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Hemicaloosia vagisclera n. sp. (Nematoda: Caloosiidae) from Bermuda grass in Florida and its phylogenetic relationships with other criconematids

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Summary – *Hemicaloosia vagisclera* n. sp. is described from Bermuda grass (*Cynodon dactylon*) in Florida. This new species is characterised by females with body slightly ventrally arcuate in death, face with a prominent elliptical oral disc, large amphids, slender stylet with mean length 64 μm, lateral field consisting of a single longitudinal line marked by continuous and discontinuous transverse striae, oval and full spermatheca, sclerotised *vagina vera* and tail annuli width greater than that of remaining body annuli. Diagnostic characters for the males are a C-shaped body, head with 4-5 faint lip annuli and pronounced oval oral disc, lateral field with two longitudinal lines intersected by transverse striae, tail digitate in the distal portion posterior to bursa, distinctly annulated and with a round terminus. Molecular characterisation of *H. vagisclera* n. sp. using the D2-D3 domain of 28S rRNA, partial 18S rRNA and ITS rRNA gene sequences is also provided. The phylogenetic relationships of this species with other representatives of the suborder Criconematina are presented and indicate that *H. vagisclera* n. sp. has sister relationships with *Caloosia longicaudata* supporting the classification of *Caloosia* together with *Hemicaloosia* as separate genera in the family Caloosiidae. A diagnostic PCR-ITS-RFLP profile for *H. vagisclera* n. sp. is also given together with an identification key for seven known species of *Hemicaloosia*.

Keywords – *Cynodon dactylon*, D2-D3, description, *Hemicaloosia*, ITS-rDNA, key, molecular, morphology, morphometrics, new species, sod grasses, USA.

Hemicaloosia Ray & Das, 1978 contains warm climate species occurring in Africa, Asia (India) and the Americas. These species can be confused with sheath nematodes (*Hemicycliophora* sp.) because they possess a double cuticle. However, this second cuticle is membranous and fits tightly to the body, except in the postvulval body portion, unlike the detached and loose second cuticle of sheath nematodes. Furthermore, this extra cuticle consists of the outermost layer(s) of the body cuticle and it is not a duplication of the whole cuticle that has given rise to a sheath as in *Hemicycliophora* spp. According to Siddiqi (1980), this outermost layer is thinner than the body cuticle and difficult to discern in some species. Also, in contrast to *Hemicycliophora* spp., *Hemicaloosia* females have a closed vulva and longer tail than the males, which, in turn, have long and straight spicules compared with C-shaped spicules in sheath nematode males. Females of *Hemicaloosia* differ from those of *Caloosia* Siddiqi & Goodey, 1964 by the extra cuticle that is lacking in the latter genus and by lip annuli that are poorly differentiated from body annuli. Raski and Luc (1987) considered these characters insufficient for the separation of these two genera and ranked *Hemicaloosia* as a junior synonym of *Caloosia*.

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Siddiqi (2000) recognised *Hemicaloosia* as a valid taxon, which contains seven species listed in the identification key. In this study, we follow the classification proposed by Siddiqi for this genus. In continental USA, species of *Hemicaloosia* and *Caloosia* have been detected by R.P. Esser (Lehman, 2002) in Florida. However, the reports of *Caloosia* species in Florida need to be confirmed. Unidentified *Hemicaloosia* juveniles were found in 2003 in the Wisconsin Arboretum (Madison, WI, USA) and fragments of 18S rRNA gene sequences were deposited in GenBank by Powers *et al*. (2010). The presence of amphimictic *Hemicaloosia* species in Florida was observed by R.P. Esser for many years in samples from tree farms, pasture lands and sod grass operations (records of the Florida Department of Agriculture and Consumer Services). These amphimictic *Hemicaloosia* were tentatively identified as *H. americanae* Ray & Das, 1978 or *H. paradoxa* (Luc, 1958) Ray & Das, 1978. Another report of the non-amphimictic *H. luci* Dhanachand & Jairajpuri, 1980 by Esser (Lehman, 2002) needs to be confirmed. Our present morphological analyses of Florida amphimictic *Hemicaloosia* populations indicated discrepancies between the morphology of these populations and that of *H. americanae* in India (Ray & Das, 1978) and *H. paradoxa* in Ivory Coast (Luc, 1958), suggesting that they belong to an undescribed species. These Florida populations have males and parasitise Bermuda grass, *Cynodon dactylon* (L.) Pers., in many localities. There is a lack of information on the molecular characteristics of *Hemicaloosia* species, and their phylogenetic relationships to *Caloosia* and *Hemicycliophora* species have not been studied. Only fragments of 18S rRNA gene sequences of the unidentified *Hemicaloosia* from a prairie in the Wisconsin Arboretum are available.

The major objectives of this work were: *i*) to characterise, morphologically and morphometrically, Florida *Hemicaloosia* populations from Bermuda grass and describe them as a new species, namely *Hemicaloosia vagisclera* n. sp.; *ii*) to characterise molecularly *H. vagisclera* n. sp. using the D2-D3 domain of 28S rRNA, ITS1-5.8S-ITS2 rRNA, and partial 18S rRNA gene sequences; *iii*) to reconstruct and test the phylogenetic position of *H. vagisclera* n. sp. within the Criconematina using analysis of the genes; and *iv*) to provide an identification key for the known species of the genus *Hemicaloosia*.

Materials and methods

Nematode population, glasshouse cultures, light and scanning microscopy

Two sod grass sites in Lake Wales and Citra, both in central and north Florida, and a tree farm nursery in Ocala, central Florida, were surveyed during all seasons in 2010-2011. More than 60 composite root and soil samples were collected in three operations. Nematodes were extracted from soil by the sieving, decanting and centrifugal flotation method (Jenkins, 1964). The sampling started in November 2010 in Lake Wales where a male-less population of *Hemicaloosia*, consisting mainly of females with a functional spermatheca filled with sperm, was collected from a stand of Saint Augustine grass, *Stenotaphrum secundatum* (Walter) Kuntze, associated with Bermuda grass. Subsequent sampling in the Ocala and Citra sites indicated that large *Hemicaloosia* populations (16 nematodes per 100 cm^3 of soil) with males were concentrated in patches with Bermuda grass stands. These populations were associated with the ring nematode *Criconemoides ornatus* Raski, 1958 and *Peltamigratus christiei* (Golden & Taylor, 1956) Sher, 1964. Plugs of soil and roots collected from nematode-infested field stands of Bermuda grass were maintained in pots in a glasshouse for 11 months as a source of specimens for our studies. Nematode specimens were killed and fixed in hot aqueous 2% formaldehyde + 1% propionic acid, dehydrated in ethanol vapour and mounted in dehydrated glycerin (Hooper, 1970). Measurements of specimens were made with an ocular micrometer and drawings with a *camera lucida*. Abbreviations used are defined in Siddiqi (2000). Photographs were taken with Leica (Wild MPS 46/52 and Leica DFC 320) cameras mounted on Nikon (Optiphot) and Leica DM 2500 compound microscopes. Additional light microscopic photographs of females and males were taken with an automatic Infinity 2 camera attached to an Olympus BX51 microscope equipped with Nomarski differential interference contrast. Heat-killed specimens fixed in FAA were processed for scanning electron microscopy according to Chitambar (1992).

DNA extraction, PCR, PCR and sequencing

DNA was extracted from several dead specimens using the proteinase K protocol. Detailed protocols for DNA extraction, PCR, cloning and sequencing were as described by Tanha Maafi *et al.* (2003). The following primers were used for amplification of three rRNA gene fragments: ITS-rRNA-TW81 (5′-GTTTCCGTAGGTGAACCTGC-

3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) Tanha Maafi *et al.* (2003); D2-D3 of 28S rRNA-D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (Subbotin *et al.*, 2006); partial 18S rRNA-G18SU (5′-GCTTGTCTCAAA GATTAAGCC-3′) and R18Tyl1 (5′-GGTCCAAGAATTT CACCTCTC-3′) (Chizhov *et al.*, 2006) primers. The newly obtained sequences have been submitted to the GenBank database under the numbers JQ246422-JQ246429.

RFLP-ITS-rRNA

The PCR product of the ITS-rRNA was purified using the QIAquick Gel Extraction Kit (Qiagen). A volume of 3-7 μl of purified product was digested by one of the following restriction enzymes: *Ava*I, *Bsh*1236I, *Dra*I, *Hin*fI, *Hin*6I and *Msp*I in the buffer stipulated by the manufacturer. The digested DNA was run on a 1% TAE buffered agarose gel, stained with ethidium bromide, visualised on a UV transilluminator and photographed. The length of each restriction fragment from the PCR products was obtained by a virtual digestion of the sequences using WebCutter 2.0 (www.firstmarket.com/cutter/cut2.html).

Phylogenetic analyses

The newly obtained sequences for each gene were aligned using ClustalX 1.83 (Thompson *et al.*, 1997) with default parameters and corresponding published gene sequences (Subbotin *et al.*, 2005, 2006; Bert *et al.*, 2008; Holterman *et al.*, 2009; Van den Berg *et al.*, 2011). Outgroup taxa for each dataset were chosen according to the results of previously published data (Subbotin *et al.*, 2005, 2006; Bert *et al.*, 2008; Holterman *et al.*, 2009). Sequence datasets were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) under the GTR + I + G model as described in Van den Berg *et al.* (2011). Posterior probability (PP) values more than 70% are given on appropriate clades.

Results

Hemicaloosia vagisclera[*] n. sp.
(Figs 1-5)

[*] Specific epithet derived from the Latin anatomical term *vagina* and the Greek term σκληρος = hardened.

Measurements

See Tables 1 and 2.

Description

Female

Body slightly arcuate ventrad. Second membranous cuticle fitting tightly to body and thinner than body cuticle. Lateral field consisting of a single longitudinal line marked by continuous and discontinuous transverse striae with breaks and anastomoses, and extending from near anterior end of body to mid-tail without marking the terminal and tapered portion of the body. SEM *en face* view showing a head with a prominent, elevated and elliptical oral disc. Oral disc attached ventrally and dorsally to a lower ring-like labial plate (see Geraert (1997) for terminology). Thick, ring-like labial plate positioned slightly posterior to internal margin of first lip annulus. Two large, open semicircular amphids delimited by oral disc and a large portion of ring-like labial plate. First lip annulus narrower than second. Cephalic framework weakly sclerotised. Stylet slender, elongated, slightly curved dorsally. Stylet knobs round, often sloping posteriorly, conferring a wrench-like shape to stylet base in two-dimensional view. Distance of dorsal pharyngeal gland duct orifice from stylet base (DGO) slightly longer in some populations (Citra) than in others. Basal pharyngeal bulb almost pear-shaped, fused with broad isthmus. Nerve ring at level of isthmus and basal bulb fusion. Hemizonid two annuli long, located 1-2 annuli anterior to excretory pore. Excretory pore located from three annuli anterior to three annuli posterior to pharyngo-intestinal junction. Body annuli 2.4-2.9 μm wide at mid-body, becoming wider (3.5 μm) in attenuated portion of tail. Annuli margins prominent, their cuticle marked by irregularly-spaced longitudinal ridges. Transverse striae with occasional anastomoses visible on tail annuli. Vulva a transverse, almost C-shaped slit, 10-11 μm long with a slightly overhanging anterior lip. Vagina almost sinusoidal. *Vagina vera* heavily sclerotised. Spermatheca prominent, oval, filled with round sperm. Tail elongate, tapering uniformly in anterior fourth, then somewhat more abruptly to a slight constriction and continuing as an attenuated, narrower portion to a finely rounded to acute terminus.

Male

Body C-shaped. Head rounded, slightly set off by interruption in body annuli in 60% of specimens and marked by 4-5 faint lip annuli in remaining 40%. SEM

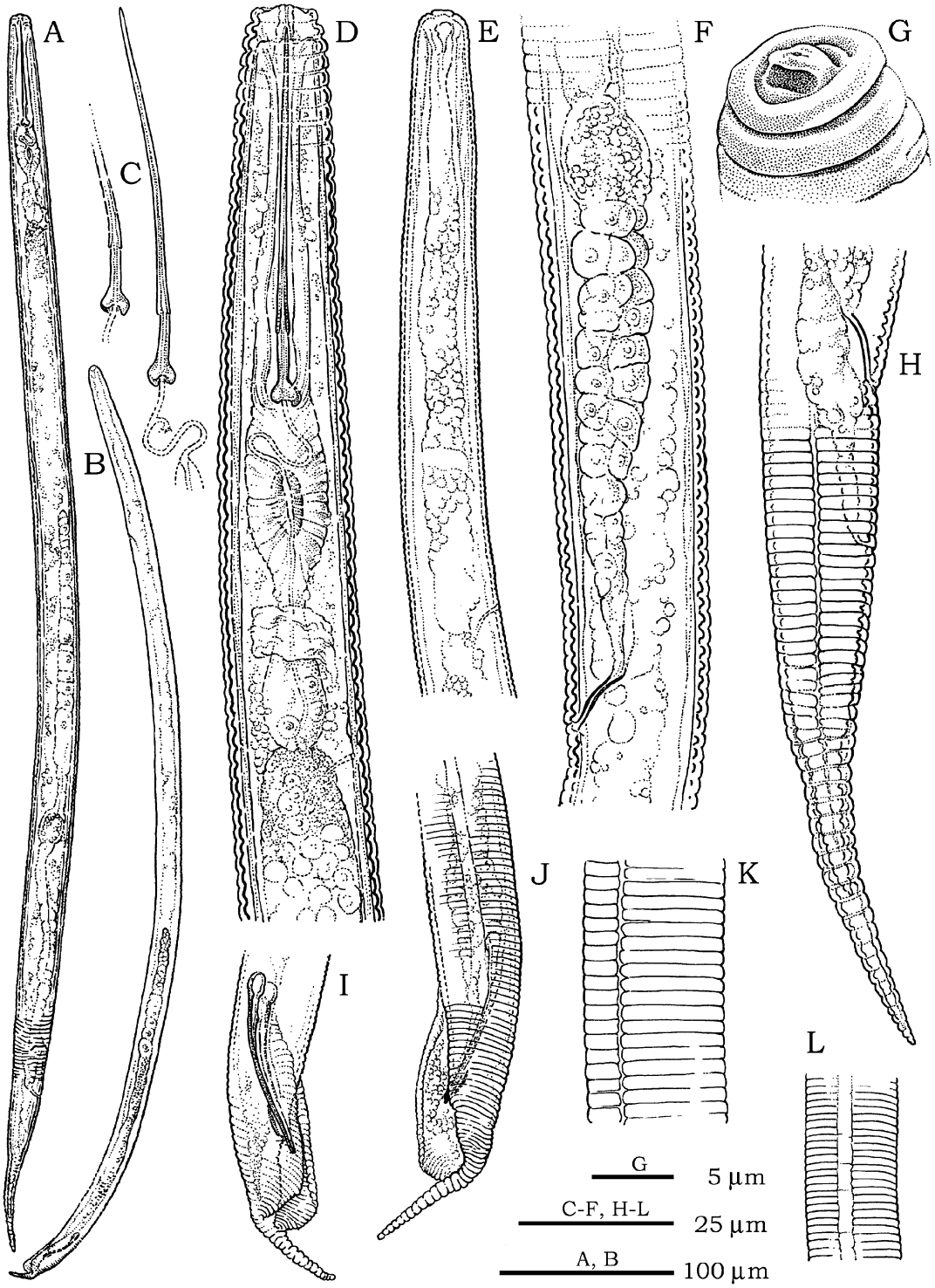


Fig. 1. Camera lucida *line drawings of* Hemicaloosia vagisclera *n. sp. A: Entire female; B: Entire male; C: Female stylet; D: Female pharyngeal region; E: Male pharyngeal region; F: Female vulval region with spermatheca; G: Schematic view of face pattern of female; H: Female posterior region; I, J: Male tail; K: Female lateral field; L: Male lateral field.*

Fig. 2. *Light micrographs of* Hemicaloosia vagisclera *n. sp. female. A: Pharyngeal region; B: Entire body; C: Vulval region showing sclerotised* vagina vera*; D: Lateral field; E: Tail; F: Posterior body portion. (Scale bars: A, C, D, F = 25 μm; B = 50 μm; E = 20 μm.)*

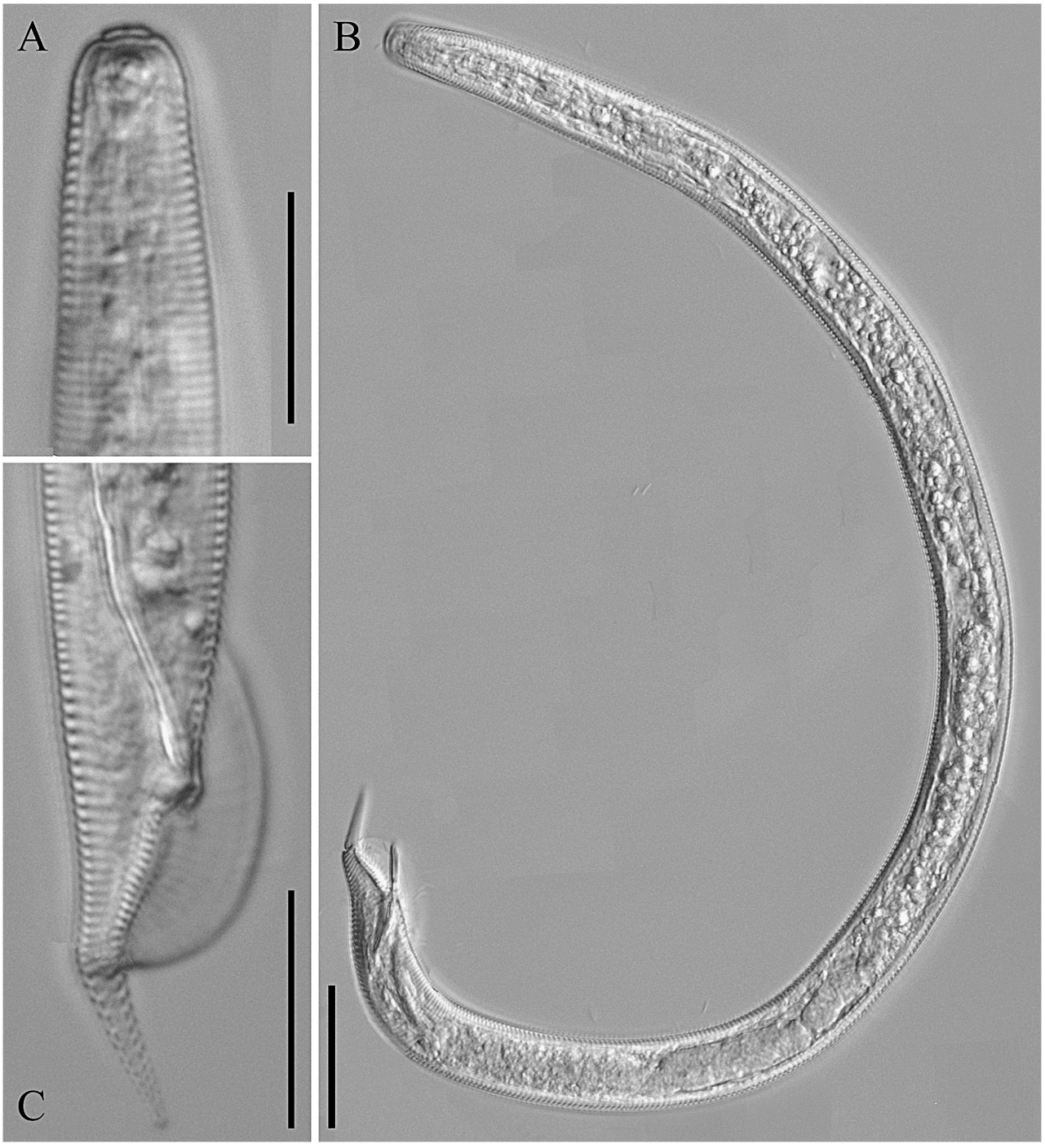


Fig. 3. *Light micrographs of* Hemicaloosia vagisclera *n. sp. male. A: Anterior end showing prominent oral disc; B: Entire body; C: Copulatory apparatus. (Scale bars: A, B = 20 μm; C = 35 μm.)*

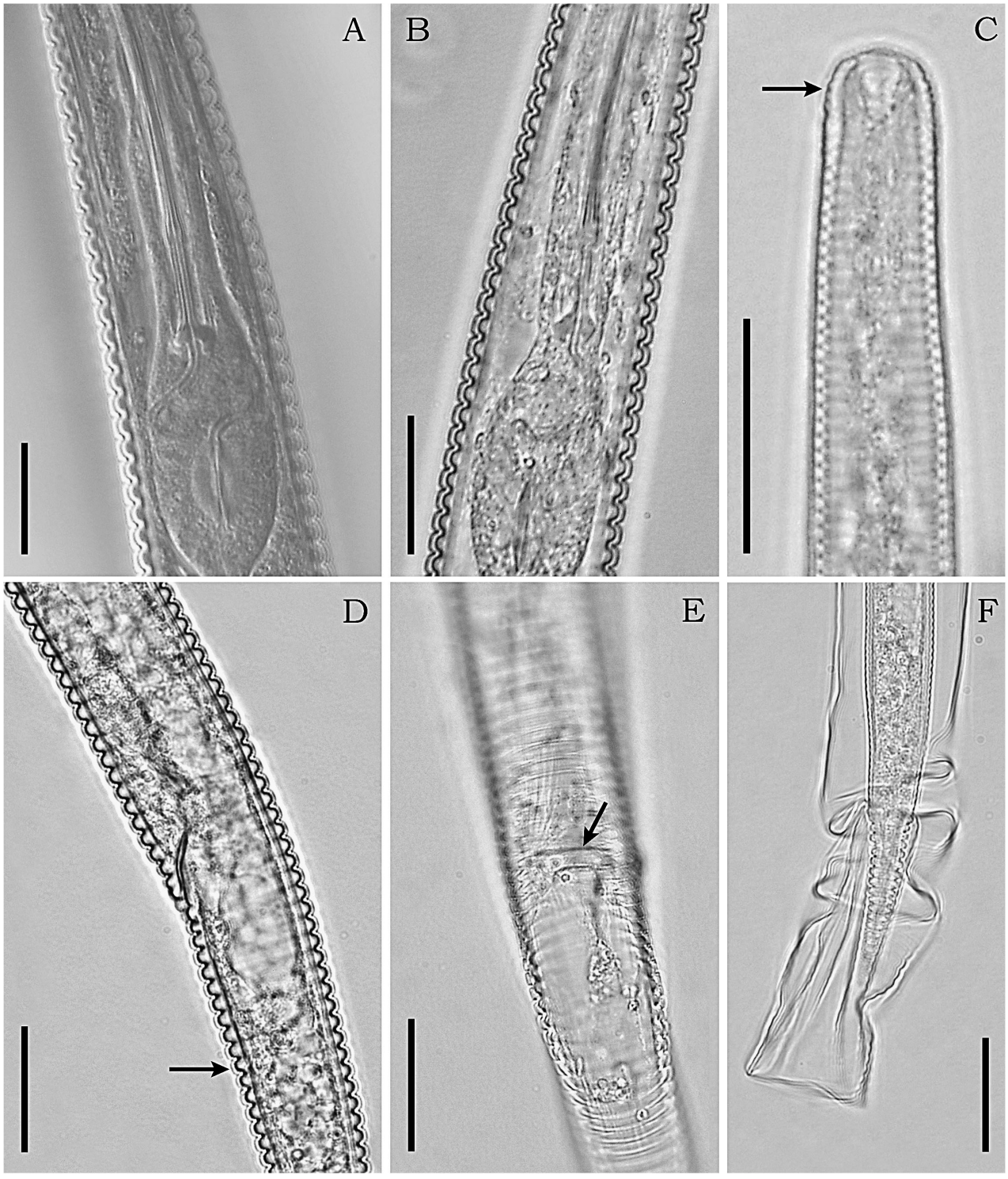
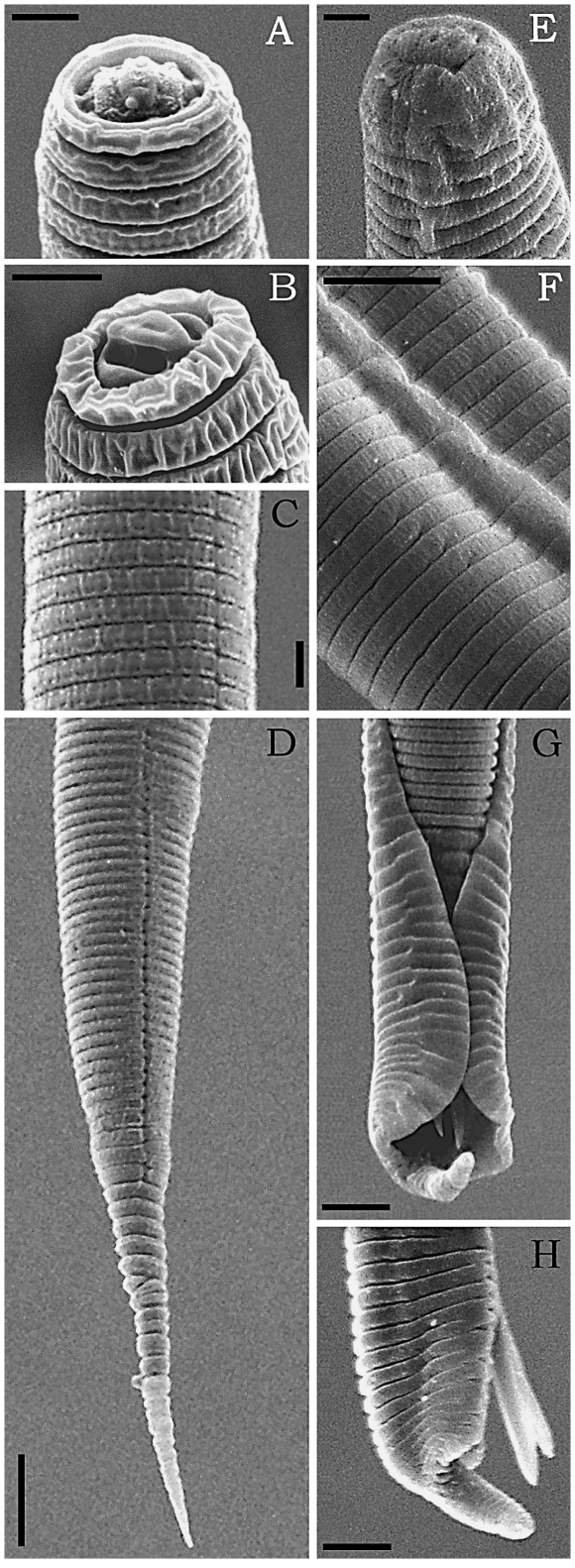


Fig. 4. *Light micrographs of* Hemicaloosia vagisclera *n. sp. A, B: Female pharyngeal region showing double cuticle (A) and wrench-like stylet knobs; C: Male anterior end showing faint lip annuli (arrow); D, E: Female vulval region showing sclerotised* vagina vera *(D), anus position (arrow) and vulval slit (E, arrowed); F: Moulting juvenile posterior end within moulted cuticle. (Scale bars = 20 μm.)*



en face view showing a prominent oval oral disc similar to that of female. SEM micrographs showing amphids covered by exudates and obscuring view of labial plate and 4-5 faint lip annuli, which were visible only with LM observations in 40% of the specimens examined. Labial framework visible. Stylet absent. Pharynx degenerate. Excretory pore distinct. Body annuli 0.5 μm thick at mid-body, 1 μm thick in distal portion of tail posterior to bursa. Each annulus marked by irregularly-spaced longitudinal ridges and thickened margins. Lateral field with two longitudinal lines intersected by transverse striae dividing field into a single row of blocks. Width of each block equalling that of two adjacent body annuli. Bursa peloderan, marked by transverse striae with anastomoses and enveloping 54% of tail. Spicules straight, proximal portion of shaft attached to slightly curved manubrium. Gubernaculum distinct. Distal tail portion posterior to bursa digitate, with distinct annuli and round terminus.

Juveniles

Juveniles in soil samples easy to recognise as usually wrapped in moulted cuticle. During moulting, the external membranous cuticle is shed, remaining loosely attached to nematode body and protecting it during regeneration of new membranous cuticle. Second-stage juveniles (J2) not observed and only moulted cuticle of this stage was observed attached to third-stage juveniles (J3). Difference in stylet length among J2, J3 and fourth-stage juveniles (J4) allowed for separation of juvenile life stages. Stylet lengths of J3 and J4 were 49.5-53.0 and 57 μm, respectively. Moulted cuticle of J2 attached to J3 often contained moulted stylet of J2, which was 40-43 μm long.

Fig. 5. *Scanning electron micrographs of* Hemicaloosia vagisclera *n. sp. A: Female head patterns with prominent and elliptical oral disc. Note ring-like labial plate encased inside first lip annulus (amphids obscured); B: Female head patterns showing a prominent and elliptical oral disc attached ventrally and dorsally to an oblong and ring-like labial plate. Note large amphids and flattened first lip annulus narrower than second; C: Female lateral field; D: Female posterior end. Note anus and large annuli in posterior half portion of the tail; E: Male anterior end showing prominent oral disc and undefined lip annuli; F: Male lateral field; G: Ventral view of male posterior end showing folded bursa alae and digitate terminal portion of tail; H: Lateral view of male tail showing spicules and digitate terminal portion of tail. (Scale bars: A-C, F-H = 5 μm; D = 15 μm; E = 2.5 μm.)*

Table 1. *Morphometrics of females of* Hemicaloosia vagisclera *n. sp. from Florida and related species,* H. americanae *and* H. paradoxa*. All measurements are in µm and in the form: mean ± s.d. (range).*

Character	*H. vagisclera* n. sp.				*H. americanae* (after Ray & Das, 1978)	*H. paradoxa* (after Luc, 1958)
	Ocala (type locality)		Citra	Lake Wales	India	Ivory Coast
	Holotype	Paratypes				
n	–	15	7	20	15	–
L	713	700 ± 55.3 (619-822)	772 ± 42.0 (714-825)	745 ± 40.0 (674-851)	790 (740-845)	680-820
a	26.9	27.4 ± 1.8 (24.6-30.3)	29.6 ± 1.4 (27.5-31.3)	29.7 ± 1.6 (26.8-32.3)	29 (27-32)	23.7-29.0
b	5.7	5.7 ± 0.3 (5.2-6.1)	6.2 ± 0.1 (6.0-6.3)	5.7 ± 0.3 (5.1-6.3)	6.5 (6.2-6.8)	4.9-5.7
c	6.9	7.0 ± 0.7 (6.2-9.3)	6.9 ± 0.4 (6.3-7.3)	7.8 ± 0.4 (6.8-8.6)	7.7 (6.6-10.4)	–
c′	4.9	5.0 ± 0.4 (4.2-5.8)	5.2 ± 0.2 (5.1-5.5)	4.6 ± 0.3 (4.2-5.2)	–	–
V	81.6	82 ± 1.3 (80-85)	82 ± 0.6 (81-83)	83 ± 0.7 (82-84)	83 (81-87)	78.1-84.0
DGO from stylet base	6.8	6.5 ± 0.4 (5.9-7.1)	7.9 ± 0.5 (7.0-8.9)	6.6 ± 0.4 (6.0-7.2)	–	–
Stylet	64.5	63.5 ± 2.2 (61-69.5)	66.5 ± 0.8 (65-67.5)	64.5 ± 2.3 (60.5-68.5)	61 (60-64)	61-65
Metenchium	52.0	52.3 ± 2.0 (49.3-57.4)	54.8 ± 1.1 (53.4-56.3)	–	–	–
Telenchium	12.5	11.3 ± 0.7 (10.0-12.3)	11.7 ± 0.7 (11.0-12.8)	12.4 ± 0.5 (11.0-13.3)	–	–
Stylet knob height	4.0	4.3 ± 0.3 (3.9-4.9)	3.9 ± 0.2 (3.4-4.0)	4.3 ± 0.4 (3.9-5.0)	–	–
Stylet knob width	5.8	5.6 ± 0.3 (5.0-5.9)	5.4 ± 0.5 (4.8-5.9)	5.8 ± 0.3 (5.2-6.9)	–	–
Lip region height	5.5	5.4 ± 0.5 (4.5-6.4)	5.4 ± 0.5 (4.8-5.9)	5.6 ± 0.5 (4.9-6.9)	–	5.78
First lip annulus diam.	11.4	11.3 ± 0.5 (10.8-12.3)	11.7 ± 0.2 (11.4-11.9)	11.7 ± 0.6 (10.8-12.8)	–	–
Second lip annulus diam.	14	13.2 ± 0.8 (11.8-14.3)	13.3 ± 0.5 (12.6-13.9)	13.3 ± 0.6 (12.6-15.0)	–	–
First body annulus diam.	15.5	14.9 ± 0.8 (13.8-16.8)	14.9 ± 0.4 (14.8-15.8)	14.7 ± 0.7 (13.8-16.3)	–	–
Second body annulus diam.	16.5	15.7 ± 0.8 (14.8-17.8)	15.8 ± 0.5 (15.0-16.8)	15.4 ± 0.7 (14.7-17.3)	–	–
Pharynx	124	121 ± 5.4 (111-134)	124 ± 6.8 (112-130.5)	128.5 ± 4.7 (121-137)	–	129*
Excretory pore-anterior end dist.	111	111 ± 10.1 (95-134)	118.5 ± 7.6 (106-129)	126 ± 7.7 (117-143.5)	148	131*
Mid-body diam.	26.5	25.5 ± 1.6 (22.6-27.7)	25.7 ± 0.9 (24.7-26.7)	25.0 ± 1.2 (23.0-28.0)	–	30*
Vulval body diam.	23.3	21.3 ± 0.8 (20.2-23.2)	22.9 ± 0.5 (22.2-23.7)	22.4 ± 0.9 (20.7-24.2)	–	27*
Anal body diam.	20.8	19.5 ± 1.0 (17.8-21.2)	20.6 ± 0.5 (19.8-21.2)	20.5 ± 1.1 (17.8-22.7)	–	26*
Vulva to anterior body	582	574 ± 52.2 (495-684)	634 ± 39.6 (578-683)	620 ± 34.2 (553-708)	–	–
Vulva to anus	29.8	26.5 ± 2.6 (22.6-30.6)	27.8 ± 2.2 (24.7-30.6)	29.7 ± 3.6 (24.0-35.6)	–	–
Vulva-tail terminus dist.	134	126 ± 7.9 (110-137.5)	138 ± 2.9 (134.5-141.5)	126 ± 5.8 (118-137)	110.6*	130*
Spermatheca length	18	17.9 ± 2.2 (14.0-21.7)	20.9 ± 3.5 (16.0-25.7)	19.5 ± 1.9 (16.0-22.7)	–	–
Spermatheca width	12	10.3 ± 1.4 (7.0-11.8)	12.9 ± 1.5 (10.8-14.8)	11.2 ± 1.5 (9.0-13.8)	–	–
Spermatheca to vulva	101	81 ± 12.8 (54.5-100)	90 ± 16.0 (60-102)	87 ± 12.2 (70-117)	–	–
Sclerotised *vagina vera* length	11.5	12.0 ± 0.9 (10.8-13.8)	14.3 ± 0.9 (13.0-15.8)	13.2 ± 1.4 (10.0-14.8)	–	–
Tail	103	99 ± 7.9 (80-110)	110 ± 1.2 (109-112)	95 ± 3.6 (83-109)	82.7*	105*

Table 1. *(Continued).*

Character	*H. vagisclera* n. sp.				*H. americanae* (after Ray & Das, 1978)	*H. paradoxa* (after Luc, 1958)
	Ocala (type locality)		Citra	Lake Wales	India	Ivory Coast
	Holotype	Paratypes				
Hyaline portion	38	24.3 ± 5.8 (14.8-37.6)	23.7 ± 2.7 (19.8-26.7)	19.2 ± 3.6 (8.4-23.7)	–	30*
R	279	277 ± 10.8 (264-294)	277 ± 6.1 (259-285)	268 ± 6.9 (259-284)	256 (231-270)	256-263
RSt	26	25 ± 1.1 (24-27)	24 ± 0.9 (23-25)	24 ± 1.4 (21-26)	–	23-25
ROes	47	–	45 ± 3.8 (41-53)	–	–	42-45
Rex	43	44 ± 1.5 (42-48)	43 ± 3.4 (39-50)	45 ± 2.6 (42-50)	50 (48-53)	45*
RV	50	51 ± 1.9 (47-55)	49 ± 1.8 (47-52)	47 ± 2.1 (44-52)	41 (28-43)	50-56
RVan	12	11 ± 1.1 (9-13)	11 ± 1.3 (9-14)	11 ± 1.2 (9-13)	11 (10-13)	9*
Ran	38	39 ± 1.9 (35-43)	38 ± 1.6 (36-41)	35 ± 2.0 (32-40)	30 (18-33)	40*
VL/VB	5.8	6.0 ± 0.2 (5.7-6.4)	5.9 ± 0.1 (5.8-6.1)	5.6 ± 0.2 (5.2-6.0)	–	5*
PV/anal body diam.	6.4	6.4 ± 0.4 (5.8-7.1)	–	–	–	4.8*

* Values calculated from figures in original descriptions.

Type host and locality

Bermuda grass, *Cynodon dactylon* (L.) Pers., roots and associated soil collected from a tree farm in Ocala, Marion County, FL, USA (latitude 28°98′59.8″N; longitude 82°14′24.3″W). The soil type is sandy, the annual precipitation 1500 mm and the climate is subtropical.

Other hosts and localities

Saint Augustine grass, *Stenotaphrum secundatum* (Walter) Kuntze, roots and associated soil collected in Lake Wales, Polk County, FL, USA (latitude 27°56′51″N; longitude 81°26′21″W) and Bermuda grass, in Citra, Marion County, FL, USA (latitude 29°24′30″N; longitude 82°09′55″W). Soil type, annual precipitation and climate are as in type locality.

Type material

Holotype female, 30 females and 21 male paratypes deposited at the Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (CNR), U.O.S. di Bari, Italy (collection numbers IPP-K-1056 to K-1068). Additional paratypes were distributed to the United States Department of Agriculture Nematode Collection, Beltsville, MD (collection number IPP-K-1069), University of California Riverside Nematode Collection, Riverside, CA (collection number IPP-K-1070) and WaNeCo, Plant Protection Service, The Netherlands, Wageningen (collection number IPP-K-1067 and 1068).

Diagnosis and relationships

Hemicaloosia vagisclera n. sp. female is characterised morphologically by the body being slightly arcuate ventrally, slender stylet, stylet knobs round, posteriorly sloping with a distinct cavity, conferring a wrench-like shape to the stylet base in two-dimensional view. Lateral field consisting of a single longitudinal line marked by continuous and discontinuous transverse striae with breaks and anastomoses, oval spermatheca filled with round sperm, a slit-like vulva, a sclerotised *vagina vera* and annuli in the attenuated portion of the tail thicker than body annuli. Males are characterised by the C-shaped body, head set off by an interruption in body annuli, set off by body constriction and showing 4-5 faint lip annuli (visible only in 40% of the specimens) and a pronounced oval oral disc, annuli marked by irregularly-spaced longitudinal ridges, lateral field with two longitudinal lines intersected by transverse

Table 2. *Morphometrics† of males of* Hemicaloosia vagisclera *n. sp. from Florida and related species,* H. americanae *and* H. paradoxa*. All measurements are in μm and in the form: mean ± s.d. (range).*

Character	*H. vagisclera* n. sp.		*H. americanae* (after Ray & Das, 1978)	*H. paradoxa* (after Luc, 1958)
	Ocala (type locality) Paratypes	Citra	Orissa, India	Adiopodoumé, Ivory Coast
n	17	20	7	–
L	560 ± 28.5 (500-607)	610 ± 26.3 (563-674)	640 (615-660)	548-663
a	30.4 ± 1.7 (27.8-34.1)	35.7 ± 1.9 (31.9-38.6)	40 (35-42)	26.4-30.0
b	4.9 ± 0.3 (4.5-5.4)?	5.0 ± 0.3 (4.6-5.6)?	6.6 (6.1-6.9)?	–
c	18.3 ± 1.2 (16.3-21.0)	18.9 ± 1.0 (17.4-21.3)	12.7 (10.6-14.1)	12.7-18.5
c′	2.9 ± 0.2 (2.2-3.4)	2.7 ± 0.1 (2.5-3.1)	–	2.4*
DGO from stylet base	–	–	–	–
Stylet	–	–	–	–
Metenchium	–	–	–	–
Telenchium	–	–	–	–
Stylet knob height	–	–	–	–
Stylet knob width	–	–	–	–
Lip region height	5.2 ± 0.6 (4.2-5.9)	5.2 ± 0.4 (4.6-5.9)	–	–
Lip region width	8.8 ± 0.3 (7.9-9.1)	8.9 ± 0.3 (8.1-9.7)	–	–
Second lip diam.	–	–	–	–
First body annulus diam.	–	–	–	–
Second body annulus diam.	–	–	–	–
Pharynx	118 ± 7.2 (100-126)?	120 ± 7.0 (107-134)?	–	–
Excretory pore-anterior end dist.	98.5 ± 5.7 (90-109)	105 ± 4.8 (98-113)	97.5*	141*
Mid-body diam.	18.4 ± 0.9 (16.8-19.8)	17.1 ± 0.7 (15.8-18.8)	–	19*
Anal body diam.	10.4 ± 0.5 (9.8-11.8)	11.6 ± 0.4 (10.9-12.3)	–	14*
Testis length	176 ± 25.2 (134-226)	150 ± 29.7 (91-192)	–	–
Gubernaculum	5.7 ± 0.4 (5.1-6.9)	5.4 ± 0.3 (5.0-6.0)	6 (5-7)	5.7*
Spicule	32.9 ± 1.7 (29.5-35.6)	32.1 ± 1.5 (30.2-34.1)	34 (33-36)	30-35
Bursa length	33.3 ± 2.1 (29.7-36.6)	32.7 ± 1.4 (30.6-36.6)	38.2*	47.7*
Tail	30.6 ± 2.3 (23.7-33.6)	32.2 ± 1.1 (30.2-34.6)	34.2-46.5*	36.2*
Tail portion after bursa	14.0 ± 0.9 (12.1-14.8)	14.9 ± 1.3 (12.0-17.3)	23.5-26.9*	15*

Question marks indicate tentative measurements due to the almost indistinct pharyngo-intestinal valve.
* Values calculated from figures in original descriptions.

striae, distal portion of tail posterior to bursa digitate with distinct annuli and a round terminus.

The amphimictic reproductive habit, presence of males and a prominent spermatheca filled with sperm biologically and morphologically separate *H. vagisclera* n. sp. from other male-less *Hemicaloosia* species with a non-functional spermatheca, such as *H. delpradi* (Maas, 1970) Siddiqi, 1980, *H. langola* (Pramodini, Mohilal & Gambhir, 2007) Van den Berg, Tiedt & Subbotin, 2011, *H. luci* and *H. psidii* Gambhir & Dhanachand, 1997. The new species differs from the amphimictic *Hemicaloosia* by the following characters: from *H. americanae* by females having a longer tail and greater number of tail annuli (Ran = 32-43 *vs* 18-33) and by males having an elevated and elliptical oral disc *vs* not elevated, distinct lateral field, greater ratio c values (16.3-21.3 *vs* 10.6-14) and shorter distal tail portion after the caudal alae (12.0-17.3 *vs* 23.5-26.9 μm); from *H. nudata* (Colbran, 1963) Ray & Das, 1978 by the shorter female stylet (60.5-69.5 *vs* 94-109 μm) and shorter male spicules (29.5-35.6 *vs* 37-45.1 μm); from *H. paradoxa*, the most similar species, by females having a sclerotised *vagina vera* and narrower body diam. at mid-body, vulva and anus (22.6-28.0, 20.2-24.2 and 17.8-22.7 *vs* 30*, 27* and 26* μm, respectively)

and by males having a more anteriorly located excretory pore (90-113 *vs* 140* μm), greater c values (16.3-21.3 *vs* 12.7-18.5), smaller body diam. at mid-body and anus (16.8-19.8 and 9.8-12.3 *vs* 19* and 14* μm, respectively), shorter tail (23.7-34.6 *vs* 36.2*) and shorter bursa (29.7-36.6 *vs* 47.7*). Asterisks indicate measurements taken from illustrations in the original descriptions. Stylet knobs sloping posteriorly are often observed in *H. vagisclera* n. sp. specimens, but are not reported in *H. paradoxa*.

Morphological considerations for the identification of *Hemicaloosia* species

The separation of amphimictic from non-amphimictic species of *Hemicaloosia* is easy and reliable as it is based on sound biological and morphological characters such as presence *vs* absence of males and presence *vs* absence of a functional spermatheca. A reliable separation of the non-amphimictic species is also achievable because these species can be separated by the stylet length, which is longest in *H. delpradi* (75-78 μm) and shortest in *H. psidii* (46-58 μm). The remaining two species *H. langola* and *H. luci* have stylet length values that overlap (63-69 and 64-74 μm, respectively). They can be reliably separated by R and RVan values which are greater for *H. luci* (278-336 and 11-18, respectively) than for *H. langola* (243-259 and 8-10, respectively). The differentiation of the four amphimictic species of *Hemicaloosia* is certain for *H. nudata* as this is based on the stylet length, which is longer (94-109 μm) in *H. nudata* than in *H. americanae*, *H. vagisclera* n. sp. and *H. paradoxa*. These latter three amphimictic species have stylet length values that overlap (60-64, 50-69 and 46-58 μm) making their differentiation difficult. The insufficient information on the variability of many characters of *H. americanae* reported in the original description (only 15 females and 7 males were examined) and the inconsistency of the configuration of some characters shown in the illustrations of the original description of this species complicate the morphological separation of *H. vagisclera* n. sp. from *H. americanae*. The variability of the characters of *H. paradoxa* is reported in re-descriptions of populations from different locales, which often do not match the characteristics provided in the original description. Useful morphometric data on additional *H. paradoxa* populations from the type locality were published by Fortuner (1993), but these values were restricted to the females only. Taking into consideration the uncertainty of these additional morphological data available in the literature, we decided to compare the characters of the new *Hemicaloosia* from Florida with those provided only in the original descriptions of *H. americanae* and *H. paradoxa* without taking into consideration re-descriptions or additional morphological data reported in the literature. A major task in the description of this new species was the collection of information on the variability of a large number of diagnostic characters, which were examined in three populations in different locales of Florida. The numerous specimens examined and the amount of morphological data obtained allowed a good separation of the new species from *H. americanae* and *H. paradoxa* and provided sufficient material for its molecular characterisation. Without molecular data the description of this new species would have been questionable. We cannot exclude the possibility that *H. vagisclera* n. sp. could be regarded as a junior synonym of the other two species if they share similar molecular characters. Unfortunately, we were not able to obtain material for molecular studies of *H. americanae* and *H. paradoxa*. A key for the morphological identification of *Hemicaloosia* is included at the end of this paper.

Molecular characterisation and phylogenetic relationships of *Hemicaloosia*

Amplification of the ITS-rRNA gene using TW81 and AB28 primers from a specimen of *H. vagisclera* n. sp. sample yielded a single fragment of *ca* 778 bp in length. The PCR-ITS-RFLP diagnostic profile for *H. vagisclera* n. sp. generated by six restriction enzymes is given in Figure 6 with approximate sizes of the fragments as follows: *Ava*I, 778 bp (not restricted); *Bsh*1236I, 778 bp (not restricted); *Dra*I, 778 bp (not restricted); *Hin*fI, 614,

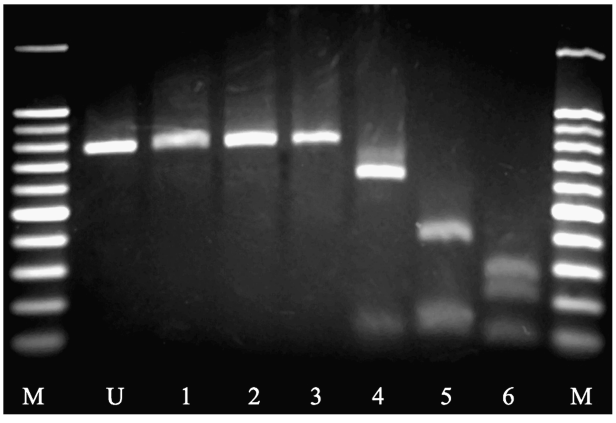


Fig. 6. *Diagnostic PCR-ITS rRNA-RFLP profile for* Hemicaloosia vagisclera *n. sp. Lanes: M = 100 bp DNA marker (Promega), U = unrestricted PCR product; 1 =* Ava*I, 2 =* Bsh*1236I, 3 =* Dra*I, 4 =* Hin*fI, 5 =* Hin*6I, 6 =* Msp*I.*

104 and 60 bp; *Hin*6I, 385, 150, 114, 105 and 24 bp; and *Msp*I, 279, 219, 102, 91 and 87 bp.

Alignment of the D2-D3 of 28S rRNA gene contained 38 sequences and was 587 bp in length. The phylogenetic tree reconstructed by the BI method is presented in Figure 7. *Hemicaloosia vagisclera* n. sp. formed a clade with *Caloosia longicaudata* (PP = 78). Alignment of partial 18S rRNA gene includes 21 sequences and it was 807 bp in the length. The 50% majority consensus BI tree is given in Figure 8. *Hemicaloosia vagisclera* n. sp. clustered with *C. longicaudata* with PP = 99. Comparison of fragments of 18S rRNA gene sequences (573 bp) of *Hemicaloosia* sp. (HM116020, HM116021) from a prairie and housed at the Wisconsin Arboretum with those of *H. vagisclera* n. sp. revealed differences in 12 bp (2.1%) that might indicate that these nematodes represent different species.

Systematics of *Hemicaloosia*

In phylogenetic trees, *H. vagisclera* n. sp. has sister relationships with *C. longicaudata* and thus supports the classification proposed by Siddiqi (1980, 2000), who regarded *Caloosia* and *Hemicaloosia* as members of the family Caloosiidae. The Caloosiidae formed a major clade (PP = 96-100) with representatives of the Hemicycliophoridae and *Criconemoides*; however, relationships between these taxa are not well resolved. Several biological, morphological and morphometric characters allow the differentiation of the seven species reported in the literature. However, the morphological separation of some amphimictic species mentioned remains unreliable without the support of molecular analysis. Detailed studies are necessary to estimate variability of morphological characters of diagnostic value and to confirm the validity of some species.

Brief biological notes on *Hemicaloosia vagisclera* n. sp.

Nematode surveys conducted in Florida during this study indicate that *H. vagisclera* n. sp. occurs commonly in the state. Uncultivated and cultivated land with Bermuda grass are inhabited by this species, which occurs especially in sod grass operations and golf courses where sod grasses, such as Saint Augustine grass, are often grown in association with Bermuda grass. These sites are commonly infested with other nematode parasites of grasses, such as lance, ring, spiral and sting nematodes, which are associated with *H. vagisclera* n. sp. The recorded population densities of *H. vagisclera* n. sp. were around 16 nematodes per 100 cm^3 of soil. The male/female ratio was 1 male per 8 females.

Concluding remarks

The results of the phylogenetic analysis conducted in this study suggest that *Caloosia* and *Hemicaloosia* are closely related. This close relationship is supported morphologically by the similar configuration of their lip patterns. Both genera share an elliptical oral disc attached dorsally and ventrally to a ring-like labial plate, two very large rectangular or semicircular amphids and also a *vagina vera* with thick cuticle. However, in *H. vagisclera* n. sp. this organ is strongly sclerotised and its dark colour stands out from the other body organs in specimens observed in lateral position at high magnification and under oil immersion. The inconsistent presence of males in the populations we studied is an aspect of the biology of *H. vagisclera* n. sp. that was not clarified during our field observations. More than 20 samples collected from a sod grass farm in Lake Wales lacked males, despite the fact that females possessed a spermatheca full of sperm. In contrast, males were present in all the samples collected at the other two sites.

The role played by *H. vagisclera* n. sp. in suppressing the growth of Bermuda grass is not clear. The host-parasite relationship of this nematode needs further investigations.

Key to species of *Hemicaloosia*

Asterisks indicate measurements taken from illustrations in the original descriptions.

1. Stylet length $\geqslant$ 90 μm . . . . . *H. nudata*
   – Stylet length < 90 μm 2
2. Stylet length $\geqslant$ 75 μm . . . . . *H. delpradi*
   – Stylet length <75 μm 3
3. Males known, spermatheca filled with sperm 4
   – Males unknown, spermatheca usually without sperm 5
4. Female with excretory pore 148 μm from anterior body end, *vagina vera* not sclerotised, Ran = 30 (18-33), RVan = 11 (10-13) (male with 4 lip annuli, excretory pore 97.5* μm from anterior body end, bursa 38.2* μm long, ratio c values 10.6-14.1) . . . . . *H. americanae*

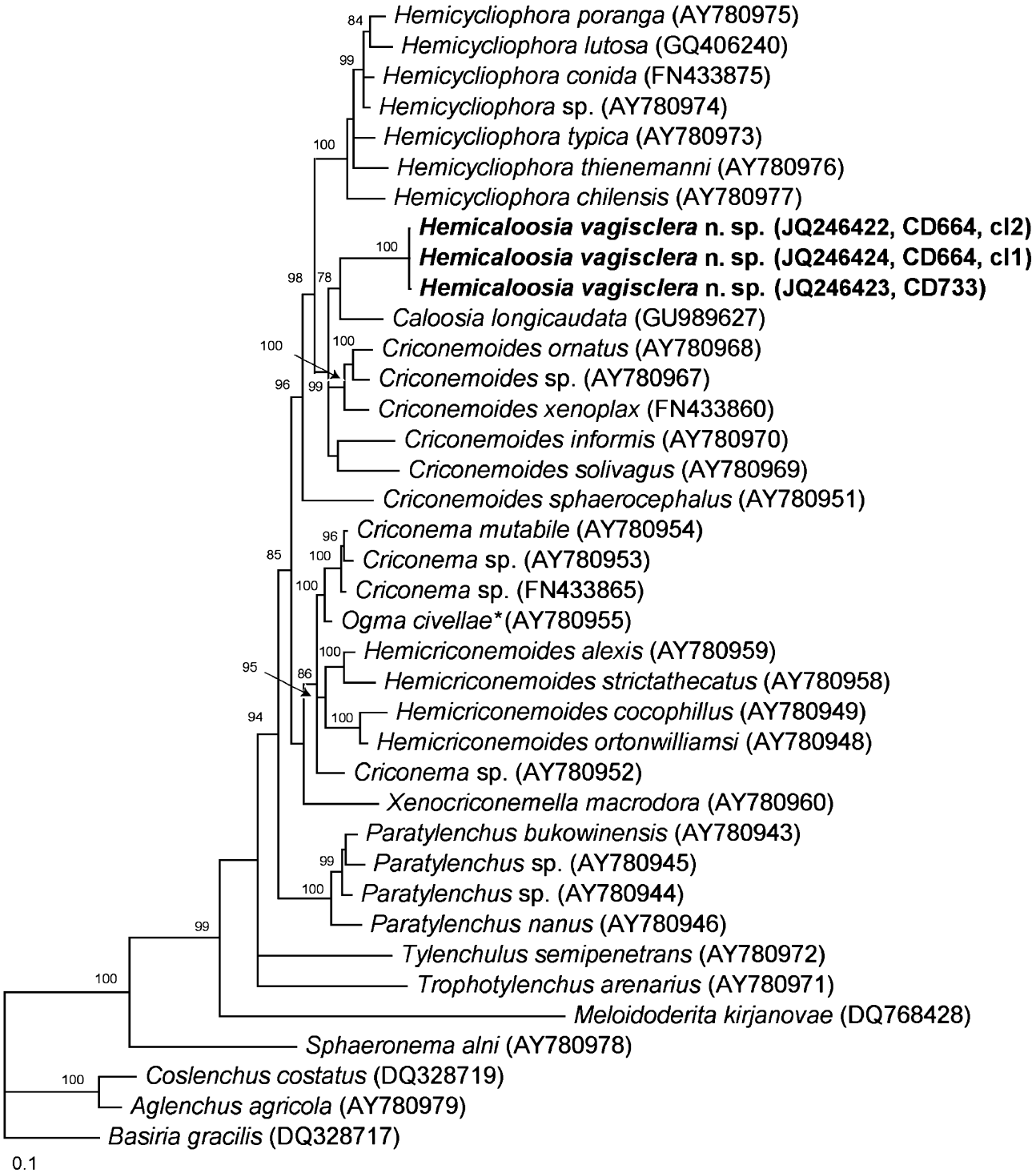


Fig. 7. *Phylogenetic relationships of* Hemicaloosia vagisclera *n. sp. with other representatives of Criconematina as inferred from Bayesian analyses of sequences of the D2-D3 of 28S rRNA gene using GTR + I + G model of DNA evolution. Posterior probability values more than 70% are given on appropriate clades. Newly obtained sequences are indicated in bold.* *Ogma civellae civellae *after Reay and Davies (1989).*

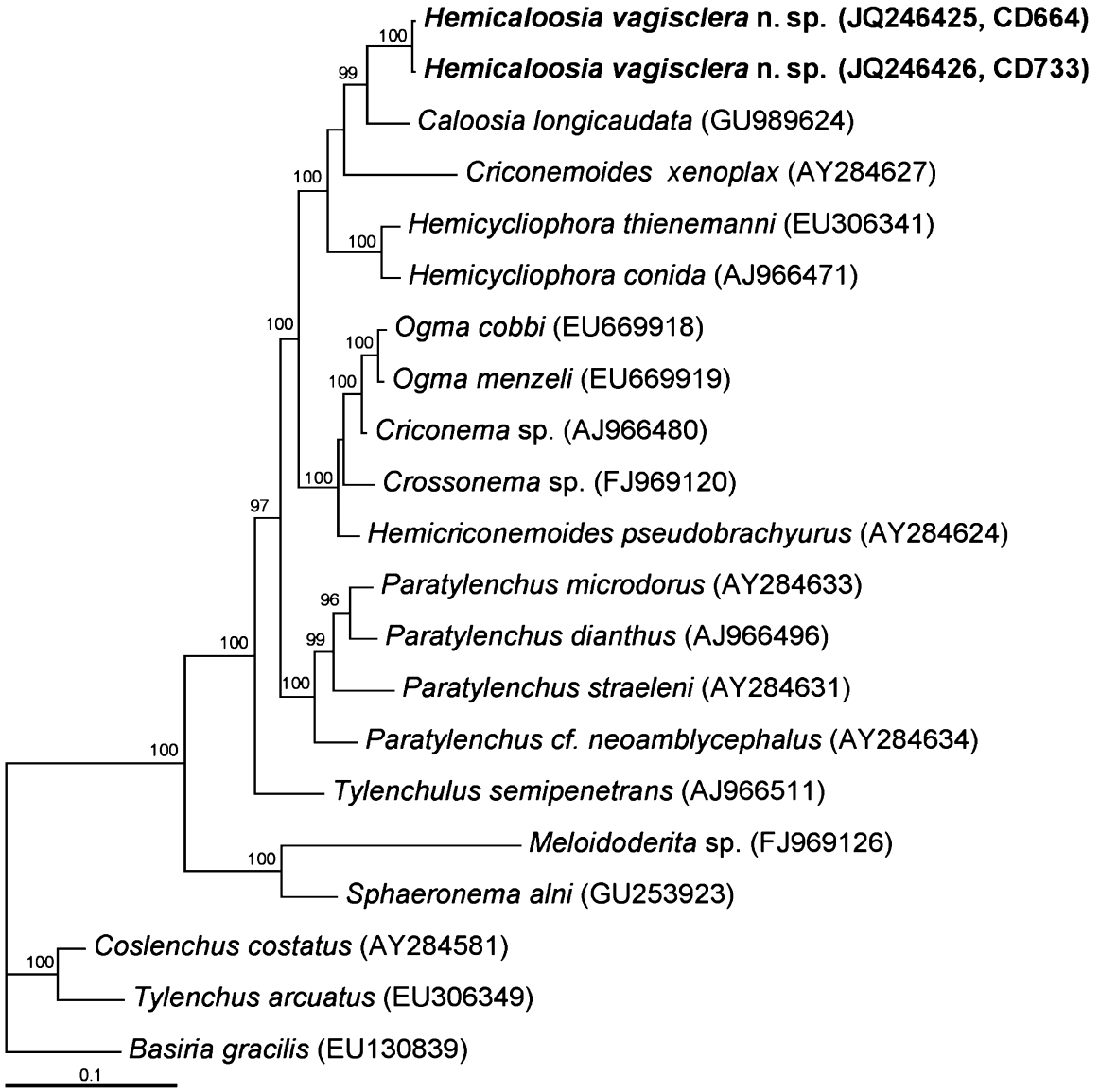


Fig. 8. *Phylogenetic relationships of* Hemicaloosia vagisclera *n. sp. with other representatives of Criconematina as inferred from Bayesian analyses of the partial 18S rRNA gene using GTR + I + G model of DNA evolution. Bootstrap values more than 70% are given on appropriate clades. Newly obtained sequences are indicated in bold.*

– Female with excretory pore 131* μm from anterior body end, *vagina vera* not sclerotised, Ran = 40* RVan = 9* (male with head marked by interruption in body annuli, excretory pore 141* μm from anterior body end, bursa 47.7* μm long, ratio c values 12.7-18.5) ..... *H. paradoxa*
– Female with *vagina vera* sclerotised, excretory pore 95-143.5 from anterior body end, Ran = 32-43, RVan = 11 (9-14) (male with 5 faint lip annuli, excretory pore 90-113 μm from anterior body end, bursa 29.7-36.6 μm long, ratio c values 16.3-21.3) ..... *H. vagisclera* n. sp.

5. R $\geqslant$ 278 ..... *H. luci*
– R < 278 6

6. Tail length $\geqslant$ 96 μm long, RVan < 17 ... *H. langola*
– Tail length < 96 μm long, RVan $\geqslant$ 17 ..... *H. psidii*

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