Morphological, Morphometric and Molecular Characterization of the Plant-Parasitic Nematode: *Hemicycliophora conida* Thorne, 1955 (Nematoda: Hemicycliophoridae) from Ethiopia

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Abstract

Nematodes are considered as one of the most difficult organisms to identify because of their morphological similarity, microscopic size, limited number of informative morphological characters, and overlapping morphometric measurements. Root ecto-parasite nematodes of the genus *Hemicycliophora* have wide distribution all over the world and feed on wide range of crop plants. The identified specimens of H. conida were found from rhizosphere of horticultural crops in the Rift Valley region of Ethiopia and were characterized morphologically and molecularly. Morphometric and morphological descriptions were done using light microscopy. *H. conida* most closely resembles *H. typica*, but it differs by lack of sperm cell in the spermathecia. Phylogenetic analysis was done using Bayesian inference method. Based on the full 18S SSU rDNA sequence, the resulted tree showed monophyly with sequence of *H. conida* deposited in the GeneBank from the Netherlands with 3bp nucleotide difference. This report represents the first comprehensive information on this species from Ethiopia.

Key words: Bayesian inference, Hemicycliophora, morphometric, morphology, phylogeny

Introduction

Plant-parasitic nematodes are known to be of economic importance to crop production in Ethiopia (Abraham 2009) but limited surveys have led to the identification of several plant-parasitic nematode genera/species associated with horticultural crops. So far the following nematode taxa were reported from Ethiopia by different authors; *Helicotylenchus* Steiner, 1945, *Hemicycliophora* de Man, 1921; *Macroposthonia* Andrassy, 1965; *Meloidogyne* Goldi,1892; *Pratylenchus* Filipjev, 1936; *Scutellonema* Andrassy, 1958; *Rotylenchus* Filipjev 1936; *Trichodorus* Cobb, 1913; *Aphelenchoides* Fischer, 1894 reported from *Ensete ventricosum by* Tessera et al 2009; Bogale et al. 2004; *Helicotylenchus multicinctus* (Cobb, 1893) Golden, 1956; *Rotylenchulus* spp., *Helicotylenchus* spp.,

Meloidogyne spp. *Radopholus similis, Helicotylenchus multicinctus, Pratylenchus goodeyi, Rotylenchulus anamictus,* reported from *Musa* sp. (Mohammed et al. 2009). Abebe and Geraert (1995) recorded one new species and other four species of plant parasitic nematodes; *viz, Discocriconemella addisababa, Criconemella parva, Paratylenchus leptus, Scutellonema brevistyletum* and *Helicotylenchus abunaamai.* O' Bannon (1975) reported, *Heterodera* spp., *Meloidogyne incognita, M. javanica, M. ethiopica,* Helicotylenchus spp., *Pratylenchus* spp., from vegetables. *Helicotylenchus multicinctus, Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961, *Helicotylenchus californicus* Sher, 1966 *Helicotylenchus gerti* Marais, Mekete & Tiedt, 2005, *Scutellonema paralabiatum* Siddiqi *et* Sharma, 1994, *Rotylenchus unisexus* Sher, 1965, *Tylenchorhynchus agri* Ferris, 1963, *Quinisulcius capitatus* (Allen, 1955) Siddiqi 1971, *Xiphinema insigne* Loos, 1949, *Xiphinema basilgoodeyi* Coomans, 1965, two species of *Xiphinema americanum sensu lato* reported from coffee crop by Mekete et al., 2008. *Pratylenchus brachyurus, P. zeae, Meloidogyne incognita, Helicotylenchus dihystera, Hoploliamus indicus, Tylenchorynchus brassicae and Rotylenchus reniformis* were recorded from Southwestern Ethiopia to be affecting soybean cultivars (Agu, 2006).

Plant-parasitic nematode surveys and identification in Ethiopia often focused on *Meloidogyne* spp. (Wondirad, Tesfamariam 2002). To our knowledge, identification of plant-parasitic nematodes in Ethiopia were mainly done based on light microscopic observations of morphological and morphometric characters. However, for some groups such as *Meloidogyne*, protein-based biochemical techniques (gel electrophoresis and immunological techniques) were used in addition to differentiate species (Wondirad, Kifle 2000).

Based on their morphological similarity, microscopic size, limited number of informative morphological characters and overlapping of morphometric measurements, nematodes are considered as one of the most difficult organisms in identification (Claudio 2011). Root ecto-parasite nematodes of the genus *Hemicycliophora* de Man, 1921 have wide distribution and feed on wide range of crop plants. Morphological characters alone are limited and insufficient to determine the species boundaries and resolve relationships. New methods using sequence analysis of the ribosomal DNA have proven to be useful for identification and molecular phylogenetic analysis. The introduction of identification tools such as bar coding and the sequencing of several nuclear and mitochondrial genes helped in differentiating phenotypically closely resembling species. In combination with morphology, molecular analyses provide a very useful identification tool (Coomans 2000, Subbotin & Moens 2006).

The objective of this study was to identify morphologically and molecularly the plant parasitic nematode (*Hemicycliophora conida)* found in the Rift Valley region of Ethiopia. The current study provides additional information on identification of plant-parasitic nematodes from Ethiopia.

Materials and Methods

Sampling

Soil samples from rhizosphere of main and permanent horticultural crops in the Rift Valley basin of Ethiopia were sampled during August, 2010 to determine the identity and population density of plant-parasitic nematodes. All sampled sites were located in the Rift Valley basin of Oromia Regional States. (Table 1). During this survey unique populations of sheath nematode (*Hemicycliophora conida*) of the species were detected in association with the surveyed plants.

Table 1. *Distribution of sampling sites, geographical locations and distance from the center*

Sampling Site	Regional state	Distance from Addis Ababa (km)	Elevation (masl)	GPS location
Melkassa	Oromiya	115	1553	8°24'N 39° 21' E
Tibila	Oromiya	175	1337	8° 26'N 39° 35' E
Wonji	Oromiya	107	1545	8° 26'N 39° 13'E

Soil samples were taken from six field sites. Each 1 kg soil sample consisted of 10-15 cores taken from the top 10-14 cm rhizosphere soil around the rhizosphere with auger. From each bulked sample, 200 g of subsample was taken for further investigation. The samples were kept in plastic bags in an ice box during transportation and taken to the laboratory for nematode extraction and identification. Nematodes were extracted using the modified Baermann method (Hooper 1985). Briefly, samples were divided in two 100 g each and placed in a 20 cm modified Baermann tray in order to hold soil above water and on top of it covered by tissue paper. Water was carefully added inside edge of tray until soil layer is wet. After 36 hours, the baskets were carefully removed and nematode suspensions were collected from tray and subdivided in two beakers (for molecular and morphological tests) for an hour to concentrate or settle the nematodes.

Morphological Observations

Formalin (4% with 1% glycerin) was heated to 70°C and added quickly to kill & fix nematodes in one step (Seinhorst, 1966). The fixed specimens were processed to anhydrous glycerin following the glycerin-ethanol method of Seinhorst (1959) and modified by De Grisse (1969). Fixed specimens were permanently mounted in anhydrous glycerol (Hooper, 1985) within a paraffin ring on thin microscope glass slides (26 mm x 76 mm) and 19 mm diameter round cover slips. For morphometric data and description, nematode specimens were drawn using Olympus BX50 and Olympus CH30 light microscopes. The morphometric data were taken according to de Man (1980) and Siddiqi (2000). Voucher specimens are deposited at Ghent university Nematode collection as UGnem-36.

DNA extraction, PCR and sequencing

For molecular characterization, half of the extract was used after preserving with DESS solution containing 20% dimethyl sulphoxide (DMSO) and 0.25 M disodium EDTA, saturated with NaCl, pH 8.0 (Seutin *et al.* 1991). This was done according to Yoder *et al.* 2006. Individual nematode from the solution was mounted on temporary slides and identified using light microscope. For DNA extraction, nematodes were mechanically disrupted in 25 µl of premixed worm lysis buffer (WLB) containing 50mM KCl, 10mM Tris, pH 8.3, 2.5mM MgCl2, 0.45% NP 40 (Tergitol Sigma), and 0.45% Tween 20 (Thomas et al., 1997) and stored at -80°C for 10 min. Then after, 1µl of proteinase K were added and incubated in the PCR machine at 65°C for one hour followed by 10 min at 95°C.

Polymerase Chain Reaction

The 18S SSU rDNA region were PCR amplified using G18S4 & 4F (forward) and 18P & 4R (reverse) primers (Blaxter *et al.* 1998). A master mix of Toptaq Qiagen was prepared (Taq DNA Polymerase, Qiagen, Germany) and DNA template was used. All PCR reactions were conducted by Thermo cycler with a cycle profile of: 1 cycle at 96°C for 4 min followed by 40 cycle at 95°C for 30 second, 54°C for 30 second, 72°C for 1 min. The last step was 72°C for 10 min. The PCR product was loaded on 1% Agarose gel and stained with Ethidium bromide.

Sequencing protocol for BigDye V3.1 on ABI 3130XL were used and cycle sequencing were performed by terminator ready reaction mix, cleaned PCR product, primers (2FX, 23R, 23F, 9FX, 9R, 26R) and sterile water. Primers used for sequencing of 18S SSU rDNA were: 9FX, 2FX, 13R, 23F, 23R 9F, 9R and 26R (Blaxter *et al.* 1998, Meldal *et al.* 2007, Bert 2008). Samples were loaded with 10µl Hi-Di Formamide and transferred from each tube to a 96 well plate and the plates were put in the sequencer and run by automated DNA sequencer. Sequencing was performed in both directions.

Phylogenetic Analysis

The sequence results of the 18S SSU rDNA region were aligned with sequences of the related species from the GenBank using ClustalW (Thompson *et al.* 1994) provided by BioEdit sequence alignment editor (Hall 1999). Phylogenetic analyses were performed by Bayesian inference (BI) method with MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). A general time-reversible model with rate variation across sites and a proportion of invariable sites (GTR + I + G) was used. Analyses were run for 3×10^6 generations and trees were generated using the last 1,000,000 generations beyond the burn-in value. Also other methods (maximum parsimony, neighbor joining, maximum likelihood) using PAUP: Phylogenetic Analysis Using Parsimony (Swofford 2002) provided the same tree topologies but is not further discussed herein.

Results and Discussions

A total of 1670 specimens' of plant-parasitic nematodes associated with horticultural crops at different localities and host plants were identified to genus level. The genera *Hemicycliophora, Xiphinema* and *Rotylenchulus* were the most abundant nematodes in the samples taken. *Hemicycliophora conida* Thorne, 1955, was characterized morphologically and studied further on the basis of molecular phylogeny.

Descriptions

HEMICYCLIOPHORA CONIDA THORNE, 1955

Materials: Fifty-one female specimens from five host crops were examined.

Table 2. *Morphometrics of* Hemicycliophora conida *Thorne, 1955 collected from ....All measurements are in µm and in the form: Mean ± standard deviation (Range)*

	Samples (per host)						Topotypes from Loof (1968)		
Characters	Guava	Citrus	Citrus	Sugarcane	Grapevine	Banana	*H. typica*	*H. conida* I	*H. conida* II
n=	7♀	6♀	10♀	10♀	10♀	8♀	26♀	100♀	83♀
L	666±52 (584-736)	687±41 (640-736)	696±44 (648-800)	702±42 (632-768)	710±23 (680-760)	701±35 (640-744)	680-850	840 (660-990)	790 (640-870)
A	19±2 (15-21)	22±1 (20-24)	22±2 (20-26)	20±1 (18-22)	22±1 (20-24)	21±2 (19-24)	21-26	23 (18-27)	23 (18-28)
B	5 (4-6)	5 (5-6)	5 (5-6)	5 (5-6)	5 (5-6)	5 (5-6)	5.1-5.7	5.2 (4.5-6)	5.1 (4.1-5.7)
C	8±1 (7-10)	9±1 (8-10)	9±1 (8-10)	8±1 (7-10)	9 (8-9)	8±1 (7-9)	8.8-10.9	11.1 (8.7-14.4)	11 (8.3-13.5)
c'	3 (2-3)	3 (3-3)	3 (3-4)	3 (3-3)	3 (3-4)	3 (3-4)			
V%	83±2 (80-85)	83±1 (81-85)	82±1 (81-84)	82±1 (80-84)	82±1 (81-84)	83±1 (81-84)	83-86	86 (84-88)	87 (83-90)
stylet	61±4 (53-66)	58±3 (54-62)	61±2 (57-65)	61±4 (55-66)	62±2 (59-64)	61±2 (59-65)	-	87 (78-96)	80 (69-86)
Pharynx length	127±8 (113-139)	127±5 (121-131)	131±3 (127-135)	129±8 (115-141)	131±3 (127-136)	130±3 (127-135)	-	-	-
Body width	35±6 (30-48)	32±3 (27-35)	32±3 (25-34)	35±2 (32-39)	33±2 (30-36)	33±3 (30-38)	-	-	-
Anal b. width	28±5 (25-39)	27±2 (25-30)	25±2 (23-28)	28±2 (26-31)	27±1 (25-28)	27±2 (25-30)	-	-	-
Tail length	80±8 (69-91)	77±8 (69-90)	80±9 (67-96)	84±10 (70-98)	83±5 (77-94)	86±9 (78-105)	-	-	-
R	203±6 (193-210)	194±8 (182-204)	208±8 (197-219)	188±26 (121-212)	201±5 (192-208)	205±6 (198-215)	193-210	245 (227-274)	204 (180-220)
Rex	39±4 (33-46)	40±1 (38-41)	41±1 (39-43)	38±2 (35-42)	39±1 (37-40)	39±2 (37-42)	37-42	48 (42-53)	40 (36-43)
Rva	11±2 (8-13)	12±2 (10-15)	13±1 (11-14)	14±1 (12-16)	14±2 (11-18)	14±1 (11-15)	11-16	15 (11-21)	13 (9-16)
Ran	34±5 (27-42)	30±4 (26-38)	34±7 (28-47)	32±4 (24-37)	31±3 (27-37)	33±3 (28-38)	28-32	33 (24-39)	27 (20-32)

n= number of specimens; L= total body length in µm; a= body length/greatest body width; b= body length/ distance from anterior end to junction of pharynx and intestine c= body length/ tail length; c'= tail length/ body width at anus V= distance of vulva from anterior end x 100/body length; R=Number of body annuli; Rex=Annuli between labial disc and first annuli after excretory pore; Ran =Number of annuli from tail terminus to anus; Rva =Number of annuli between vulva and anus

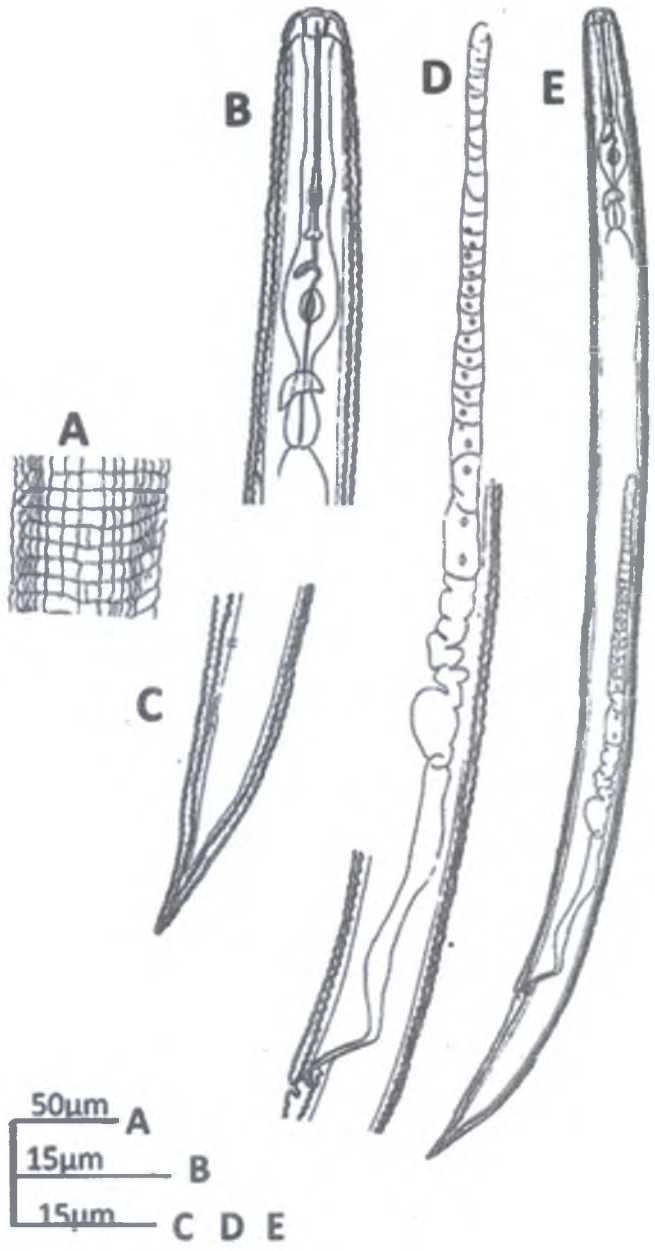

Figure 2. Light Microscopy drawings of Hemicycliophora conida. A: lateral field at mid body; B: Head region; C: tail shape; D: Female reproductive system; E: Entire body

Description

Female. Body nearly straight when relaxed, some specimens with slightly bent ventrally in the post vulvar part. Body length with a range of 666-710 µm. Double cuticle, the outer cuticle fitting close. On the distal portion of tail, very fine but distinct annules visible. Body clearly annulated with 188 to 208 annules in range of all population. Lateral field indicated

except in anterior most part and at terminal ends (tail and lip region), with two longitudinal lines, between which break by transverse striae over the whole body, outside of lateral field cuticle with numerous longitudinal lines forming regular blocks over whole length. Lateral lips lower than submedian lips. Labial disc in line with the apices of the submedian lips. Stylet mean range within 58-62µm. Basal of stylet knob directed backward and slightly elevated. Pharynx, range of mean value 127-131µm from anterior end to middle of median bulb. Excretory pore near the end of pharyngeal bulb, 38-41 annules range of mean value from anterior end. Hemizonid clearly visible near the end of pharynx and 3-4 annules anterior to excretory pore. Vulva lips modified, elongate post vulval, but irregular. Posterior vulva position (range of mean 82-83% from the anterior end of body, range mean values). Female reproductive system prodelphic, monodelphic, outstretched. Uterus long and thin with round spermatheca without sperm. Post vulvar body cylindrical, tapering at the end. Anus hardly marked. Tail (77-86 µm length) largely cylindrical, with narrow marked conoid distal end with pointed tip. (Fig. 2 &3).

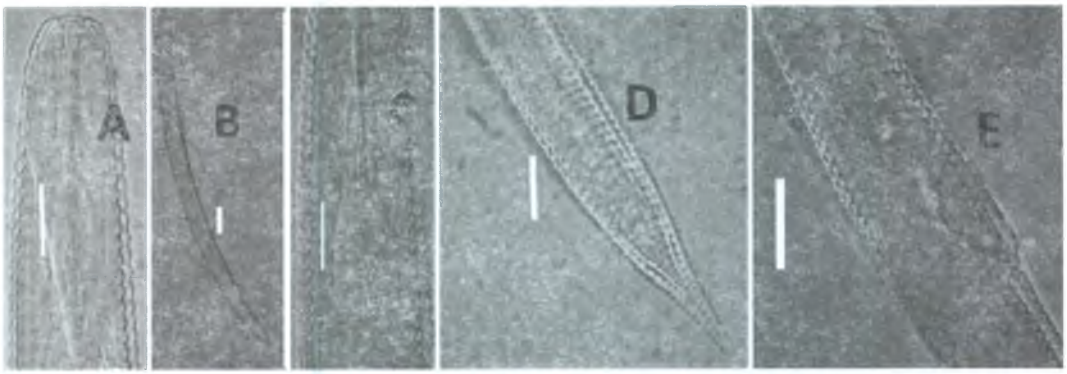

Figure 3. Light microscopy photographs of *Hemicycliophora conida*; A: Entire body, B: head region, C: Stylet knob and hemizonid; D: tail shape; E: Vulva and spermatheca (Scale bars: A, C, D, E= 20µm; B= 50µm)

Remarks

The studied Ethiopian specimens most closely resembles *H. conida* Thorne,1955 form II (according to Loof 1968) in body habitus, body length, range mean number of body annules, number of annules from anterior end to excretory pore, empty spermatheca, basal of stylet knob directed backward and slightly elevated. However, slight differences were observed between *H. conida* form II and the studied specimens in vulva position, 82-83% (range of mean) *vs* more posterior 83-90%, shorter stylet length 58-62 µm *vs* 69-86 µm. According to Thorne (1955) hemizonid were weakly developed in the case of *H. conida,* but in most specimens of studied population it was clearly visible near the end of pharynx and anterior to excretory pore. The morphometric variation of studied Ethiopian specimens with *H. conida* form II can be affected by specimen fixation and environmental conditions which is in an expected morphometric variation range over a population of different geographical locations.

Our specimens also resemble *Hemicycliophora typica* de Man, 1921 as redescribed by Loof (1968) in morphometric features such as body length, vulva position, number of lip annules and in body habitus with typical tail shape. However, our specimens differ from *H. typica* in shorter stylet 58-62 µm *vs* 63-74 µm, lower number of body annules 188-108 *vs* 193-210. Spermatheca is empty *vs* spermatheca with sperm and males are absent in our samples *vs* and males are known in *H. typica*. In all sampled populations spermatheca with sperm were not observed.

Furthermore rDNA sequences of the described material shows sister relationship with *H. conida* with lower number of nucleotide difference (3bp) and 0.2% divergence (Table 3).

H. conida form I is similar with *H. conida* form II and the studied specimen in body formation, but it differs in longer body size, longer stylet, higher number of body annules and annules from anterior to excretory pore (see table 2). \

H. conida is reported from South Africa, Ran with more annules, 33 to 55 compared with 24 to 39 (Loof, 1968), tails of the South African specimens are longer 122.7 compared with 86.9 of the Dutch specimens and also higher than Ethiopian specimens. Among species described from Africa *Hemicycliophora labiata* Colbran, 1960 was the other species which has closely related morphology and morphometric data to Ethiopian specimens but it differs mainly in having sperm cell in spermatheca (Berge, 1981).

Male: not found

Locality and host

The specimens were extracted from soil samples, collected from Wonji Shewa sugar industry farm (sugarcane), Melkassa Agricultural Research Center (*Citrus*, grapevine (*Vitis* sp.) Guava (*Psidium* sp.), Banana (*Musa* sp.) and Upper Awash Agro Industry Enterprise farm (Citrus) (Table.1).

Phylogeny

Bayesian Inference 50% majority rule consensus tree of *Hemicycliophora* species specimen under study shows monophyly with *H. conida* (AJ966480) with posterior probability support of (PP:81) (Fig. 4) and with intraspecific divergence of 0.2% and 3bp nucleotide difference (Table 3.). The other *H. conida* with accession number (AJ966471) is paraphyletic with the studied material and showed the divergence value of 1.6%. *H. typica* is also paraphyletic with the studied specimen (Fig. 4).

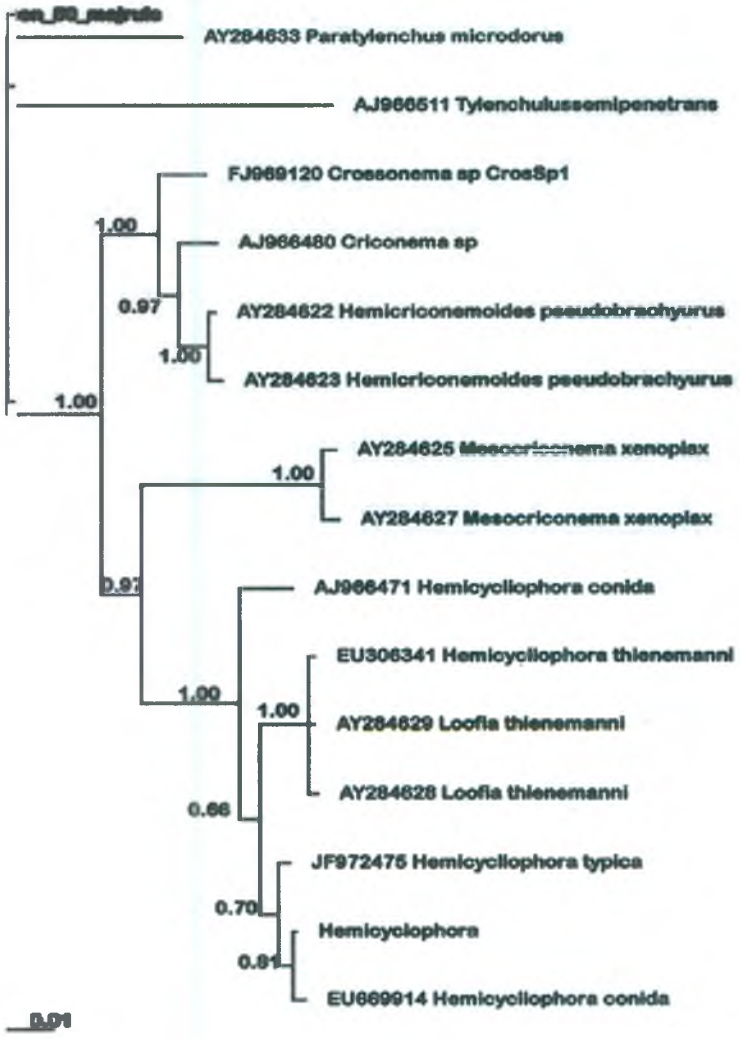

Figure 4. BI 50% majority rule consensus phylogeny of *Hemicycliophora* sp. and with 12 other related species from GenBank sequences based on SSU 18S rDNA data analysed with a GTR+I+G model as estimated by PAUP/Mr Modeltest 1.0b. Branch support is indicated by posterior probably (PP). *Pratylenchus microdorus* and *Tylenchulus semipenetrance* are outgroups.

PAIRWISE SIMILARITY COMPARISON OF SSU RDNA

Table 3. *Sequence Identity Matrix of* Hemicycliophora *species and closely related taxa with two out groups*

Sequen->	Hemic	AJ966	EU669	EU306	AJ966	AY284	AY284	FJ969	AY284	AY284	AY284	AY284	JF972	AY284	AJ966
Hemicyc	1,000	0,957	0,998	0,984	0,983	0,983	0,983	0,960	0,958	0,947	0,906	0,900	0,476	0,887	0,921
AJ966480	---	1,000	0,956	0,957	0,954	0,956	0,956	0,983	0,986	0,949	0,930	0,900	0,467	0,895	0,926
EU669914	---	---	1,000	0,982	0,982	0,982	0,982	0,959	0,957	0,946	0,905	0,899	0,475	0,886	0,919
EU306341	---	---	---	1,000	0,979	0,998	0,998	0,957	0,961	0,945	0,908	0,897	0,470	0,886	0,919
AJ966471	---	---	---	---	1,000	0,978	0,978	0,958	0,955	0,945	0,903	0,898	0,470	0,890	0,921
AY284629	---	---	---	---	---	1,000	0,997	0,956	0,959	0,944	0,906	0,896	0,470	0,885	0,918
AY284628	---	---	---	---	---	---	1,000	0,956	0,959	0,944	0,907	0,896	0,470	0,886	0,919
FJ969120	---	---	---	---	---	---	---	1,000	0,982	0,952	0,925	0,902	0,469	0,894	0,923
AY284622	---	---	---	---	---	---	---	---	1,000	0,949	0,939	0,900	0,468	0,895	0,927
AY284625	---	---	---	---	---	---	---	---	---	1,000	0,897	0,938	0,462	0,879	0,909
AY284623	---	---	---	---	---	---	---	---	---	---	1,000	0,952	0,495	0,947	0,879
AY284627	---	---	---	---	---	---	---	---	---	---	---	1,000	0,490	0,933	0,866
JF972475	---	---	---	---	---	---	---	---	---	---	---	---	1,000	0,481	0,445
AY284633	---	---	---	---	---	---	---	---	---	---	---	---	---	1,000	0,880
AJ966511	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1,000

Key for codes in the table

Hemicycliophora
AJ966480_Criconema_sp
EU669914_Hemicycliophora_conida
EU306341_Hemicycliophora_thienemanni
AJ966471_Hemicycliophora_conida
AY284629_Loofia_thienemanni
AY284628_Loofia_thienemanni
FJ969120_Crossonema_sp_CrosSp1
AY284622_Hemicriconemoides_pseudobrachyurus
AY284625_Mesocriconema_xenoplax
AY284623_Hemicriconemoides_pseudobrachyurus
AY284627_Mesocriconema_xenoplax
JF972475_Hemicycliophora_typica
AY284633_Paratylenchus_microdorus
AJ966511_Tylenchulus semipenetrans

Conclusions

Although morphological description is mostly affected by environmental conditions, it is still a very important feature of systematic (Luc *et al.*, 2010; Coomans, 2002). The molecular phylogeny confirms the sister relationship of the described material with *H. conida* sequence deposited in the GenBank from The Netherlands by Holterman *et al.* (2008). Although the information on different forms of species (I *vs* II) submitted sequence was not described in the authors' publication, *H. conida* type II is most common and widespread species in The Netherlands (Loof, 1968).

In general, though the described specimen showed some morphological differences with original description of *H. conida* form II, it has more closely related morphological & morphometric appearances with molecular phylogenetic support. So the described material can be suggested as *H. conida* Thorne, 1955. The topologies of tree, agree with previously done phylogenetic tree used SSU rDAN by Megen *et al.* (2009), Bert et al. (2008) and Subbotin *et al.* (2005). This is the first comprehensive report on the *Hemicycliophora* species from Ethiopia.

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