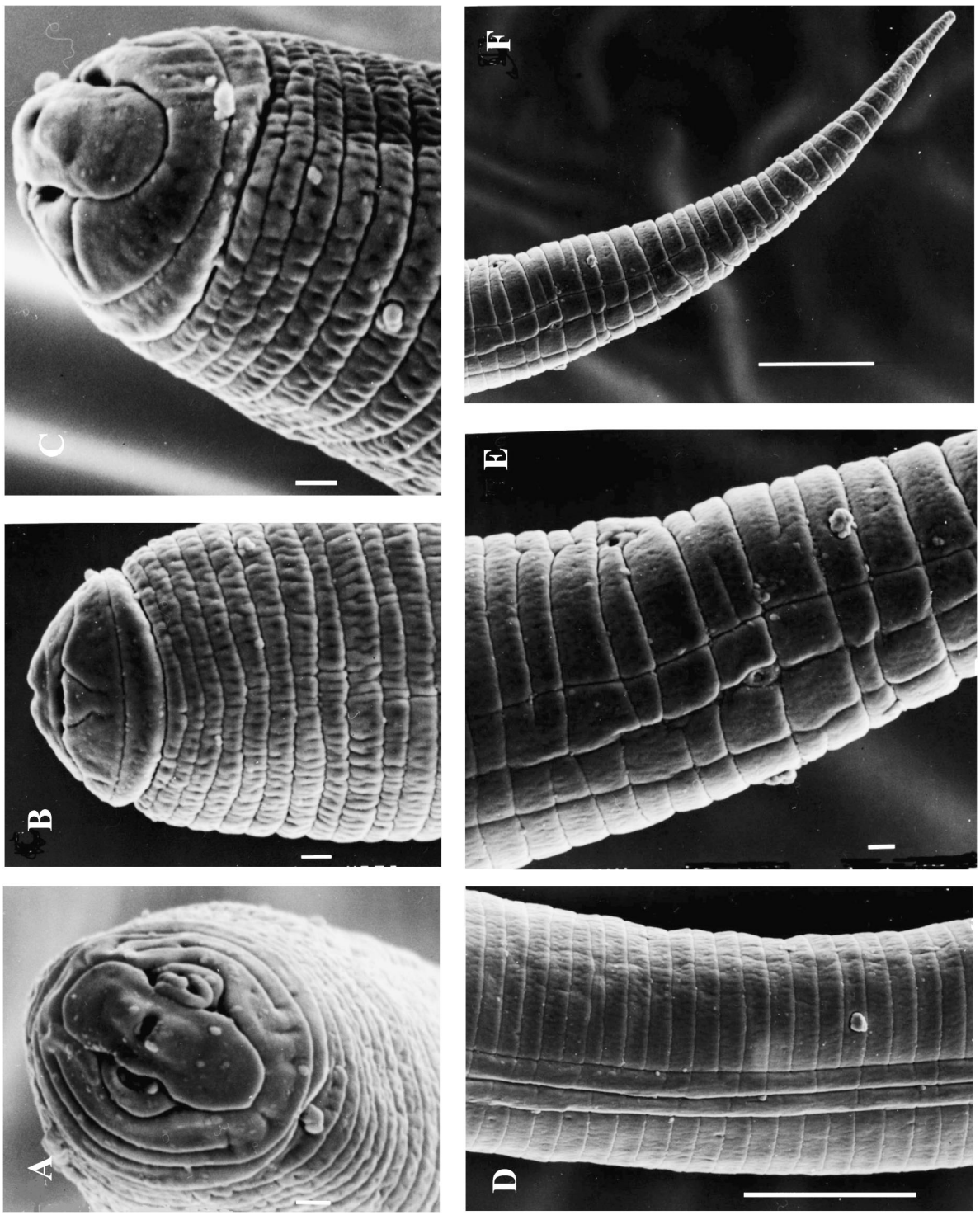

Fig. 3. Heterodera goldeni *(Iranian population). SEM photos of second-stage juveniles. A-C: Anterior end; D: Lateral field; E: Anus and phasmid; F: Tail. (Scale bars: A-C, E = 1 µm; D, F = 10 µm.)*

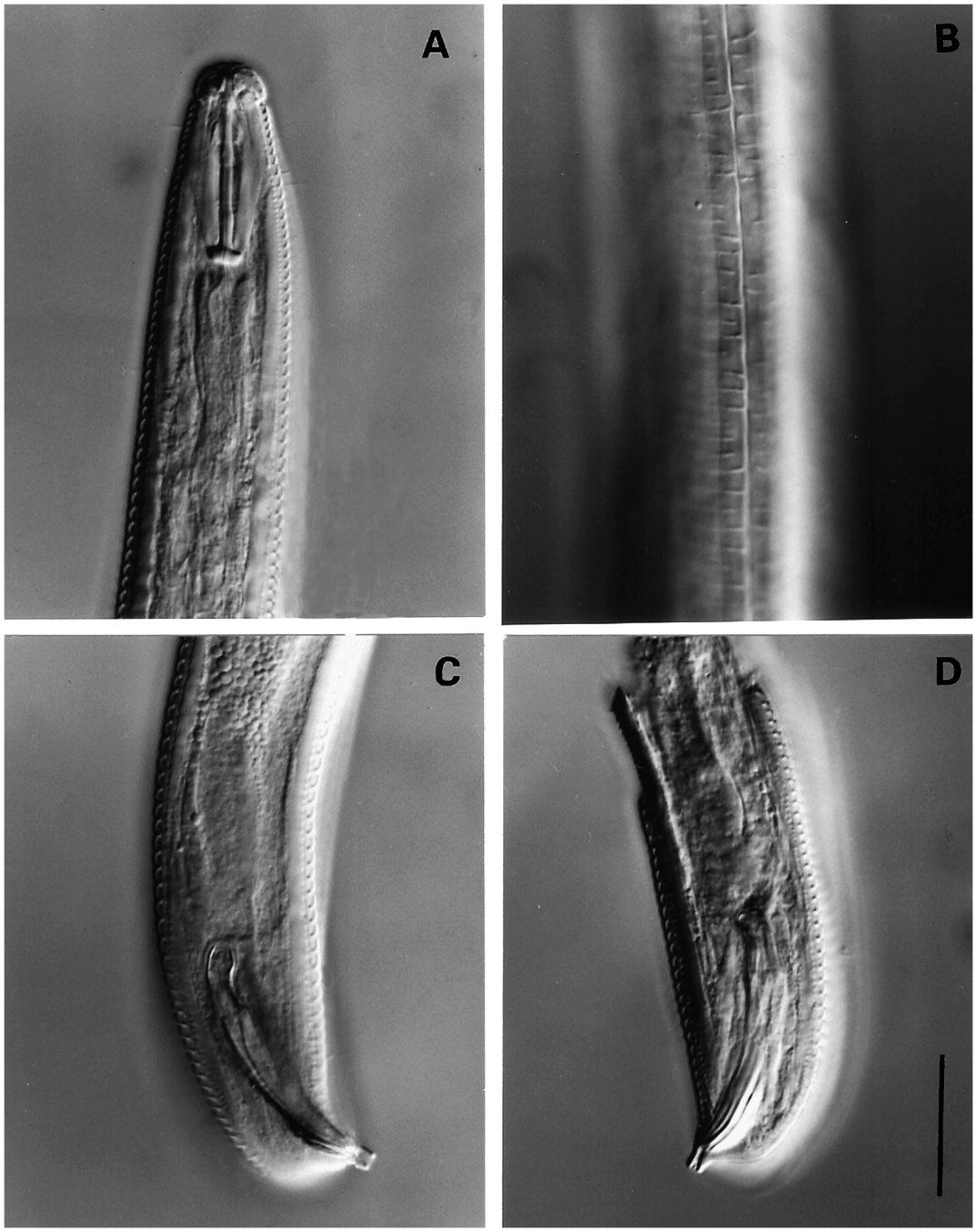

Fig. 4. Heterodera goldeni *(Israeli population). Male. A: Anterior end; B: Lateral field in mid-body region; C, D: Posterior ends. (Scale bar = 20 μm.)*

like in the second-stage juveniles and situated closely posterior to the anus but absent in males. Males have a penial tube. Hosts are Poaceae and Juncaceae. Based on molecular studies *H. sorghi* Jain, Sethi, Swarup & Srivastava, 1982 has been placed, together with the *H. sacchari* species complex, into the *sacchari*-group (Subbotin *et al*., 2001; Tanha Maafi *et al*., 2003). In most morphological characters this species resembles

the other three species in this group, but the strongly developed underbridge lacks finger-like projections and the phasmids in the second-stage juveniles are punctiform (confirmed by our studies). *Heterodera oryzae*, although sometimes found with *H. sacchari* on the same host and at the same location, is not included in the comparison here because of its morphological differences, including absence of finger like projections in the underbridge of the cyst vulval cone, shape and length of tail and stylet length in juveniles.

The three species in the s*acchari*-group are very close in morphometrics and other morphological characteristics, a fact which does not allow clear morphological distinction between the individual species, particularly after our present studies have emended the original description of *H. leuceilyma*. There appear to be only minor differences in cyst size and in morphometrics of the second-stage juveniles. *Heterodera sacchari* has generally smaller cysts than *H. goldeni* and *H. leuceilyma* (mean values 590-735 × 430-470 *vs* 707-766 × 437-481 and 830 × 480 μm, respectively) and shorter fenestral length (average 40-52 *vs* 60-61 μm and range of 48-50 μm, respectively). Second-stage juveniles of *H. leuceilyma* and *H. goldeni* differ from those of *H. sacchari* by having a slightly longer tail (means 67 and 59-65 *vs* 50-62 μm) and longer hyaline region (means 36.5-38 and 34-38.4 *vs* 26-31.9 μm). The stylet of *H. sacchari* females (23-25 μm) is slightly longer than that of *H. goldeni* females (21-23 μm) and the stylet of *H. leuceilyma* males (28-30 μm) is slightly longer than that of the other two species (24-30 μm and 26-29 μm). Distinct differences in the shape of the stylet knobs do not appear to exist.

Further and more extensive morphological studies, including SEM, of specimens in a broader spectrum of habitats is needed to further clarify the relationships and identities of these three species. A molecular evaluation of *H. leuceilyma* is also necessary.

Molecular characterisation of *H. sacchari* and *H. goldeni*

PCR amplified products were *ca* 1090 bp in length for all studied samples. Comparison of all available sequences for both species generated alignment in 1100 bp. Sequences of *H. sacchari* and *H. goldeni* differed from each other by six insertion/deletion events and by 22 nucleotides, 18 of them situated in the ITS1 and four in ITS2. The ITS sequences for two populations of *H. sacchari* from different hosts from Ghana and Côte d'Ivoire were almost identical. The difference between ITS sequences for *H. goldeni* samples varied from 1-6 nucleotides (*i.e.*, 0.1-0.5%).

Phylogenetic relationship within the *sacchari*-group

Mulvey (1972) placed *H. sacchari* and *H. leuceilyma* in the *schachtii*-group, based on vulval slit length, ambifenestrate fenestration of the cyst vulval cone and strong underbridge. Stone (1975) grouped both species in the *avenae*-group based on the lip region pattern of the second-stage juveniles although he later erected the *oryzae*-group, based on host range, and placed *H. sacchari* and *H. leuceilyma*, together with *H. oryzae* and *H. zeae* parasitising Gramineae, in this group (Stone, 1979). Sturhan (1998) proposed the *bifenestra*-group for all *Heterodera* species with three lateral lines in the second-stage juveniles and having Poaceae, Cyperaceae and other monocotyledonous plants as hosts. Subbotin *et al.* (2001), based on the results of phylogenetic analysis of cyst-forming nematodes using ITS-rDNA sequences, erected the *sacchari*-group with two members, *H. sacchari* and *H. sorghi*. Later, Tanha Maafi *et al.* (2003) considered another species as a member of the *sacchari*-group, *Heterodera* sp. 2, which is identified herein as *H. goldeni.* Relationships of the *sacchari*-group with other cyst nematodes was analysed using the ITS-rRNA gene sequences and the ITS-PCR-RFLP data (Rivoal *et al*., 2003). It has been shown that this group has close relationships with the *avenae*-group in all molecular phylogenetic trees (Subbotin *et al.*, 2000; Tanha Maafi *et al.*, 2003). In our present study, using trees yielded by two phylogenetic methods, *H. sacchari* and *H. goldeni* formed a highly supported clade with a basal position for *H. sorghi* within the group (Fig. 5).

Distribution and hosts of *H. sacchari*, *H. goldeni* and *H. leuceilyma*

Heterodera sacchari has been found in several African countries, *viz*., Congo-Brazzaville, Nigeria, Côte d'Ivoire, Liberia, Benin, Burkina Faso, Senegal, Cameroon, Gambia,
Ghana, Guinea, Togo (Luc & Merny, 1963; Jerath, 1968; Merny, 1970; Luc, 1986; Nobbs *et al.*, 1992; Coyne *et al*., 1996; Evans & Rowe, 1998; this paper). In Asia it is recorded from India (Swarup *et al*., 1964) and Pakistan (Maqbool, 1981). It has also been reported from Trinidad (Singh, 1974). Rice and sugarcane are the major field crops infected by this nematode. In addition to these hosts,

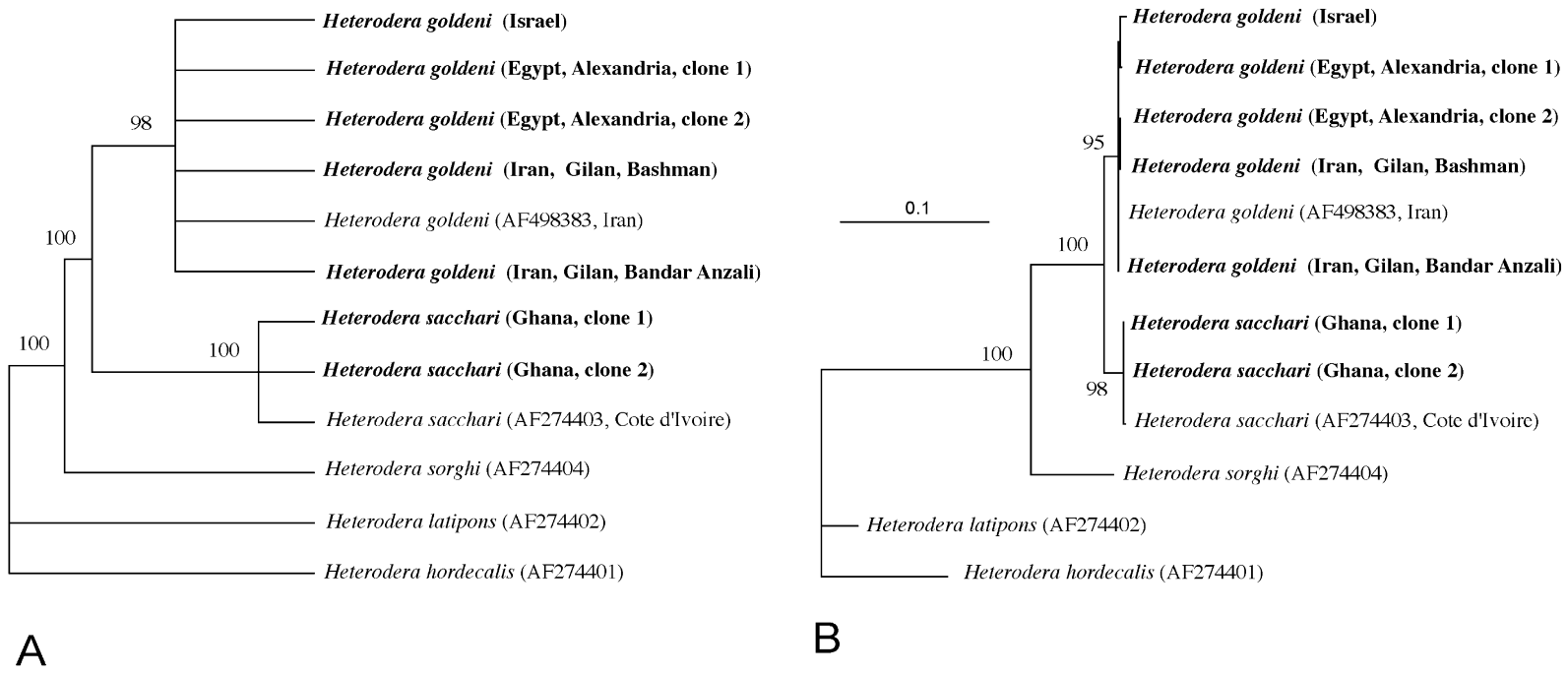

Fig. 5. *Phylogenetic relationships within the* sacchari*-group as inferred from the ITS1-5.8S-ITS2 sequences of rRNA. A: Strict consensus of 50 maximum parsimonious trees (number of parsimony-informative characters = 135; tree length = 295; CI = 0.9458; HI = 0.0542); B: One of six maximum likelihood trees (Ln L = −2866.55) obtained using GTR+I+G model of DNA evolution. Bootstrap values more than 70% are given on appropriate clade. Newly obtained sequences are indicated by bold letters.*

several wild grasses were considered to be hosts by Odihirin (1975) in Southern Nigeria, namely sour grass (*Paspalum conjugatum*), bermudagrass (*Cynodon dactylon*), carpet grass (*Axonopus compressus*), *Mariscus umbellatus*, *Brachiaria brizantha* and *Elesune indica*. As information on the morphology of the populations isolated is lacking, the status of these plants as hosts for *H. sacchari* should be confirmed with more precise identification of the nematodes concerned.

Heterodera goldeni was originally described from Alexandria, Egypt (Handoo & Ibrahim, 2002). The three populations from Iran used in this paper were found along the coast of the Caspian Sea at Nashtaroud, Mazandaran province, and at Bashman and Bandar Anzali, Gilan province (Tanha Maafi, 2002). The species was also identified at an additional coastal site west, and another east, of Bandar Anzali and on the coast east of Tonekabon, Mazandaran province (Sturhan, unpubl.). The soil at all sampling sites was sandy. The population from Israel was collected at Arava in the south of the country and the species was also identified from two sites 10 km and 20 km north of Tel Aviv. All records are from light soils. In Israel, *H. goldeni* was found around *Pennisetum clandestinum* and was reared on this plant in the glasshouse. In Egypt, the nematode was associated with *Panicum coloratum* whereas in Iran it was found on common reed, *Phragmites australis*, and in the rhizosphere of *Juncus acutus* and various unidentified Poaceae and Juncaceae. The population from Gilan (Bashman) was tested in the glasshouse on sugarcane ratoon seedlings and successfully multiplied on this plant after 2 months.

Heterodera leuceilyma is known from several localities along the southern east coast of Florida, USA (Di Edwardo & Perry, 1964). The type host is the grass *Stenotaphrum secundatum* on which it is considered to be a severe pathogen (Di Edwardo & Perry, 1964). Various specimens in the USDA Nematode collection at Beltsville, MD, USA, are recorded from bermudagrass (*C. dactylon*).

According to our present knowledge, *H. sacchari* is definitely present in tropical Africa, *H. goldeni* is found in the 'oriental' region and *H. leuceilyma* occurs in the subtropical south-east of North America. The species identity of *H. sacchari* records from other Asian countries, as well as from Trinidad, West Indies, may need confirmation. In addition, studies are required to see whether differences among the three species in host ranges and preferred hosts actually exist.

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