First Report of Carrot Cyst Nematode Heterodera carotae Jones 1950 from a New Host Brassica oleracea var. viridis L. in Türkiye: Comporation of Morphological and Morphometrical Characters with World Populations

Bugra GUVERCIN
Ordu University
Faruk AKYAZI (farukakyazi@hotmail.com)
Ordu University

Research Article

Keywords: Cabbage, Diagnosis, Heterodera carotae, Morphometrics, ITS, Phylogeny

Posted Date: August 10th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3227662/v1

Abstract

In October 2021, root and soil samples were collected from cabbage (*Brassica oleracea* var. *viridis* L.) growing fields in the Black Sea region of Turkiye. Cyst forming nematodes were detected in soil and root samples. Cysts were extracted from soil using Cobb's sieving method. Obtained nematodes were subjected to morphological and molecular analysis. For molecular analysis, the ribosomal DNA region spanning the internal transcribed spacers (ITS1, 5.8S, ITS2) gene and D2-D3 expansion segments of the 28S ribosomal RNA (rRNA) were amplified using primer sets TW81/AB28 and D2A/D3B, respectively. Also, Cytochrome Oxidase I (cox1) region of mitochondrial DNA (mtDNA) using species-specific primer set (Car-F/Car-R) were used to clarify the identification. PCR product was sequenced and then compared with sequences of *Heterodera* species available in the GenBank database. The NCBI BLAST analysis of Turkiye population sequences showed 99.82% similarity with *Heterodera carotae* Jones 1950 sequences registered in GenBank. The newly obtained sequences were submitted to the GenBank database under accession number OP602412.1. The present study includes scanning electron micrographs (SEM) of the external morphology of the cyst cone, second stage juvenile and male. The results obtained from morphological and molecular studies showed that the cysts nematode population was *H. carotae*. This is the first report of *H. carotae* detected in Türkiye.

Introduction

Cyst nematodes (*Heterodera* spp.) are distributed in warm regions (Imren, 2020). Their great potential as highly specialized parasites around the world makes it particularly important (Madani, 2004). Over 100 species of cyst nematodes have been identified (Subbotin et al., 2010). *Heterodera carotae* Jones 1950 is one of the crucial species from *Heterodera* genus. This nematode has a very narrow host range that includes carrot (*Daucus carota* and *D. pulcherrima*). This species causes damage to plants such as irregular growth, yellowish leaves, chlorosis, stunted growth, wilted plants, taproot decay and early lignification. These damages renders affected carrots unmarketable (Anonymous, 2019). This species causes significant yield losses in carrot production in Italy. It can reason about 20 to 90% yield loss in carrots (Greco et al., 1994). Although this species was first found in the carrot (*Daucus carota* subsp. *sativus*) soils in Spain (Isle of Ely) by Jones (1950), it was also recorded that it found on *Daucus carota* L. (wild and cultivated), *Daucus pulcherrimus* K., *Torilis arvensis* (hedgeparsley) and *Torilis lepyophylla* L. (Yu et al., 2017; Goodey et al., 1965; Escobar-Avila et al., 2018).This species also has been reported from Europe, India, Cyprus, Southern Africa, North America and Mexico on carrot (Berney and Bird, 1992; Subbotin et al., 2010; Escobar-Avila et al., 2018, Shubane et al., 2021).

As in the world, cyst nematodes are one of the important nematodes that damage to many crops in Türkiye. The presence of cyst nematodes in Türkiye has been determined by many researcher and so far: *H. avenae* (Wollenweber, 1924), *H. ciceri* (Vovlas, Greco and di Vito 1985) from *Cicer arietinum* and *Lens culinaris, H. cruciferae* (Franklin, 1945) from *Brassica sp.*, H. *filipjevi* (Madzhidov, 1981) from *Triticum aestivum*, *H. goettingiana* (Liebscher, 1892) from *Solanum tuberosum*, *H. latipons* (Franklin, 1969) from *Triticum aestivum*, *H. mani* (Mathews, 1971), *H. schachtii* (Schmidt, 1871) from *Beta vulgaris* L. spp.

vulgaris var altissima Doell., *H. fici* (Kir'yanova, 1954) from *Ficus carica* and *F. domestica*, *H. humuli* (Filipjev, 1934) from *Urtica dioica* species have been found (Yüksel, 1973; Di Vito et al., 1994; Yüksel, 1966; Yüksel, 1981; Rumpenhorst et al., 1996; Enneli and Öztürk, 1996; Rumpenhorst et al., 1996; Enneli et al., 1994; Diker, 1959; Akyazi et al., 2019). Although many host have been detected in the world, *H. carotae* hasn't been found in cabbage (*Brassica oleracea* var. *viridis*) plant root. Moreover, In a 2018 study by Escobar-Avila et al., some *B. oleracea* species were tested for host range. But, it hasn't occured, infection in the roots.

In this study, we further characterized the *Heterodera* population extracted from cabbage fields (*Brassica oleracea* var. *viridis* L.) in Turkiye using morphological, morphometric and molecular features. This population was identified as a first report of *H. carotae* in Türkiye. We report here on the results of our morphological, morphometric and genetic characterization of the first report species infecting cabbage from World and Türkiye.

Material and Methods

Soil sampling and nematode extraction

Soil samples were collected from at depth of 20 to 40 cm from the base of the cabbage plants using a hand shovel in October 2021 in the Ordu province (40° 58' 0.84" N, 37° 30' 16.56" E), (40°58'03"N, 38°03'04"E), and in Giresun province (41° 3' 25.1634" N, 39° 8' 39.984" E) Blacksea region of Türkiye (Figs. 1a, b). Cysts were extracted from soil (100 cm^3) using Cobb's sieving method (Cobb, 1918). Cyst obtained from a 60-mesh seive were handpicked under a stereo microscope at 40X magnification (Leica S8 APO). Infective second stage juveniles (IJ_2s) which hatches from an egg inside the cyst were used to investigate.

To demonstrate reproduction on cabbage plants (*B. oleracea* var. *viridis*) 5-week-old seedlings were planted into plastic pots containing a mixture of autoclaved sand and soil (Fig. 1b). The seedling was inoculated with about 1000 eggs obtained from cysts. Non-inoculated plants were used as control. The seedlings were grown at 25 ℃ under a photoperiod of 16 h light/8 h dark. Sixty days after inoculation, cysts were harvested from the roots (Fig. 1d), and detected by the molecular method.

Morphological and morphometric characterization

Light microscopy

Morphological study was conducted using second stage juveniles (IJ_2), males and cysts. Identical morphological characters were studied for *Heterodera carotea* (Escobar-Avila et al., 2018). For morphometric characters, second stage juveniles, males transferred to a drop of pure water on a clean glass slide on the hot plate was killed by 4–6 seconds at 55 ^oC. Then, the samples were examined on temporary slides for morphological characters and morphometric measurements. Photomicrographs and

measurements were done at 200x and 400x magnifications using a Zeiss Primo Vert microscope furnished with a dedicated camera (Zeiss Axiocam 105).

Important morphological identification characters were observed and measured from IJ_2; Body length (L), DGO, lip region diameter, lip region height, stylet length, stylet knob height, stylet knob width, median bulb lenght, median bulb width, anterior end to valve of median bulb, anterior end to excretory pore, distance from anterior to base of esophageal glands, body diameter at mid-body, body diameter at anus, body diameter at hyaline region, tail length and hyaline region length. In addition to these measurements, length, width, fenestral length, fenestral width, semifenestral height, underbridge length, vulval slit length were also measured for cyst and spicule length for male. The morphometric measurements of *H. carotae* investigated during this study was compared to those originally measured by Jones, (1950), Mathews, (1975), Escobar-Avila et al., (2018), Madani et al., (2018) and Shubane et al. (2021). Microsoft Excel was used for analysis of the allometrics variables of the second stage juveniles, males and cyst (Tables 1 and 2).

Table 1

Morphometrics of *Heterodera carotae* infective juvenile (IJ_2) from Turkiye and comparisons of measurements from different countries. All measurements are in μm and in the form: mean ± s.d. (range).

Population characters	Turkey This study	Isle of Ely, UK (Jones, 1950)	Ireland, England and Italy (Mathews,1975)	Mexico (Escobar-Avila et al., 2018)	Canada (Madani et al., 2018)	South Africa (Shubane et al. 2021)
	20	-	100	30	10	20
L	**442 ± 15.2** (410.5-469.2)	453.8 ± 1.38	422 (375–452)	394 (360–443)	413 ± 27.5 (382–446)	366.2 ± 60.8(270.3–434.0)
a	**20.5 ± 0.6** (19.6–21.8)	-	-	18.9 (18.8–20.14)	19.9 ± 0.95 (18.5–20.7)	21.7 ± 4.1 (14.7–27.8)
b	**3.1 ± 0** (2.7–3.5)	-	-	3 (2.99–3.46)	3.6 ± 0.3 (3.2–4)	3.5 ± 4.7 (1.8–23.5)
c	**8 ± 0** (7.2–8.8)	-	-	8.2 (8.18–8.52)	8.5 ± 0.9 (7.5–9.6)	7.8 ± 1.6 (5.3–11.1)
c'	**4 ± 0.2** (3.6–4.4)	-	-	3.7 (3.7–4)	3.8 ± 0.4 (3.1–4.1)	4.2 ± 0.5 (3.4–5.3)
DGO	**5.1 ± 0.4** (4.1–5.8)	-	5–6	5 (4–6)	5.1 ± 0.1 (5–5.2)	3.9 ± 1.3 (1.9–6.2)
Lip region diam.	**9 ± 0.6** (7.8–10.4)	-	10 (9–10)	9 (8–10)	9.4 ± 0.7 (9.3–10.6)	6.9 ± 1 (4.3–8.7)
Lip region height	**4.3 ± 0.4** (3.6–4.9)	-	5 (4–6)	5 (4–6)	4.7 ± 0.1 (4.5–4.9)	4.6 ± 0.9(3.1–6.2)
Stylet length	**24.8 ± 1.1** (22.9–26.9)	-	24 (22–25)	25 (24–26)	24.1 ± 0.7 (23.5–26.1)	-
Anterior end to valve of median bulb	**70.9 ± 4.5** (56.6–78.7)	-	66 (61–72)	61 (52–73)	-	-

L: body length, a = body length/maximum body width; b = body length/distance from anterior end to pharyngo intestinal junction; c = body length/tail length; c' = tail length/body width at anus

Population characters	Turkey This study	Isle of Ely, UK (Jones, 1950)	Ireland, England and Italy (Mathews,1975)	Mexico (Escobar-Àvila et al., 2018)	Canada (Madani et al., 2018)	South Africa (Shubane et al. 2021)
Anterior end to excretory pore	**99.1 ± 6.4** (56.6–78.7)	-	99	87 (78–98)	98.9 ± 2.9 (95.4–103)	110 ± 14.9 (92.4–145)
Oesophageal length	**145.3 ± 9.7** (120.8-158.6)	-	-	131 (104–148)	-	-
Body diameter at mid-body	**21.6 ± 0.5** (20.6–22.4)	-	20 (19–21)	21 (19–22)	21.5 ± 1.6 (19.5–24.0)	17 ± 2 (14.9–21.1)
Body diameter at anus	**13.6 ± 0.4** (12.9–14.4)	-	12.4 (11.1–13.4)	13 (11–14)	12.5 ± 1.1 (1.3–14)	11.8 ± 2.4 (8-19.5)
Body diameter at hyaline region	**7.6 ± 0.5** (6.6–8.4)	-	7.1 (5.9–8.3)	8 (7–9)	-	-
Tail length	**55 ± 2.4** (49.3–59)	-	51.8 (43.5–58.6)	48 (44–52)	46.7 ± 3.6 (40.4–51)	47.6 ± 5.9 (37.8–58.3)
Hyaline region length	**29.9 ± 2.8** (23.8–34.6)	-	28.3 (20.3–31.8)	26 (21–31)	28.1 ± 1.8 (26.0–30.0)	26.2 ± 2.2 (22.3–30.4)

L: body length, a = body length/maximum body width; b = body length/distance from anterior end to pharyngo intestinal junction; c = body length/tail length; c′= tail length/body width at anus

Table 2

Morphometrics of *Heterodera carotae* male, cyst and egg from Turkiye and comparisons of measurements from different countries. All measurements are in μm and in the form: mean ± s.d. (range).

Population characters	Türkiye This study	Isle of Ely, UK (Jones, 1950)	Ireland, England and Italy (Mathews,1975)	Mexico (Escobar-Avila et al., 2018)	Canada (Madani et al., 2018)	South Africa (Shubane et al. 2021)
Male	**n = 4**	-	**n = 40**	**n = 30**	**-**	**n = 4**
L	**1076.4** ± 82,7 (1013,3-1216,3)	1119 ± 14	1154 (1090–1220)	1180 (1114–1219)	-	1185 ± 26 (1147–1205)
a	**35.8** ± 2.4 (33.4–39.4)	-	-	37 (33.9–42.8)	-	40.3 ± 2.7 (38.5–44.1)
b	**6.7** ± 0 (6.3–7.2)	-	-	6.7(4.7–7.7)	-	6.6 ± 1 (6.2–7.5)
Stylet length	**29.7** ± 0.3 (29.2–30.1)	28.8 ± 0.36	35 (31–38)	32 (29–36)	-	-
Anterior end to excretory pore	**149.4** ± 2.3 (146.1-152.5)	-	153 (148–161)	157 (146–169)	-	162 ± 7 (152–168)
Body diameter at mid-body	**30.1** ± 0.7 (29-30.9)	31.4 ± 0.6	30 (27–33)	32 (26–36)	-	25.1 ± 0.4 (24.8–25.4)
Labial region height	**6.6** ± 0.4 (6–7)	-	7	5 (4–6)	-	6.6 ± 1.1 (5–8)
Labial region diameter	**11.2** ± 0.8 (10.4–12.4)	-	11	11 (9–13)	-	10,3± (8-11.1)
Anterior end to valve of median bulb	**95.7** ± 3.4 (90.4–99.4)	-	90 (85–105)	103 (101–106)	-	92 ± 8.4 (80.6–98.5)
Spicule length	**36.3** ± 0.7 (35.4–37.2)	-	34 (31–36)	36 (32–39)	-	38.6 ± 1.5 (36.5–40)
Distance from anterior to base of esophageal glands	**161.3** ± 4.3 (156–168)	-	-	175 (144–238)	-	-

L: body length, a = body length/maximum body width; b = body length/distance from anterior end to pharyngo intestinal junction, L/W = length/width

Population characters	Türkiye This study	Isle of Ely, UK (Jones, 1950)	Ireland, England and Italy (Mathews,1975)	Mexico (Escobar-Avila et al., 2018)	Canada (Madani et al., 2018)	South Africa (Shubane et al. 2021)
Anterior end to oesophageal gland base	**172.3** ± 9.1 (136.2-184.8)	-	-	203 (172–267)	-	-
Cysts	**n = 20**	**-**	**n = 150**	**n = 30**	**n = 20**	**n = 16**
Length (excluding neck)	**469 ± 39.1** (396.2-549.6)	210–680	408 (218–625)	423 (286–575)	438.3 ± 81.4 (358–619)	372 ± 49 (294–450)
Width	**375.7 ± 41.9** (303-450.5)	150–530	309 (165–500)	331 (217–472)	295 ± 53.7 (222–360)	252 ± 48.4 (179–341)
Vulval slit	**40.3 ± 3** (44.5–37.5)	-	-	-	-	40.7 ± 10.6 (28.3–54.6)
Fenestral length	**38.3 ± 1.2** (37-39.9)	-	-	-	-	37.5 ± 6.6 (26.7–54.6)
Fenastral width	**44.3 ± 4.8** (38.5–50.2)	-	-	-	-	26.3 ± 6.5 (16.7–42.7)
Underbridge length	**84.8 ± 8.9** (66-94.6)	-	-	-	-	57.6 ± 8.1 (67.6–79.7)
L/W ratio	**1.3** ± 0.1 (1.1–1.5)	-	-	1.28 (1.22–1.31)	1.5 ± 0.2 (1.3–1.7)	
Eggs	**n = 20**	-	-		**n = 10**	
Length	**100.9 ± 3.9** (91.4-108.8)				107.3 ± 4.3 (100–109)	
Width	**44.1 ± 2** (38.8–47.2)				49.7 ± 3.2 (43.5–54.0)	

L: body length, a = body length/maximum body width; b = body length/distance from anterior end to pharyngo intestinal junction, L/W = length/width

Population characters	Türkiye This study	Isle of Ely, UK (Jones, 1950)	Ireland, England and Italy (Mathews,1975)	Mexico (Escobar-Avila et al., 2018)	Canada (Madani et al., 2018)	South Africa (Shubane et al. 2021)
L/W ratio	**2.3 ± 0.1** (2.1–2.5)				2.1 ± 0.2 (1.8–2.5)	

L: body length, a = body length/maximum body width; b = body length/distance from anterior end to pharyngo intestinal junction, L/W = length/width

Table 3
Comparison of morphometry of *Heterodera carotae* infective juvenile and male from Türkiye with *H. cruciferae* measurements from different countries. All measurements are in μm and in the form: mean ± s.d. (range).

	Heterodera carotae	*Heterodera cruciferae*			
Characters	**This Study** **n = 20**	**UK population** Stone and Rowe, 1976	**Spain population** Bello et al., 1999	**Iranian population** Jabbari and Niknam, 2008	**Russia population** Chizhov et al., 2009
Infective Juvenile stylet length	**24.8** ± 1.1 (22.9–26.9)	24.1 ± 1.6	23.5 (21.9–25.4)	21 ± 0.8 (20–22.4)	23 ± 0.9 (21–25)
Infective Juvenile Hyaline region length	**29.9** ± 2.8 (23.8–34.6)	25.2 ± 2.7	27.3 (20.7–33.9)	21.3 ± 1.9 (16–23.4)	24 ± 3.3 (17–30)
Male stylet length	**29.7** ± 0.3 (29.2–30.1)	24.9 ± 1.1	27.1 (25–28)	23 ± 1.6 (20–24.3)	25 ± 1.7 (22–28)
B					

For the observation of the vulval cone, cysts were soaked in lactic acid 45% for 15min, transferred to water and then, the posterior region was dissected and mounted in glycerin with for its observations and analysis (S'Jacob and Van Bezooijen, 1984; De la Jara-Alcocer et al., 1994). The measurements taken were vulval slit length, fenestral length and width, semifenestral height and underbridge length.

Scanning electron microscopy (SEM) *analyses*: Females in cyst form and second-stage juveniles (IJ_{2s}) and male were prepared for scanning electron microscopy (SEM), according to the methods proposed by Eisenback (1985). TAFF fixed nematodes were transferred into phosphate buffer and rinsed in three times with 6% glutaraldehyde every 30 minutes and refrigerated for 24 hours. Several changes of 0.1 M phosphate buffer (pH 7.2) and postfixed overnight in 2.0% aqueous osmium tetroxide solution. Postfixed specimens were rinsed in cold 0.1 M phosphate buffer within 15 minutes, dehydrated through a series of

10–100% absolute ethanol. Anhydrous nematodes were placed on double-sided carbon conductive tape and coated (approximately 10 nm thick) with gold automatic Spray Coating. The sputtering process was performed using an SPC-900-C plasma sputtering coater system with a DC current of 10 mA for the sputtering time of 60 s. The samples were displayed using the Hitachi SU1510 scanning electron microscope at the Central Research Laboratory, Ordu University, Turkiye.

Molecular characterization

DNA extraction: For molecular studies, second-stage juveniles obtained from a single cyst were used. The genomic DNA were extracted from IJ_2 as follows: five specimens were placed into 10 μl of extraction buffer (10 mM Tris-HCl, pH 8.8; 1 mM EDTA) in a 1.5 ml PCR tube and then 0.1% Triton X-100 (v/v) and 0.1 mg/ml Proteinase K were added to the tube. Sample tubes were kept at -20°C for 1 night. Samples were ground using a glass capillary tube and incubated at 56°C for 1 h and subsequently at 95°C for 10 min (Pagan et al., 2015). The mixture was used as a DNA template for PCR.

PCR amplification: The ribosomal region spanning the Internal Transcribed Spacer (ITS1, 5.8S, ITS2) gene and D2-D3 expansion segments of the 28S ribosomal RNA (rRNA) were amplified using primer sets TW81 (5´- GTT TCC GTA GGT GAA CCT GC -3´)/AB28 (5´- ATA TGC TTA AGT TCA GCG GGT-3´) (Joyce et al., 1994) and D2A (5´-ACA AGT ACC GTG AGG GAA AGT TG-3´)/D3B (5´- TCG GAA GGA ACC AGC TAC TA- 3´) (De Ley et al., 1999)., respectively. Also, Cytochrome Oxidase I (cox1) region of mitochondrial DNA (mtDNA) using species-specific primer set Car-F (5´- CTT TGG TTT AAT TAG TTT AAG AG- 3´) /Car-R (5´- GAA AAA TAT CTA AAC TAG CG- 3´) (Madani et al., 2018) were used to clarify the *H. carotae* identification. The thermocycling was carried out by using a Veriti™ 96-Well Thermal Cycler (Singapure) instrument. The thermocycling reactions for ITS and D2A/D3B primers was as follows: 95°C for 4 min; followed by 37 cycles of 95°C for 30 s, 56°C for 45 s and 72°C for 2 min; and a final extension step of 72°C for 10 min. The thermocycling reactions for species specific primer set (Car-F/Car-R) was performed as recommended by Madani et al., (2018).

Electrophoresis

DNA fragments were separated by electrophoresis in 1X TAE buffer and 1.5% agarose gel, stained with ethidium bromide, visualized using ErBiyotek GEN-BOX imagER Fx and photographed. Sequence analysis of PCR products was performed at STAB VIDA in Portugal. An ABI 3730xl DNA Analyzer (Applied Biosystems) was used to perform precise sequencing operations. The new sequences obtained were compared to those deposited in the National Center for Biotechnology Information (NCBI) database using BLAST engine search for sequence homology. The sequences of ribosomal expansion region of ITS for the populations of *H. carotae* from Ordu province was deposited in the GenBank database with the accession number of OP602412.1.

Phylogenetic analysis

To perform the phylogenetic analysis, the ribosomal region spanning the internal transcribed spacer (ITS) gene sequences obtained from *H. carotae* identifed in this study and those retrieved from the GenBank from the NCBI database were aligned using Clustal W for multiple alignments of 30 nucleotide sequences using MEGA 11 software (Kumar et al. 2016). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). *Xiphinema index* (MZ677016.1) was used as outgroup for constructing the phylogenetic tree based on the sequences form the ITS. The phylogram was generated using 1000 bootstrap replicates.

Result

Morphological and morphometrical studies

The morphological details of *H. carotae* cysts, infective juveniles and males obtained from the cabbage field (*B. oleracea* var. *viridis*) in Türkiye observed by light and scanning electron microscope (SEM) are given in Figs. 3–8. The morphometric characteristics of *H. carotae* stages were examined and measured are listed in Tables 1 and 2. Moreover, the morphometric measurements of the Türkiye population were compared to the United Kingdom population described by Jones (1950), the England population described by Mathews (1975), Mexico population detected by Escobar-Avila et al. (2018), the Canada population described by Madani et al. (2018) and the South Africa by Shubane et al. (2021).

Second Stage Juvenile

Vermiform and body slender. Second stage juveniles are folded four times within the egg. Body length 442 µm, body diameter 21.6 µm (at mid body), 13.6 µm (at anus) and 7.6 (at hyaline region). Head slightly offset with four indistinct post-labial annules (Fig. 7a). Medial lips and labial disc indistinct in cephalic region (Fig. 7a). Lip region 7.8–10.4 μm diameter and 3.6–4.9 μm height. Cephalic framework well developed heavily sclerotized (Fig. 3b, c). Stylet very strong 22.9–26.9 μm, stylet knobs round (Fig. 3c). Median bulb oval with distinct valve (Fig. 3b). Position of excretory pore (EP) ranging 89.5-112.7 μm from anterior end. Pharyngeal glands elongated, tapering posteriorly, overlapping intestine ventrally (Fig. 3a). Lateral field with four incisures forming three bands (Fig. 7c, 7d). Tail acutely conical with prominent terminal hyaline part (Fig. 3d, e; Fig. 7e, f).

Male

Body vermiform with a short-rounded tail, cuticle annulated. Male twisted in posterior half when killed by gentle heat (Fig. 4a). Head offset, 7 µm long, labial disc slightly oval with 6–8 indistinct post-labial annules (Fig. 8c). Cephalic framework robust (Fig. 4b). The stylet is strong with well-developed knobs (Fig. 4b). Lateral field with four incisures forming three bands, outer bands areolated (Fig. 8d). Lateral lines start from behind the head incisure line and continue about to the tip of the tail (Fig. 8f). Oesophagus well developed, median esophageal bulb oval with poorly developed valve plates which are 90.4–99.4 (av. 95.7) µm from anterior end. Excretory pore 146.1-152.5 (av. 149.4) µm from anterior end. Single testis uniformly packed with sperm and averaging 59% of total body length. Spicules arcuate with

a bulbous anterior part and tubular mid-part tapering into a twisted posterior section (Fig. 8f). Gubernaclum slightly curved. Phasmids adanal.

Cyst

The collected cyst color changes from white to russet brown. Mature cysts, lemon-shaped with distinct neck and vulval cone. Cyst usually filled with eggs. The eggs were oval, 91.4-108.8 (av. 100) μm long and 38.8–47.2 (av. 44) μm wide. The main morphological characteristics of the cyst vulval cone were shown in Fig. 5a-d. Wall-pattern consists of irregular zig-zag lines forming. Vulval bridge broken in some specimens Fig. 5d. Bullae absent. Underbridge 85 µm long. Vulval slit, 37.5–44.5 (av. 40) µm long, often appears partly open. Vulval lips sometimes flat. The morphological and morphometric features were in agreement with

Molecular characterization

The amplifcation of the D2/D3 LSU rDNA expansion segments yielded one 760 bp fragment based on jel electrophoresis (Fig. 2a). The amplifcation of the ribosomal region spanning the internal transcribed spacer gene expansion segments yielded one 1100 bp fragment based on jel electrophoresis (Fig. 2b). The amplifcation of the specific primer expansion segments yielded one 355 bp fragment based on jel electrophoresis (Fig. 2c).

The comparison of ribosomal region spanning the internal transcribed spacer (ITS1-5.8S-ITS2) gene sequences of PCR products of Turkiye *H. carotae* with those of the GenBank database showed similarity (99.82%) to *Heterodera carotae* (MG563237) and *H. cruciferae* (MK848393, MG298948, ON007089) species. Phylogenetic relationships of the turkish population of *H. carotae* with other populations using ITS region is shown in Fig. 8. *Heterodera cruciferae* was the closest species to *H. carotae* in phylogenetic tree, because both are in the Goettingiana group.

Discussion

A cyst-forming nematode population was collected from roots and soil from the rhizosphere of *Brassica oleracea* var. *viridis* in Ordu and Giresun provinces. Comparing its morphology and the ITS sequence of this population with published data allowed us to conclude that the species was *H. carotae*.

Heterodera carotae can be easily confused with another cyst nematode *Heterodera cruciferae*, when identified morphologically. This species was compared morphologically and morphometrically with the *H. cruciferae* species previously found by many researchers. Infective Juveniles stylet lenght (24.8 μm vs. 24.1 μm, 23.5 μm, 21 μm, 23 μm) were longer from *H. cruciferae* Uk, Spain, Iranian and Russia population, respectively. At the same time, the hyaline region was longer than *H. crusiferae*, so these two species are separated from each other. Likewise, the male of *H. carotae* differs from Spain, Iranian, UK and Russia populations by having longer stylet. However, these species can be distinguished using some morphological characteristics of individuals. Subbotin et al (2010) stated that *H. carotae* differed from *H.*

cruciferae by a longer average hyaline part of tail region in IJ_2. Jones (1950a) and Mathews (1975) stated in their study that the stylets of *H. carotae* males were longer than those of *H. cruciferae*. In this study, long male stylet and hyaline part showed clear differences in *H. carotae* species from *H. cruciferae*. Similarly, Chizhov et al., (2009), Jabbari and Niknam, (2008), Bello et al., (1999) found the stylet length of *H. cruciferae* males to be shorter than 28 µm in their studies. Moreover, molecular differentiation of *H. carotae* from *H. cruciferae* was carried out by using the species-specific primers Car-F/Car-R of the COI region used in this study. In this study, phylogenetic analysis showed that the cyst nematode population from Türkiye grouped together in a clade with *H. carotae* and *H. cruciferae*.

This species was compared to the United Kingdom population described by Jones (1950), the Ireland, England and Italy population detected by Mathews (1975), Mexico population detected by Escobar-Avila et al. (2018), the Canada population detected by Madani et al. (2018), the South African population described by Shubane et al. (2021).

Escobar-Avila et al. (2018) and Madani et al. (2018) reported that *H. carotae* cysts are light brown to slightly dark brown. The cysts obtained in this study were found in similar colors.

Second-stage juveniles from the Turkiye population has a slightly longer mean body length than that of specimens from Mexico, Ireland, England, Italy, Canada and South Africa (442 *µ*m vs. 394, 422, 413 and 366 µm) respectively. However, it is slightly shorter than the United Kingdom (442 *µ*m vs. 453.8 µm). Their heads are almost the same (7.8–10.4 *µ*m vs. 8–10, 9–10 and 9.3–10.6 µm) except South African population (4.3–8.7 *µ*m). The male stylet is smaller in the Türkiye specimens (29.2–30.1 µm vs. 29–36 *µ*m in Mexico and 31–38 *µ*m in Ireland, England and Italy), but is nearer to that of United Kingdom males (28.5–29.6 *µ*m vs. 28.8 *µ*m).The spicules of the Türkiye males were furthermore slightly longer than that of Ireland, England and Italy specimens (35.4–37.2 *µ*m vs. 31–36 *µ*m) but compared with that of the Mexican and South African males (32–39 *µ*m and 36.5–40 *µ*m), it is shorter.

Conclusion

In conclusion, *H. carotae* was recovered from *Brassica oleracea* var. *viridis*, Ordu and Giresun provinces, Eastern Blacksea region of Turkiye. This is the first report of *H. carotae* infecting cabbage (Kale) in the world. There should be some future studies focusing on the control strategies of *Heterodera* in cabbage. Therefore, this first record of *H. carotae* in cabbage field could be the good source of nematological studies such as developing resistant varieties. As a result, it is necessary to work on control strategies for *Heterodera* species in order to increase yield in cabbage fields. We emphasize that future research on the genus *Heterodera* is still needed to prevent yield loss in cabbage.

Declarations

- Competing Interests

- All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.
- The authors declare that they have no competing interests.

- Funding Information

- No funds, grants, or other support was received.

- Author contributions

The contributions of all authors must be described in the following manner:

The authors confirm contribution to the paper as follows:

study conception and design: F. AKYAZI,

Sample collection and carried out the extraction and identification of nematodes,: B. GUVERCIN; F. AKYAZI,

analysis and interpretation of results: F. AKYAZI, B. GUVERCIN

draft manuscript preparation: F. AKYAZI, B. GUVERCIN

All authors reviewed the results and approved the final version of the manuscript.

- Data Availability Statement

- The data presented in this study are available on request from the corresponding author.

- **Research Involving Human and /or Animals**

- All *research* studies *involving* the use of invertebrate *animals (Nematodes)*

- **Informed Consent**

- Not applicable.

References

1. Akyazi F, Felek AF, Čermák V (2019) First report of the hop cyst nematode *Heterodera humuli* from common nettle *Urtica dioica* in Turkey. *Plant Disease*, 103, 161, DOI:10.1094/PDIS-04-18-0634-PDN.
2. Anonymous, (2019) *Heterodera carotae*, http://nemaplex.ucdavis.edu/Taxadata/G060s2.aspx
3. Berney MF, Bird GW (1992) Distribution of *Heterodera carotae* and *Meloidogyne hapla* in Michigan carrot production. *Journal of Nematology* 24, 776-778.

4. Di Vito M, Greco N, Ores G, Saxena MC, Singh KB, Kusmenoglu I (1994) Plant parasitic nematodes of legumes in Turkey. *Nematologia mediterranea*, 245-251.
5. Eisenback JD (1985) Techniques for preparing nematodes for scanning electron microscopy. In: Barker KR, Carter GG, Sasser JN (eds) An advanced treatise on Meloidogyne, Volume II: Methodology. Department of Plant Pathology, North Carolina State University, Raleigh, U.S.A., pp 79–105.
6. Escobar-Avila IM, López-Villegas EÓ, Subbotin SA, Tovar-Soto A (2018) First report of carrot cyst nematode in Mexico: Morphological, molecular characterization, and host range study. *Journal of Nematology* 50, 229-242, DOI:10.21307/jofnem-2018-021.
7. Filipjev IN (1934) Nematodes harmful and useful in agriculture. OGIZSekhozgiz, Moscow-Leningrad, USSR. 440
8. Franklin MT (1945) On *Heterodera cruciferae* n. sp. of *brassicas*, and on a *Heterodera* strain infecting clover and dock. *Journal of Helminthology* 21, 71-84, DOI: https://doi.org/10.1017/S0022149X00031965
9. Franklin MT (1969) *Heterodera latipons* n. sp., a cereal cyst nematode from the Mediterranean region. *Nematologica* 15, 535-542.
10. Goodey JB, Franklin MT, Hooper DJ (1965) T. Goodey's' The nematode parasites of plants catalogued under their hosts', 214.
11. Greco N, D'addabbo T, Brandonisio A, Elia F. (1994) Damage to Italian crops caused by cyst-forming nematodes. *Journal of Nematology* 25, 836.
12. Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95|98|NT. Nucleic Acids Symposium series 41: 95-98.
13. Hooper DJ (1986) Extraction of free-living stages from soil. In: Southey JF (ed) Laboratory Methods for Work with Plant and Soil Nematodes. London (GB), Her Majesty's Stationary Office, pp 5-30.
14. Imren M (2020) The cyst nematode *Heterodera latipons* Franklin, 1969 (Nemata: Heteroderidae): hatching behavior and development on wheat in arid and semiarid regions in Turkey. *Turkish Journal of Agriculture and Forestry* 44, 331-338, DOI: https://doi.org/10.3906/tar-1906-16
15. Jenkins DW (1964) A rapid centrifugal-flotation technique for separating nematodes from soil. *Plant Disease Reporter* 48, 692.
16. Jones FGW (1950) Observations on the beet eelworm and other cyst-forming species of Heterodera. *Annals of Applied Biology* 37, 407-440, DOI: https://doi.org/10.1111/j.1744-7348.1950.tb00966.x
17. Joyce SA, Burnell AM, Powers TO (1994) Characterization of *Heterorhabditis* isolates by PCR amplification of segments of mtDNA and rDNA genes. *Journal of Nematology* 26, 260-270.
18. Kirjanova ES (1954) Summaries and perspectives in the develop-ment of phytonematology in USSR. Zoological Institute of the Academyof Sciences, USSR, Turdy Prob. i Tematicheskikh Soveshchanii 3:9–47.
19. Kumar S, Stecher G, Tamura K (2016) Mega7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33, 1870-1874. DOI:

https://doi.org/10.1093/molbev/msw054

20. Madani M, Vovlas N, Castillo P, Subbotin SA, Moens M (2004) Molecular characterization of cyst nematode Species (*Heterodera* spp.) from the Mediterranean Basin using RFLPs and sequences of ITS-rDNA. *Journal of Phytopathology* 152, 229-234, DOI: https://doi.org/10.1111/j.1439-0434.2004.00835.x
21. Madani M, Palomares-Rius JE, Vovlas N, Castillo P, Tenuta M (2018) Integrative diagnosis of carrot cyst nematode (Heterodera carotae) using morphology and several molecular markers for an accurate identification. *European Journal of Plant Pathology* 150, 1023-1039, DOI: 10.1007/s10658-017-1342-2
22. Madzhidov AR (1981) New species of Bidera filipjevi sp. nov. Heteroderina: Tylenchida) from Tadzhikistan. Izvectija Akademii Nauk Tadzjikiskov SSR, *Otdelenie Biologitseskich Nauk* 2, 40-44.
23. Mathews HJP (1975) *Heterodera carotae*. CIH Description of plant parasitic nematodes. Set 5, No. 61 St Albans, UK, Common wealth Institute of *Helminthology* 1-4.
24. Pagan C, Coyne D, Carneiro R, Kariuki G, Luambana N, Afokpon A, Williamson VM (2015) Mitochondrial haplotype-based identifcation of ethanol-preserved root-knot nematodes from Africa. *Phytopathology* 105, 350-357, DOI: https://doi.org/10.1094/PHYTO-08-14-0225-R
25. Rumpenhorst HJ, Elekcioglu IH, Sturhan D, Öztürk G, Enneli S (1996) The cereal cyst nematode *Heterodera filipjevi* (Madzhidov) in Turkey. *Nematologia mediterranea* 24, 135-138.
26. Schmidt A (1871) Über den Rüben-Nematoden (*Heterodera schachtii* A.S.) Zeitschrift Des Vereines Für Die Rübenzucker-Industrie Im Zollverein, 21, 1-19.
27. Shepherd AM (1986) Extraction and estimation of cyst nematodes. In: Southey J.F. (Ed.). Laboratory methods for work with plant and soil nematodes. London, UK: Her Majesty's Stationery Office, pp. 51-58.
28. Subbotin SA, Mundo-Ocampo M, Baldwin JG (2010) Systematics of cyst nematodes. Nematoda: Heteroderinae). In: Hunt, D. J. & Perry, R. N. (Eds.), Nematology Monographs and Perspectives, 8B, The Netherlands; Leiden, pp.512.
29. Swart A, Knoetze R (2020) Interesting new facts on *Heterodera schachtii* (Nematoda: Heteroderidae) from South Africa. *South African Journal Plant and Soil* 37, 326-329. DOI: https://doi.org/10.1080/02571862.2020.1761469
30. Yu Q, Ponomareva E, Van Dyk D, McDonald M R, Sun F, Madani M, Tenuta M (2017) First report of the carrot cyst nematode (*Heterodera carotae*) from carrot fields in Ontario, Canada. *Plant Disease* 101, 1056-1056, DOI: https://doi.org/10.1094/PDIS-01-17-0070-PDN
31. Yüksel H (1966) *Heterodera schachtii* Schmidt 1971 ile Türkiye'de yeni bulunan *Heterodera cruciferae* Franklin kist nematodları üzerinde mukayeseli morfolojik çalışmalar. *Atatürk Un. Ziraat Fakultesi Arastırma Ens. Teknik Bülteni* (8), 23.
32. Yüksel H (1973) Studies on the morphological and biological differences of *Heterodera* species (Nematoda: Heteroderidae) in Turkey. *Journal of Ataturk University Agriculture Faculty* 4, 15-20. (Turkish)

33. Yuksel H (1981) *Heterodera fici* Kirjanova 1954 in Aegean Region. *Journal of Turkish Phytopathology* 10, 45-51.
34. Wollenweber HW (1924) Zur Kenntnis der Kartoffel Heteroderen. *Illustrierte Landwirt schaftliche Zeitung* 44, 100-101.
35. Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10:512–526, DOI: https://doi.org/10.1093/oxfordjournals.molbev.a040023
36. Tamura K, Stecher G, Peterson D, Filipski A, Sudhir K (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* 30, 2725-2729.
37. Wang H, Qi M, Cutler AJ (1993) A simple method of preparing plant samples for PCR. *Nucleic Acids Research*, 21, 4153-4154, DOI: https://doi.org/10.1093/molbev/mst197
38. Wehunt EJ, Golden AM, Robbins RT (1989) Plant nematodes occurring in Arkansas. *Supplement of Journal of Nematology* 21 (45): 677-681.
39. Win PP, Kyi PP, Maung ZTZ, De Waele D (2013) Population dynamics of *Meloidogyne graminicola* and *Hirschmanniella oryzae* in a double rice-cropping sequence in the lowlands of Myanmar. *Nematology* 15: 795-807.
40. Vovlas N, N Greco, M Di Vito (1985) Heterodera ciceri sp. N. (Nematoda: Heteroderidae) on Cicer arietinum from Northern Syna. Nematologia mediterranea 13,239-252.
41. Van Berda de Haan J (1902) Een aaltjesziekte va de rijst, “omo mentek” of “omo bambang”. *Mededelingen uit’s Lands Plantentuin* 53: 1-65.

Figures

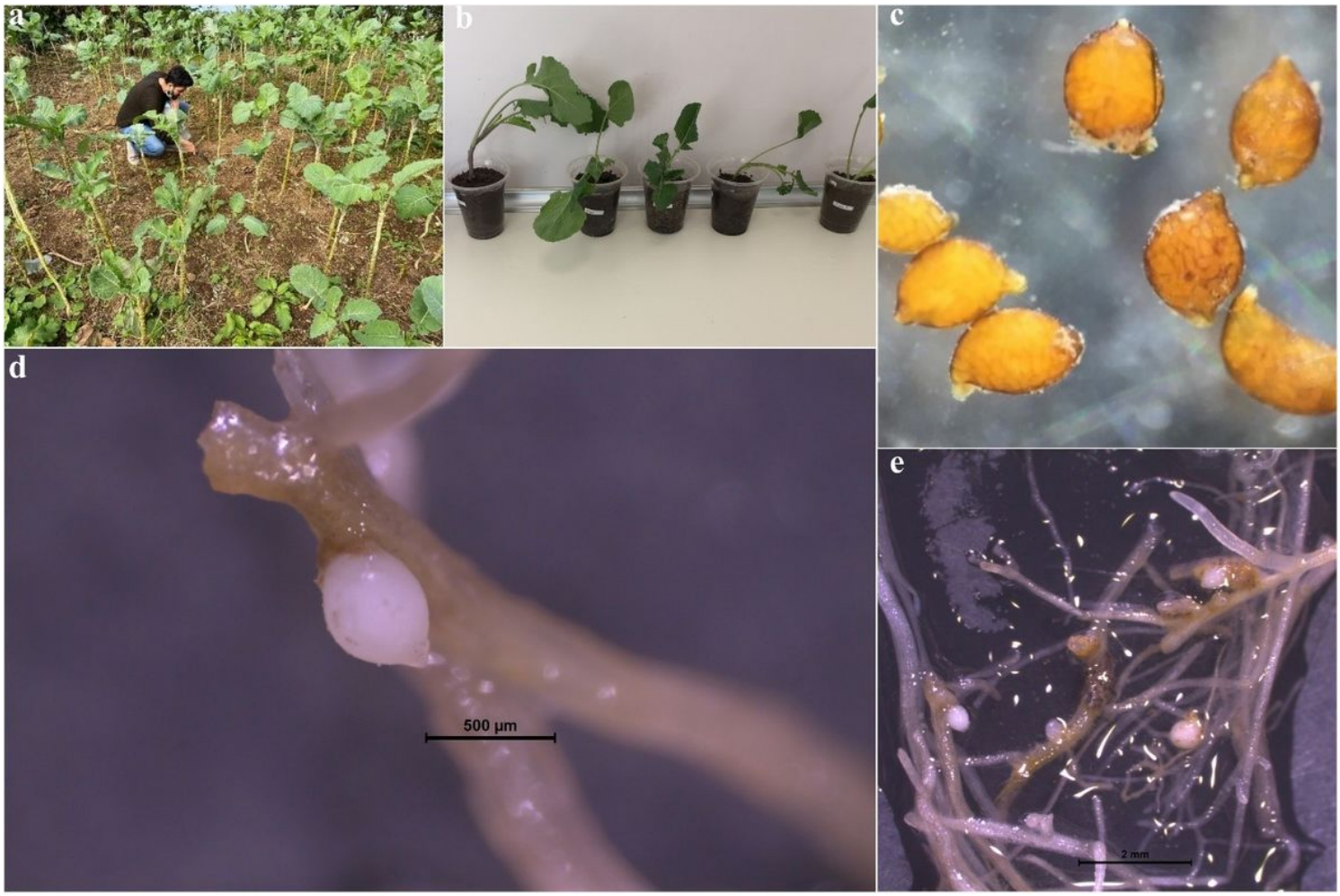

Figure 1

(a) Cabbage field infected with *Heterodera carotae*; (b) inoculated kale seedlings (c) cysts with eggs (d), White female on Cabbage roots; (e) secondary roots with white females and cysts on cabbage plant roots.

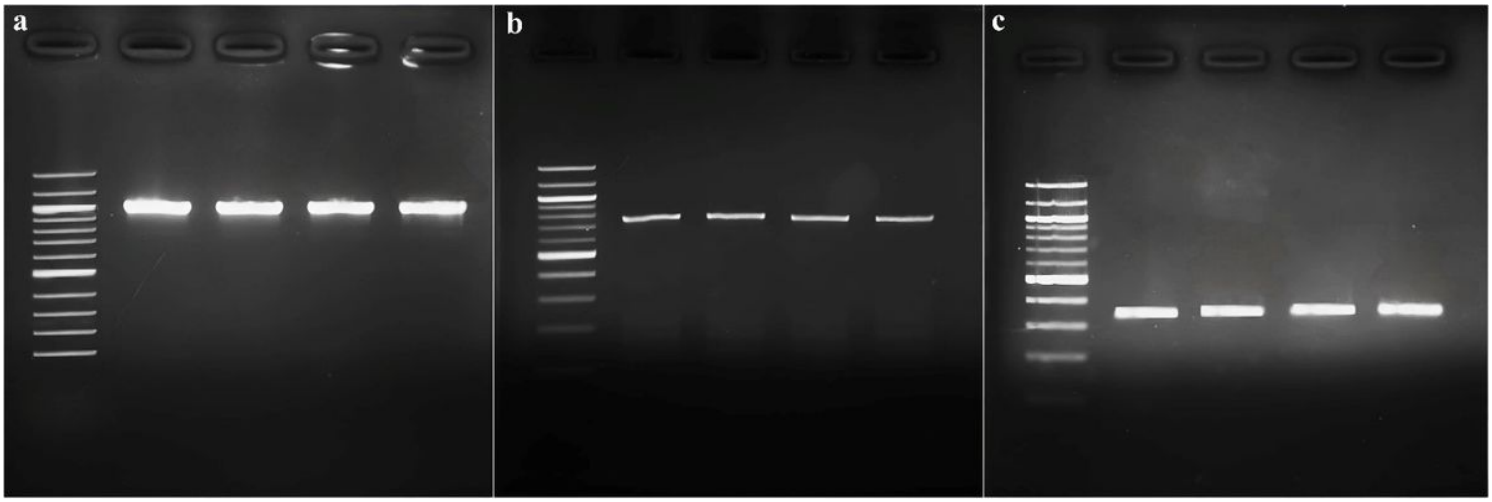

Figure 2

PCR products of Heterodera carotae species. (a) Fragments of D2/D3 region of 28S rDNA using D2A/D3B primer pair (Line1-4); (b) Fragments of internal-transcribed spacer (ITS) (ITS1-5.8S) region of rRNA using TW81/AB28 primer pair (Line1-4); (c) Fragments of Cytochrome Oxidase I (cox1) of mitochondrial DNA (mtDNA) using Car-F/Car-R primer pair (Line1-4); M, 100 bp DNA marker ladder.

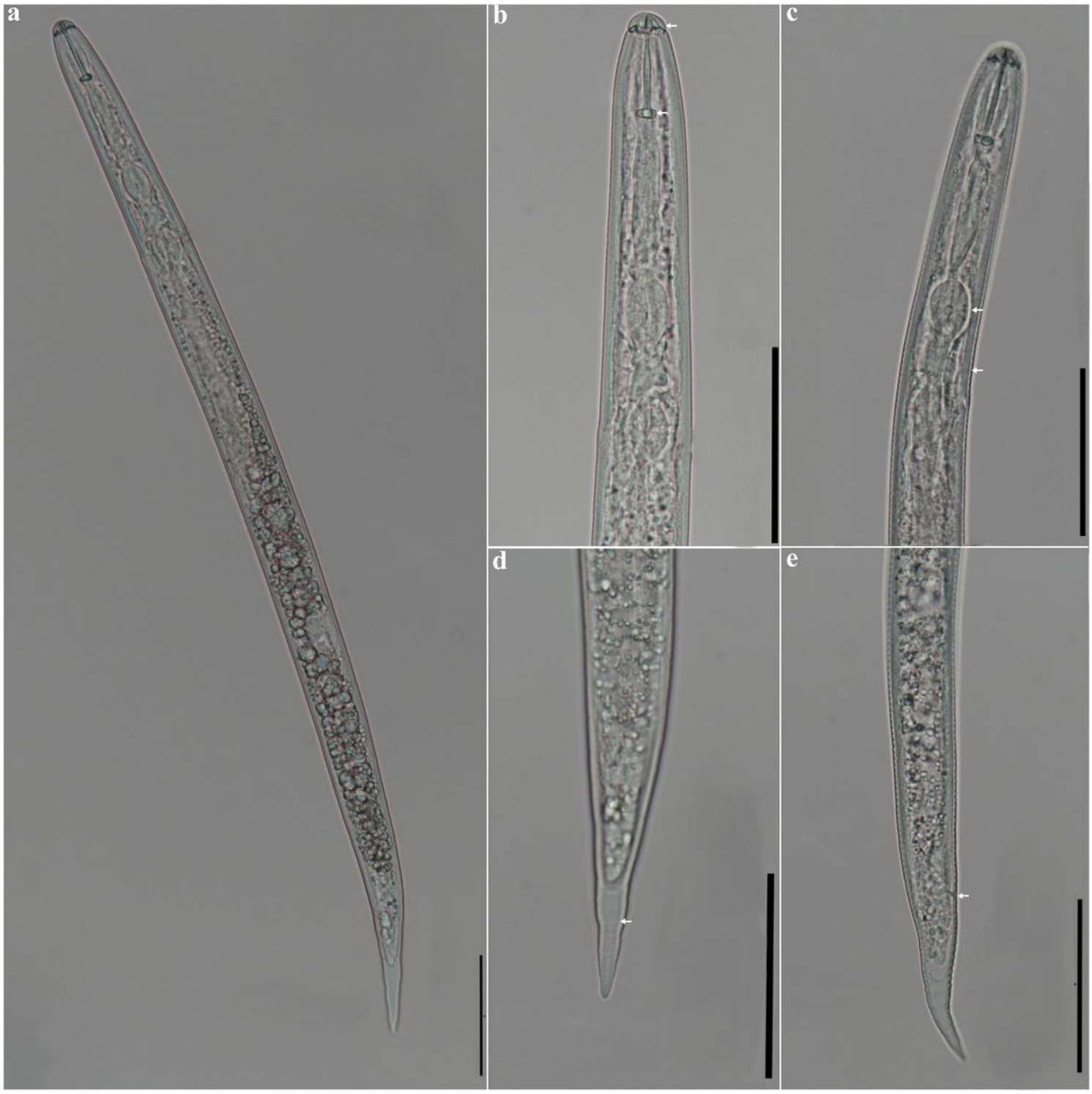

Figure 3

Photomicrographs of *Heterodera carotae* second-stage juvenil. (a) Female whole body; (b), (c) Anterior region (end); (d), (e): Tail region. Scale bars: 20 μm.

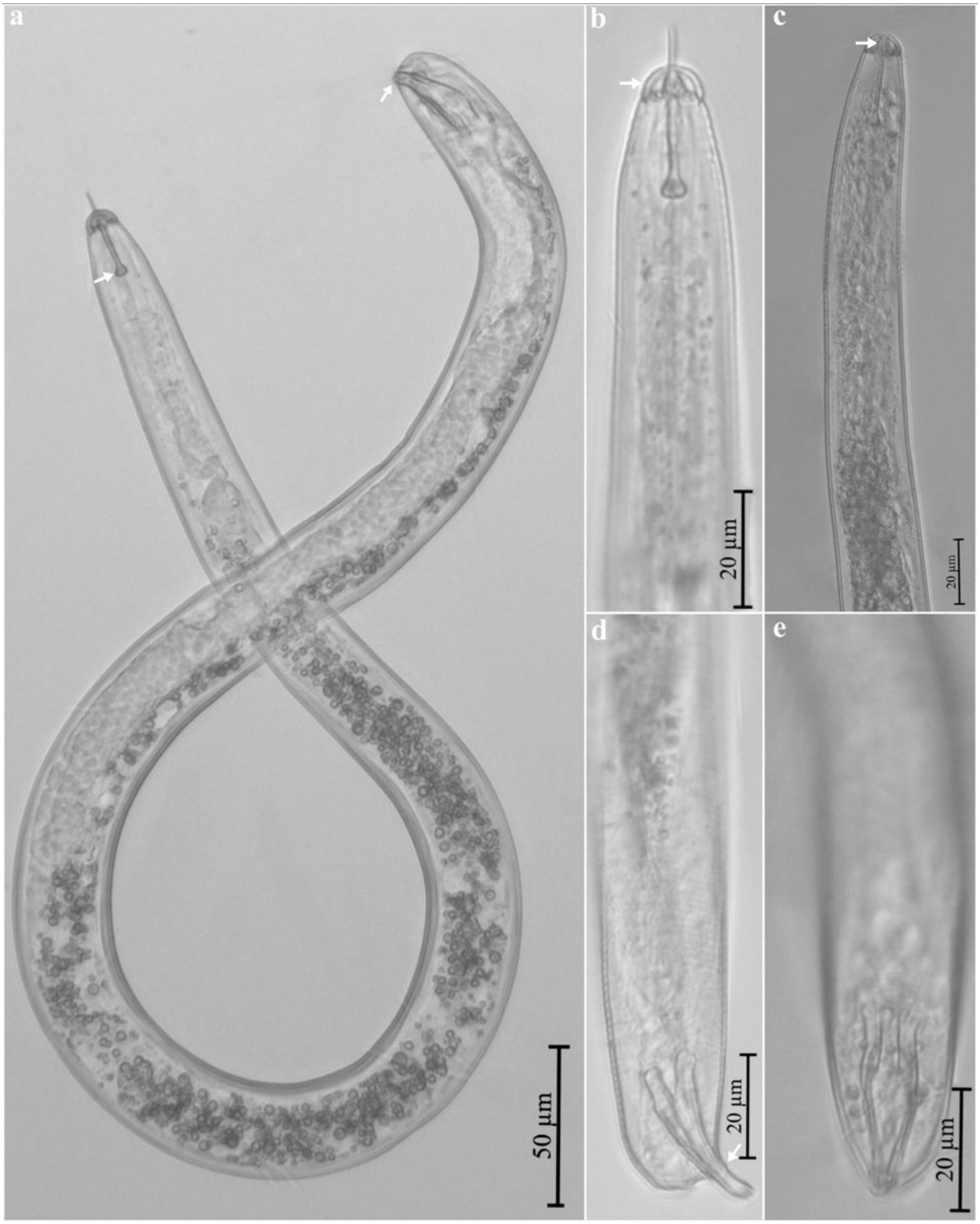

Figure 4

Photomicrographs of *Heterodera carotae* male. (a) Whole body; (b) Head region; (c) Anterior region; (d) Posterior region and characteristics tail tip; (e) Spiculae.

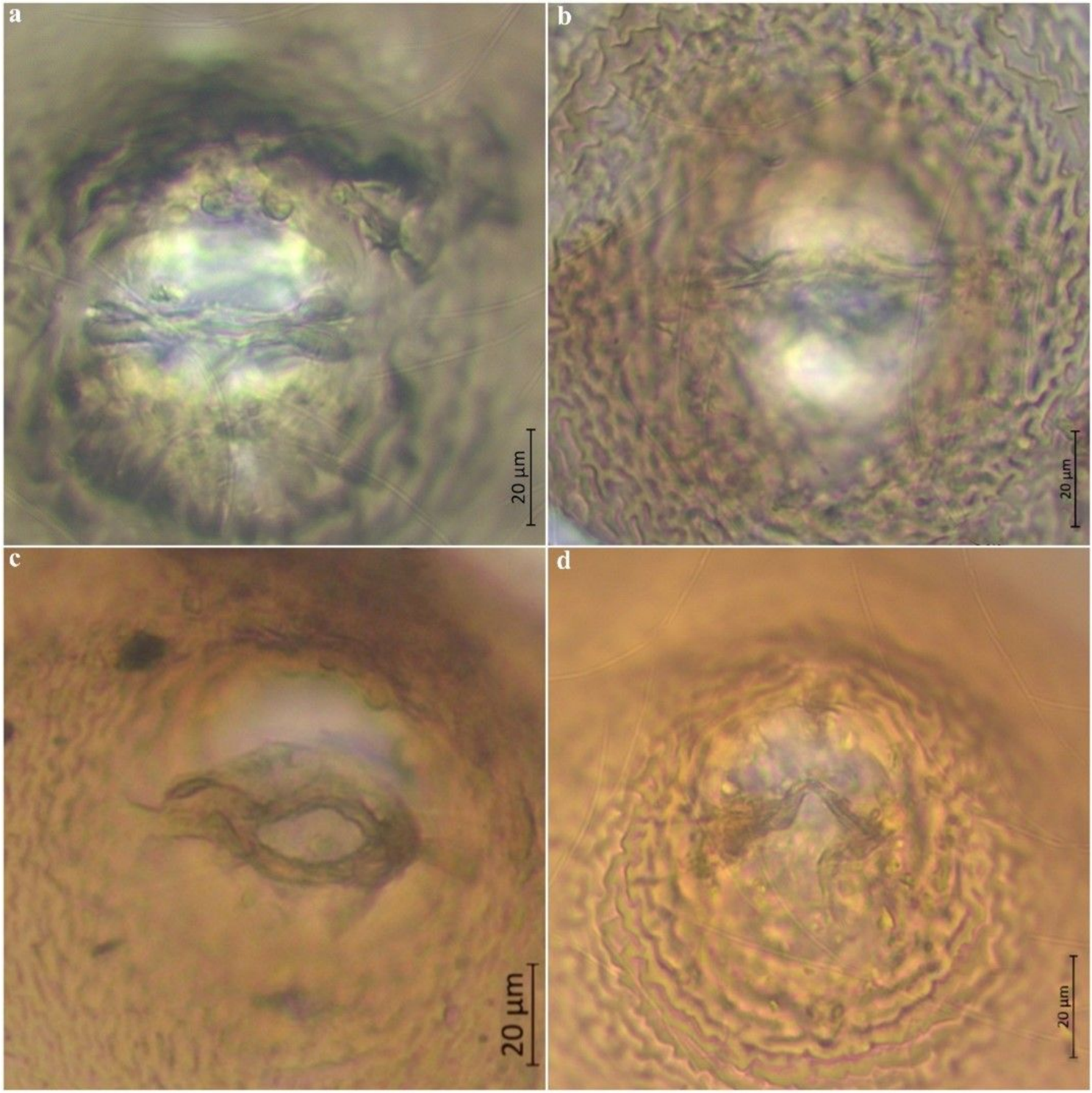

Figure 5

Light mycroscopy micrographs vulval structure of *H. carotae*. (a) Underbridge; (b) Fenestral and vulval slit (c) Sphincter vagina muscle (d) Vulval cone structure

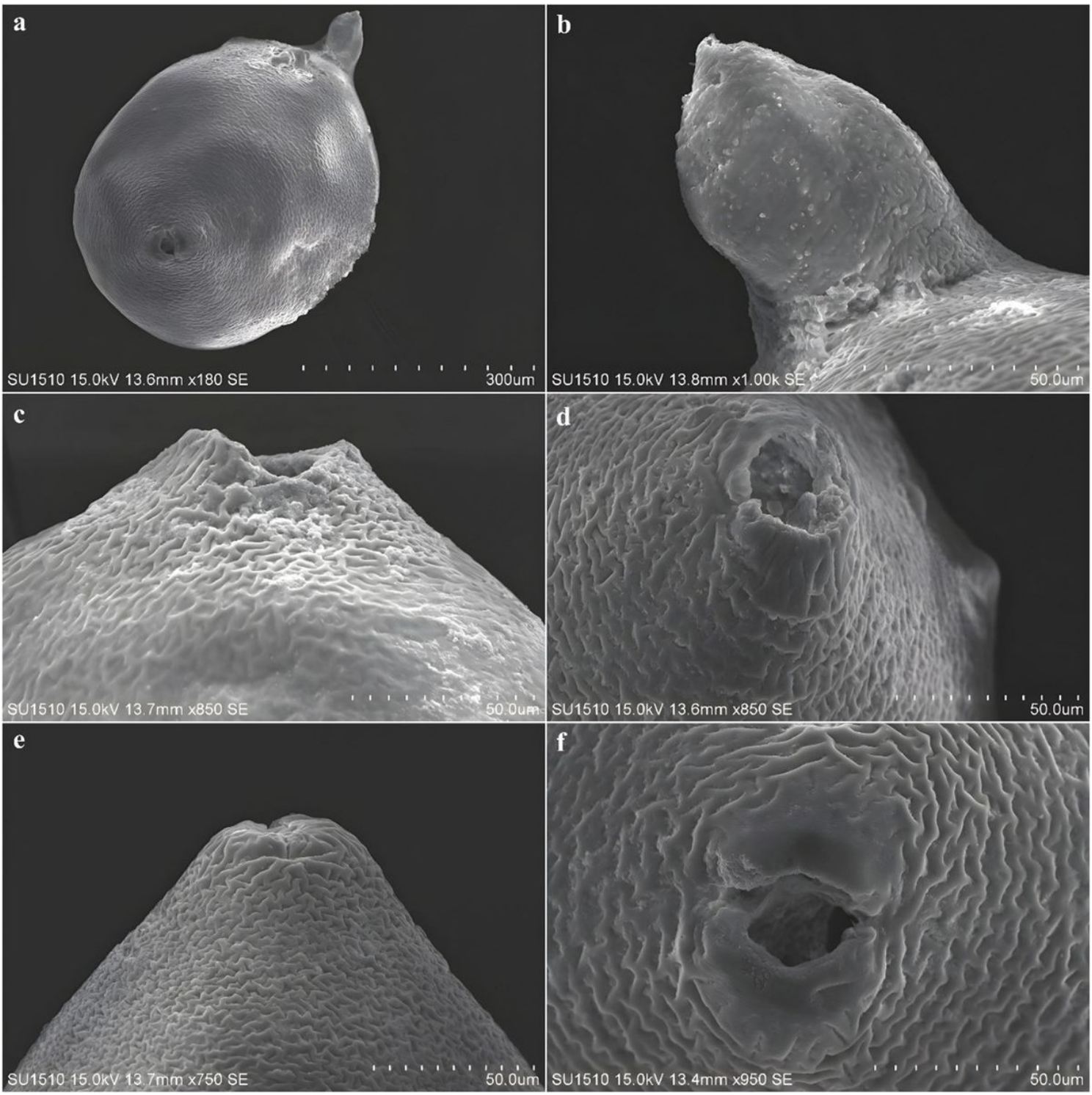

Figure 6

SEM photomicrographs of *Heterodera carotae* cyst: (a) Whole cyst; (b) Neck region; (c), (d), (e), (f) Vulval region.

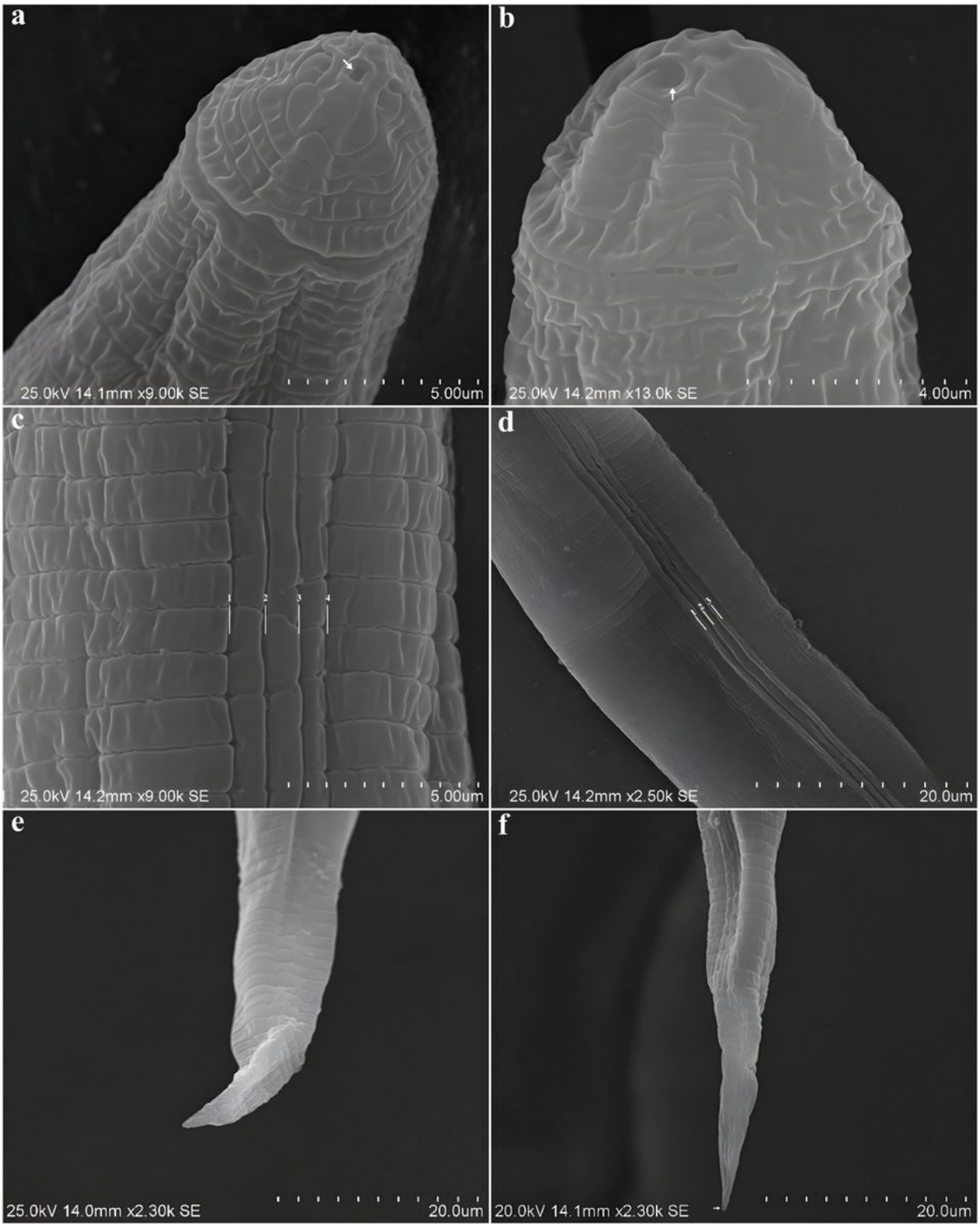

Figure 7

SEM photomicrographs of *Heterodera carotae* second stage juvenile (J_2): (a), Head region; (b), Lip region; (c) lateral line; (d) Mid-body; (e) Tail, (f) Posterior region.

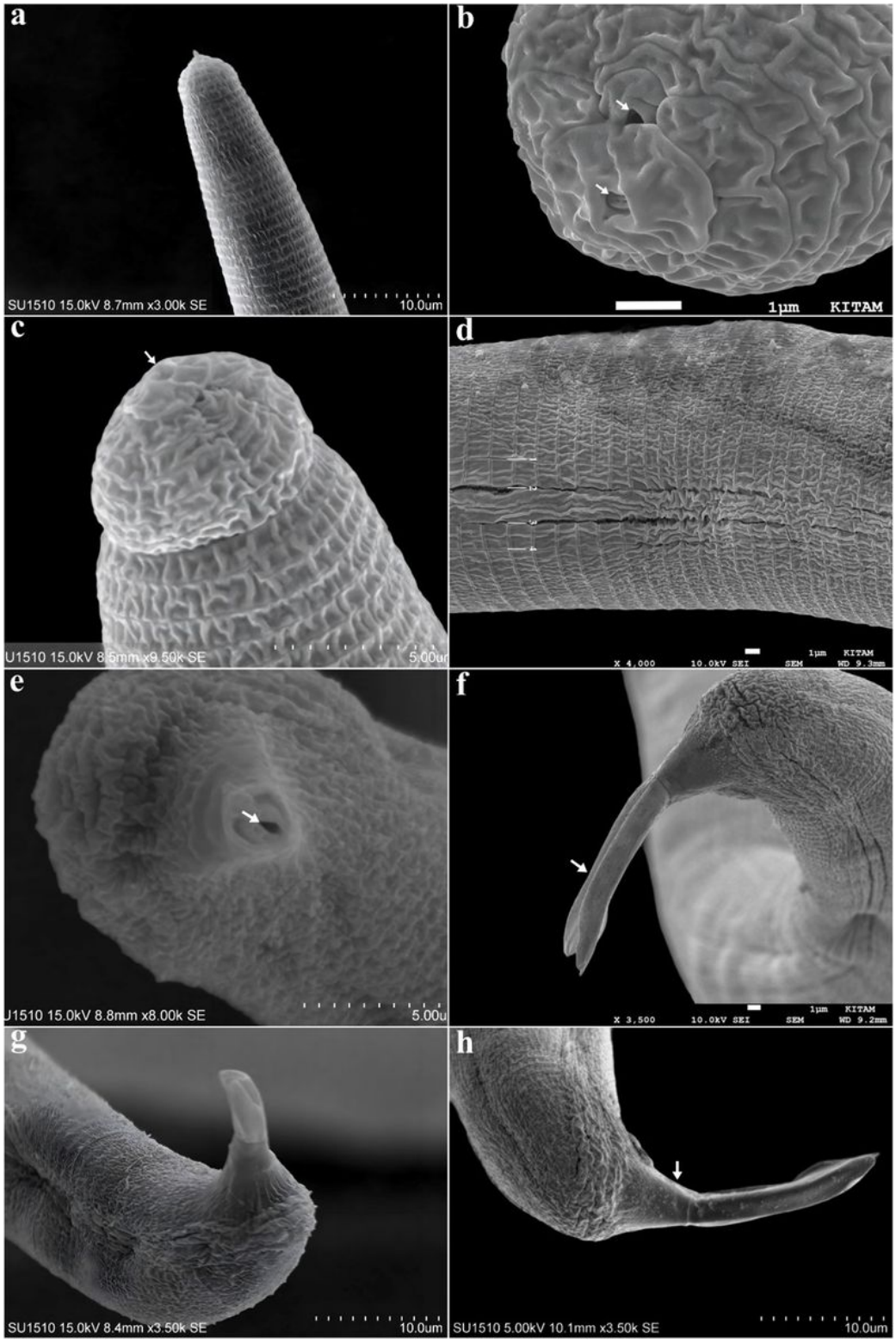

Figure 8

SEM photomicrographs of *Heterodera carotae* male: a, b, c Anterior region; d, Lateral field at mid-body; e, f, g, h, Posterior region.

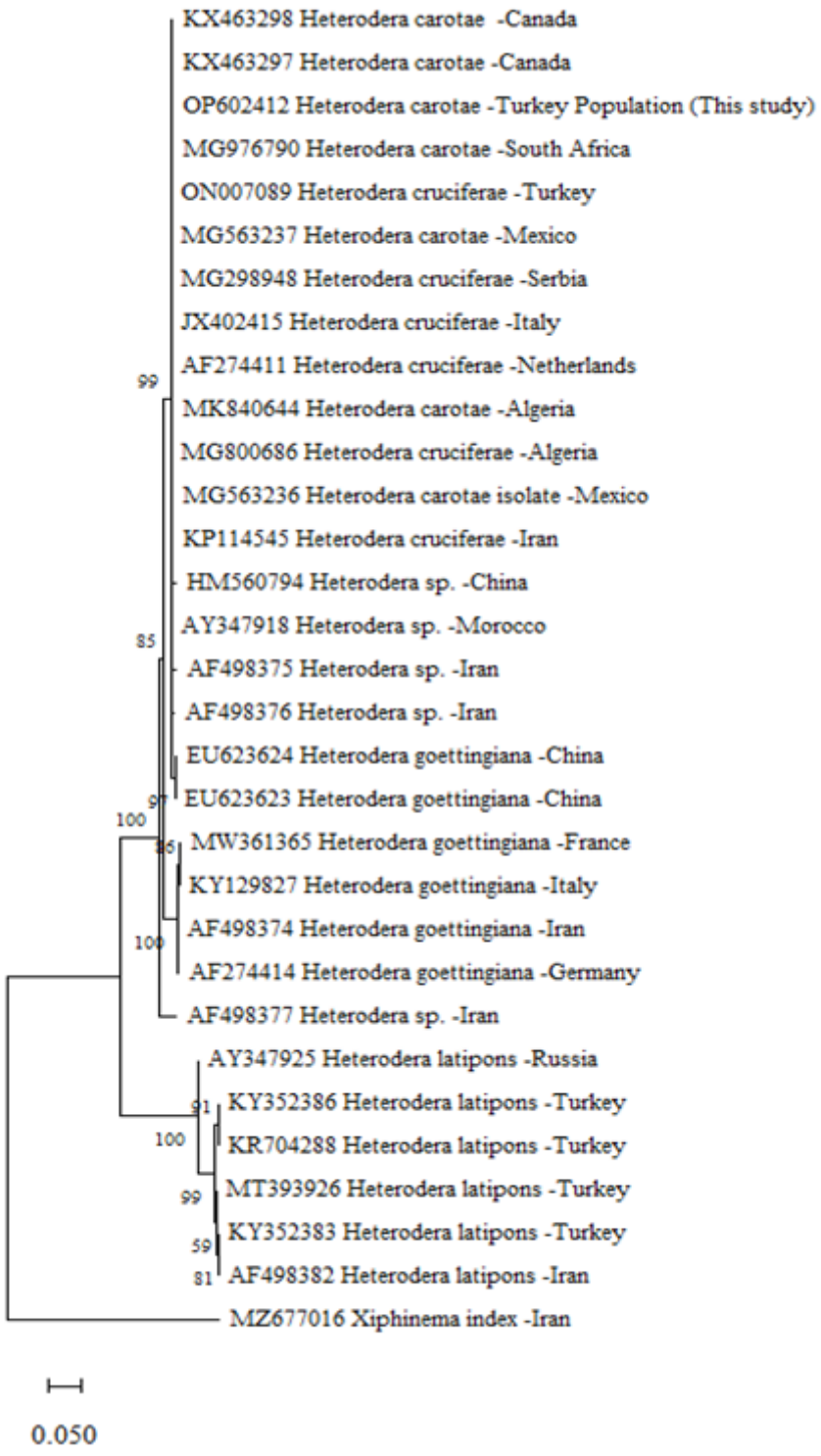

Figure 9

Phylogenetic relationship based on ITS expansion segments of ribosomal gene sequences within the genus *Heterodera*. Bootstrap support for each clade is indicated at the nodes. Accession preceded by OP602412 is a new sequence.