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RESEARCH

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Molecular and phenotypic characterization of *Hemicriconemoides rosae* (Rathour et al., 2003) from mustard rhizosphere in India

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Abstract

Background Nematodes belonging to Genus *Hemicriconemoides*, commonly known as sheathoid nematodes, damaged many fruits, vegetables and cash crops, worldwide. A survey has been conducted in the agricultural fields of Bulandshahr district to find out the plant-parasitic nematodes load.

Results A total of 85 soil samples were collected from mustard fields of Bulandshahr district of Uttar Pradesh and processed for the presence of plant-parasitic nematodes and they were isolated by 'Cobb sieving method'. Among all the collected soil samples, one soil sample found to be positive for the genus *Hemicriconemoides* and docketed as HCN. The earliest identification based on morphology revealed the species to be *Hemicriconemoides rosae* which was recovered from the mustard field for the first time. The morphology was found to be more consistent with rose population as compared to sugarcane population of the *Hemicriconemoides rosae*. Further, the *Hemicriconemoides rosae* was identified on the basis of molecular and phylogenetic analysis based on the concatenated matrix. In addition, correlation analysis of the *Hemicriconemoides rosae* based on morphometric parameters was done. The results revealed the importance of body length and its relation with other morphometric parameters, and they were found significant. Besides this, de Manian index a showed highest correlation with the body length in adult females. In all the studied ratios, a is very important for the evaluation of the females of a specific species of genus *Hemicriconemoides*. The data from the principal component analysis (PCA) revealed the high intraspecific and interspecific variations between the species of genus *Hemicriconemoides*. However, less intraspecific variations were present between the rose population and mustard population of *H. rosae*.

Conclusions The study revealed the new host, i.e. mustard crops, for *H. rosae* showing dissimilarity in morphology with the sugarcane population.

Keywords *Hemicriconemoides rosae*, Mustard, Morphology, Taxonomy, Correlation, PCA

Background

There are some nematodes which show association with the plants over millions of years, have resulted in the evolution of plant-parasitic nematodes (PPNs). They have been evolved in such a way that they have developed an accessory structure known as stylet with the help of which they are associated with the plants and able to derive the nutrients from them. Genus *Hemicriconemoides* described by Chitwood and Birchfield (1957) comprises of the species which does not belong to either the genus *Hemicycliophora* (De Man, 1921) or *Criconemoides* (Taylor, 1936). The demarcating characteristics possessed by members of this genus are the presence of an extra cuticular sheath in the mature females, presence of large body annules and having lateral and

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sublateral ridges throughout the length of males giving the appearance of slight caudal alae. These nematodes are commonly known as 'sheathoid' nematodes due to the presence of an outer accessory sheath with smooth annuli.

The species of this group have been reported worldwide; however, some of them are native (Geraert, 1966). The nematodes of this genus showed obligatory ectoparasitism (Geraert, 1966). Though they are obligate parasites, they could be migratory, semi-sedentary and sedentary depending upon the environmental conditions (Geraert, 1966).The nematodes of this group were found to be associated with higher plants, mainly woody perennials, vines, etc.(Christie, 1953; Jenkins & Taylor, 1967), and several other plants as a result of which rot, necrosis of leaves, suppressed root growth, root lesions and coarse roots, etc., are common symptoms observed in case of these nematodes.

The genus *Hemicriconemoides* (Chitwood & Birchfield, 1957) comprises of 54 species (Khan et al., 2019; Maria et al., 2018). Besides this, there were some species which was synonymized by the Geraert (2010). It is interesting to note that 13 species have been reported from the agricultural rich fields of India, i.e. *Hemicriconemoides asymmetricus* recovered from rose (Rathour et al., 2003; Uttar Pradesh), *H. communis* isolated from the orange, mango, mulberry, litchi and peach plants (Edward & Misra, 1964; Uttar Pradesh), *H. conicaudatus* recovered from peach fruits rhizosphere (Phukan & Sanwal, 1983; Assam), *H. dipterocarpus* recovered from *Dipterocarpus tuberculatus* (Mohilal et al., 2004; Manipur), *H. doonensis* from litchi (Srivastava et al., 2000; Uttar Pradesh), *H. longistylus* from tree fern (Rahman, 1990; Assam), *H. mangiferae* from litchi, mango amla and ashoka tree (Siddiqi, 1961; all India), *H. mehdii* from grasses (Suryawanshi, 1971; Maharashtra), *H. neobrachyurus* from oak, rice pomegranate and rose (Dhanachand & Jairajpuri, 1980; Manipur), *H. rosae* from rose and sugarcane (Khan et al., 2019; Rathour et al., 2003; Uttar Pradesh), *H. rotundoides* from *Hibiscus rosasinensis* (Geraert, 2010;Uttar Pradesh), *H. sunderbanensis* from mangrove (Ganguly & Khan, 1982; West Bengal) and *H. variabilis* from peach (Rahman & Ahmad, 1995; Assam). It is noteworthy to mention that only three species out of thirteen have been reported with males, i.e. *H. communis*, *H. mangiferae* and *H. variabilis*. The morphological identification of these species is a tedious task and requires expertise. In some *Hemicriconemoides* species, males are absent which increases the need for the proper identification of the females and juveniles (if present) (Ashokkumar & Vadivelu, 1990).

There are various species of genus *Hemicriconemoides* which were encountered from the rhizosphere of various plants irrespective of fruit and vegetable (Edward & Misra, 1964; Edward et al., 1965; Dasgupta et al., 1969; Crozzoli et al., 1998; Siddiqi, 2000; Jain, 2009; Van den Berg et al., 2015). Among all the *Hemicriconemoides* species, *H. rosae* was first described from the rose fields of Bareilly district, Uttar Pradesh, India (Rathour et al., 2003), then reported from the sugarcane fields of Meerut district, Uttar Pradesh, India, by Khan et al. (2019). A survey was conducted in the agricultural fields of Bulandshahr district (28°40′ North and 77°86′ East at an altitude of 209 m ASL), Uttar Pradesh, India. One species of the genus *Hemicriconemoides* recovered from the rhizosphere of mustard fields. The earliest identification based on morphological and some morphometric characters revealed that the isolated population could be *H. rosae* (Rathour et al., 2003), as it showed resemblance with the original description. The occurrence of *H. rosae* in the sugarcane and rose fields is very common. However, the species was first time reported from the mustard fields. For more precise identification, molecular and phylogenetic analysis was done. Furthermore, to look for the variations between the species of genus *Hemicriconemoides* reported worldwide with the studied isolate, principal component analysis (PCA) was also done.

Methods

Collection and nematode isolation

The present investigation was carried out in the fields of Bulandshahr district, Uttar Pradesh. The soils were collected from the meadows, pastures, gardens and agricultural fields of district Bulandshahr (28° 40′ North and 77° 86′ East, elevation of 209 m ASL) of Meerut Division. A total of 85 soil samples were collected at a minimum depth of 5–10 cm (Southey, 1974) from the fields. During the collection of samples, it was made sure that the samples were collected from the previous sample at a distance of 5 km. Soil samples (250gm) were collected in the transparent polyzipper bags labelled with the date, host plant, soil type and locality. Further, the soil was brought to Nematology Laboratory, Department of Zoology, Chaudhary Charan Singh University, Meerut, India, for further processing.

Nematode extraction and identification

The nematodes were extracted from the soil, using modified Cobb's (1918) sieving method and Baermann's funnel techniques (Goodey, 1957; Southey, 1986). To isolate the plant-parasitic nematodes, 250 g of the soil was taken and then all lumps were broken gently in order to avoid the damage to nematodes. After this, 250 gm of soil was taken in a 5 L bucket which was filled up to 3–4 L water. After topping up with water, it was swirled thoroughly by hand to suspend all particles. The resulting suspension

was washed through a 2 mm aperture sieve. The mixture was allowed to settle for about 25 s sedimentation time. After this, the supernatant was decanted through a bank of sieves differing in their aperture size mainly 60 µm, 150 µm, 200 µm, 250 µm, 300 µm, 350 µm and 400 µm, respectively. Then, the residues on the sieves were washed thoroughly with a gentle jet of water and finally collected in a beaker. Water was added to the remaining heavy residues and thoroughly mixed, and again the supernatant was decanted through the same bank of sieves. The sieves were washed, and the residues on the sieves were added to the previously collected. The sievings were shaken gently to suspend all particles before pouring through a 90p aperture nylon screen supported by polyethylene ring (Flegg, 1967), which was immediately placed on a Baermann funnel containing enough water just to submerge the screen surface and debris. After 20 h, about 25 ml of water containing the extracted nematodes from the funnel was taken for examination under a stereoscopic microscope at × 25 or × 50 magnification.

Morphology and morphometry analysis

The adult females were heat killed in Ringer's solution and then fixed in 50% TAF for 24 h. They were then fixed in 100% TAF for 5–7 days (Courtney et al., 1955) and dehydrated in Seinhorst I and II (Seinhorst, 1959). Further processing was done by following the protocols of Bharti et al. (2020). To prevent nematodes from flattening, they were mounted in a small drop of glycerine on permanent clean glass slides with an extra amount of paraffin wax (Bhat et al., 2019). The phase contrast microscope's inbuilt software was used to perform observations and measurements are in micrometre (µm). De Manian indices (de Man, 1881) and other ratios were calculated. A drawing tube connected to a Nikon microscope with differential interference contrast optics was used to create line drawings. Images were captured with a Nikon Digital Sight DS-U1 camera and DIC optics on a Nikon microscope. Adobe®Photoshop®CS was used to edit the micrographs.

Molecular characterization

DNA isolation

The DNA was extracted from single virgin females (not having plug at the vulva). The single virgin female from each species/strain was first washed with Ringer's solution followed by washing in Phosphate buffer saline (pH 7.2). The female from each species/strain was transferred into a sterile Eppendorf tube (500 µl) with extraction buffer 20 µl (ddH20 17.7 µl, 10X Taq buffer with $(NH_4)_2SO_4$ 2 µl, 1% tween2 µl, and proteinase K 0.1 µl). This buffer was frozen at − 80 °C for several days before being incubated at 65 °C for 1 h and subsequently at 95 °C for 10 min. The lysates were chilled on ice and then centrifuged at 6000 × g for 2 min, and 3 µL of supernatant was used for PCR (Bhat et al., 2021). The ITS rDNA was amplified using primers 18S: 5′-TTGATTACGTCC CTGCCCTTT-3′ (forward) and 28S: 5′-TTTCACTCG CCGTTACTAAGG-3′ (reverse) (Vrain et al., 1992) and partial sequence of LSU (28S) gene, D2–D3 domains were amplified using primers, and D2A (5'-ACAAGT ACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAA GGAACCAGCTACTA-3') (De Ley et al., 1999) primer pairs were used. The 30 µl PCR product consisted of 10 µl nuclease free distilled water, 1 µl each forward and reverse primer, 3 µl DNA extract and 15 µl Dream Taq Green PCR master mix 2X (Thermo Scientific). The PCR amplifications were carried out using Verti 96-well fast thermocycler (AP Scientific) with heated lid pre-set at 95ºC and subjected to the following cycling profile: For the ITS rDNA region, PCR conditions included 1 cycle of initial denaturation at 94 °C for 5 min; followed by 35 cycles of 94 °C (denaturation) for 1 min, 55 °C (annealing) for 1 min 30 s or 52 °C (annealing) for 30 s and 72 °C (primer extension) for 1 min, followed by a final extension at 72 °C for 15 min to confirm all of the PCR products are complete length. For the D2D3 rDNA region, PCR conditions included 1 cycle of initial denaturation at 95 °C for 3 min; followed by 35 cycles of 95 °C (denaturation) for 45 s, 55 °C (annealing) for 45 s and 72 °C (primer extension) for 1 min, followed by a final extension at 72 °C for 10 min to confirm all of the PCR products are complete length. The amplified products were electrophoreses in 1% agarose (w/v) gel using 1 × TAE buffer buffered agarose gel stained with ethidium bromide (45 min, 100 V) was used to assess amplification success (Bharti et al., 2020). The amplified PCR products were purified and sequenced by Bioserve Pvt. Ltd., Hyderabad (India). BioEdit was used to edit the newly discovered sequences (Hall, 1999). Sequences were deposited under the accession numbers:

Phylogenetic analysis

The ITS (Internal transcribed spacer) and D2D3 sequences were BLAST (Basic Local Alignment Search Tool) for % similarity matches with the previously submitted sequences in the NCBI (National Centre for Biotechnology Information) (Altschul et al., 1990). The conserved regions, i.e. D2D3 and ITS, have been exploited for establishing the relationship among the *Hemicriconemoides* species. For each amplified rDNA region (ITS and LSU), an alignment of our data with sequences from other closely related species was generated using default ClustalW parameters in MEGA 7.0 (Kumar et al., 2016) and manually optimized in BioEdit (Hall, 1999). The best fit models of nucleotide substitution used for

the phylogenetic analysis were selected using jModelTest 2.1.10 (Darriba et al., 2012). Sequences were concatenated with the R package 'concatipede' v1.0.0 (Vecchi & Bruneaux, 2021).Bayesian inference (BI) was used to infer the phylogenetic trees from the datasets. *Aglenchus agricola* were utilized as out-group taxa, with all characteristics being equally weighted and gaps being treated as missing data (Subbotin et al., 2005; Van den Berg et al., 2014; Khan et al., 2019). MrBayes 3.2.7 was used to produce Bayesian phylogenetic reconstructions (Ronquist et al., 2012). The General Time Reversible substitution model with gamma distributed rate variation across sites and a proportion of invariable sites (GTR + G + I) was used as the optimal nucleotide substitution model for the analyses. One tree was preserved per 1000 generations after running Markov chain Monte Carlo generations (MCMC) for 1×10^7 cycles. The tree was visualized and saved with FigTree 1.4.4 (Rambaut, 2018).

Statistical analyses

Correlation analysis and linear regression analysis were performed to resolve the relationship between various morphometrical parameters using GraphPad Prism software (version 6.0). Principal component analysis (PCA) was done to find out the morphological variations between the isolated nematodes in the present investigation and closely related species of the nematodes. The measurements of the *Hemicriconemoides* species were collected from their original descriptions. The 2D plot was formed by using the PC1 and PC2 values of each isolate based on eigenvalues given by the software MINITAB 20 (Nisa et al., 2021).

Results

In the present investigation, HCN strain recovered from one soil sample out of 85 soil samples collected from the rhizosphere of mustard from Bulandshahr district. The strain was found to be plant-parasitic nematodes due to presence of long stylet on the labial region. The pH of the soil was 5.4, and the texture of the soil was found to be alluvial. Most of the sampling was done in the winter season mainly in January–March. The strains HCN bear double cuticle, which is a characteristic feature of *Hemicriconemoides*, thus belong to same genus.

Based on morphology and morphometry, these nematodes were found close to *Hemicriconemoides rosae*, hence described as the same. In the present study, only females of the strain HCN were recovered as noted in the original descriptions. For the morphological characterizations, permanent slides were prepared and deposited in the Nematology Laboratory, Department of Zoology, Ch. Charan Singh University, India.

Morphological characterization

Present specimen (HCN) showed close resemblances to the *Hemicriconemoides rosae* (Rathour et al., 2003) in morphological observations with respect to the presence of a prominent and elevated labial disc, long stylet, pharynx distinct and separated from intestine. Besides this, vulva was enclosed with well-developed membranous sheath, presence of conoid tail which is dorsally convex at the tail tip, and hence, it was considered as the same (Fig. 1). Line drawings were prepared for comprehensive analysis (Fig. 2). However, few morphometrical characters showed little variation from each other.

Female

Small in size 0.37–0.47 mm in length. Body straight to slightly arcuate ventrally after heat killed. Sheath closely fit to the body except on tail and frequently protruding forward over the lip region. Sheath is not annulated at vulval region. Cuticle smooth and covered by outer accessory layer/sheath with smooth annules. Body annuli distinct and rounded (Fig. 1). Lip region rounded with a prominent labial disc, not set off with two annuli, first annuli smaller than second (Fig. 1, Table 1). Stylet long (50-58 µm), well developed with stylet knobs (2.4–4.0 µm) prominently anchor-shaped anteriorly and rounded posteriorly, 4.8–6.9 µm wide (Fig. 1, Table 1). Dorsal pharyngeal gland opening situated at the base of stylet knobs. Median bulb observed with prominent valve. Procorpus and metacorpus found to be fused. Excretory pore situated from 5–11 annuli posterior to the base of pharynx. Hemizonid not found. Sheath annuli smooth, slightly indented over the whole length. Anastomoses found absent. Vulva posterior, located 8–13 annuli from terminus and 90–94% of body length (Fig. 1, Table 1). Flap like structure absent on vulva but with a prominent membranous sheath. Spermatheca well developed and oval shape filled with sperm cells (Fig. 1). Vagina observed distinct and straight. Body found to be tapering posterior to anus. Tail shape conoid and found dorsally convex with the sheath enclosing the tip, tapering gradually to a more pointed tip. Anus position was uncertain still it was located just posterior to vulva 2–5 annuli (Table 1).

Male

Males are absent.

*Comparative study of H. rosae strain (HCN) and H. rosae (*Rathour et al., 2003*)*

As far as females was concerned, most of the morphometric parameters of isolate (HCN) and original species was found to be approximately similar. The other

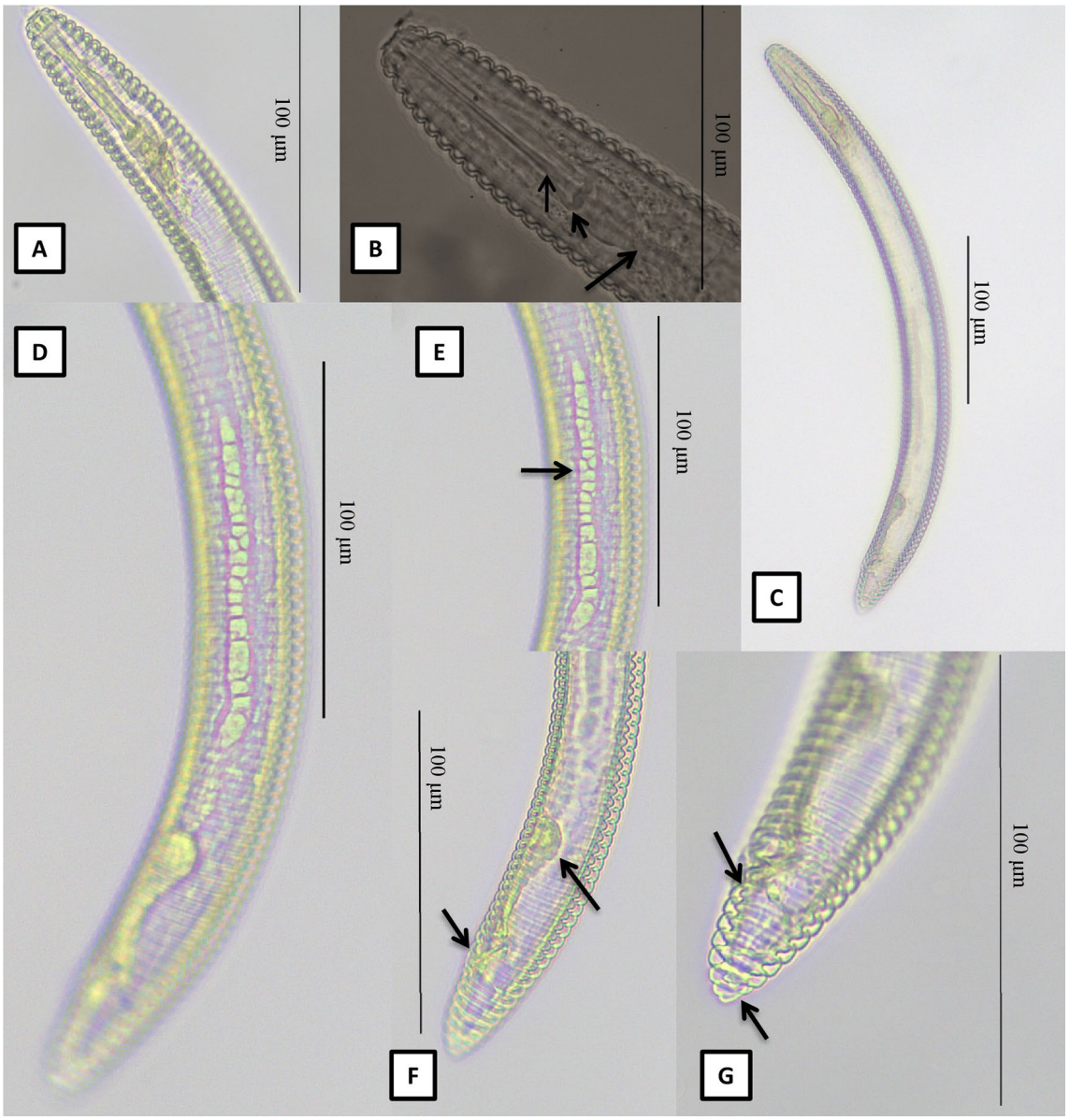

Fig. 1 Photomicrographs of *Hemicriconemoides rosae* isolate HCN. **A**, **B** Anterior region of female; **C** Whole female; **D** Posterior region of female; **E** Female gonad; **F** Vulval region; and **G** Anal and tail region. The arrows indicate the stylet, stylet knob and nerve ring (**B**), female gonad (**E**), vulval region and spermatheca of female (**F**) and anal region and tail (**G**)

measurements, i.e. a (13.6–16.9 vs 16.0–17.6), b (4.6–5.6 vs 4.9–5.4), V (90–95 vs 92–94.5), stylet length (50–58 vs 50–55 µm), metenchium length (40–49 vs 44.8 µm), telenchium length (9–11.8 vs 7.7 µm), stylet knob width (4.8–6.9 vs 6.9 µm), labial disc diameter (4.3–5.9 vs 5.1 µm), R (102–115 vs 116), Rst (12–20 vs 12–14), Roes (20–22 vs 21–26) of present strain, were found close to the original description *H. rosae* (Rathour et al., 2003). All the body parameters were found to be close except body length (376–472 µm vs 470–510 µm), the length of the pharynx (72–94 vs 102 µm) and number of annuli posterior to vulva (8–13 vs 6–9) which showed deviation from the original description.

Hemicriconemoides rosae isolate (HCN) was compared with *H. rosae* Khan et al.(2019) sugarcane population and three closely related species of *Hemicriconemoides*, and it differs in some morphological and morphometric characters with compared species. The comparison of morphometrics is presented in Tables 1 and 2 and is described as follows:

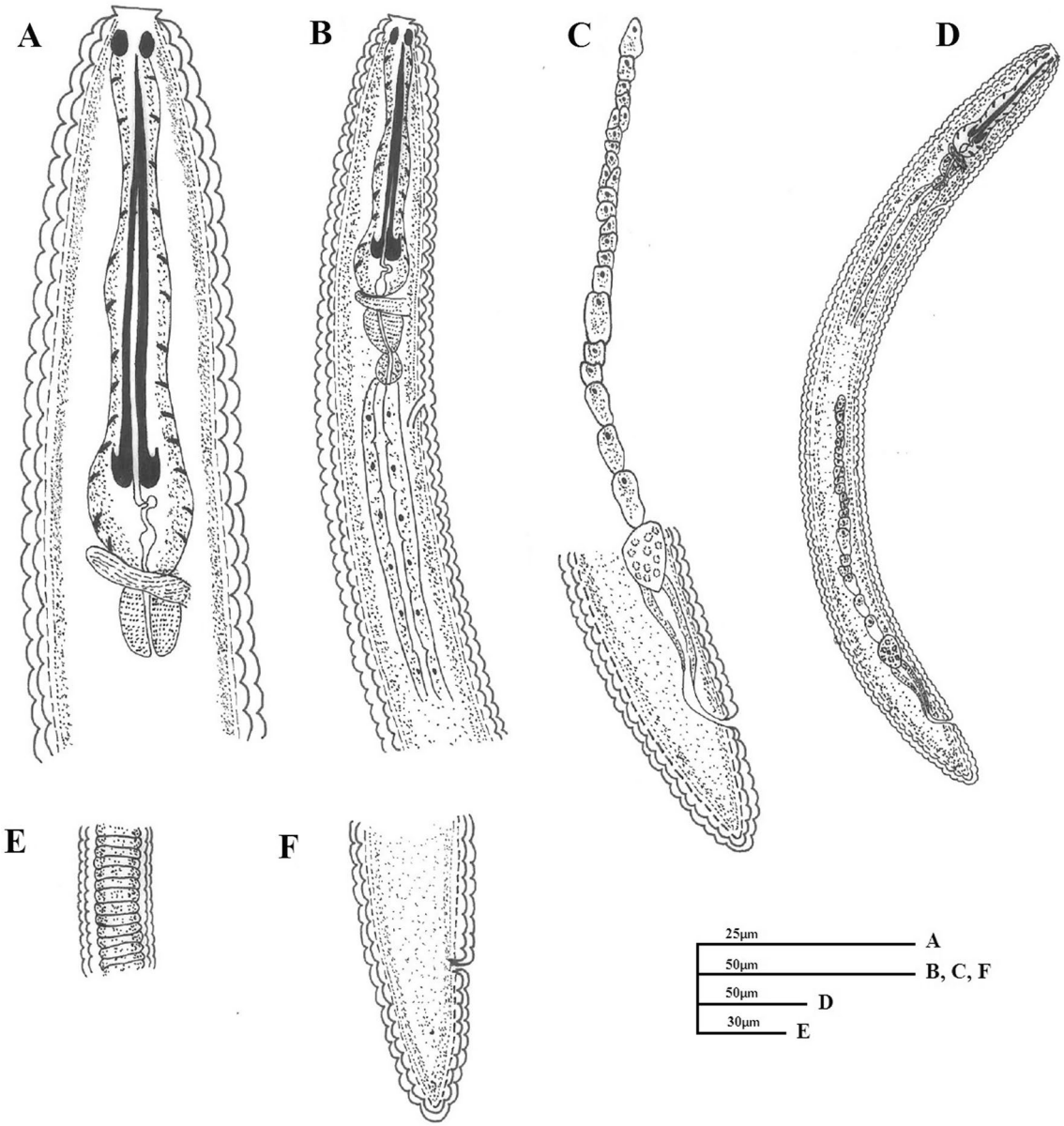

Fig. 2 Line drawings of *Hemicriconemoides rosae* from rhizosphere of mustard. **A**, **B** Anterior region of female; **C**: Female gonad and tail; **D**: Entire adult female; **E** body annuli in mid-body region; and **F** Vulva region

The present isolate HCN of *Hemicriconemoides rosae* recovered from mustard were found to show some variations from the *H. rosae* (Khan et al., 2019) sugarcane population. The parameters which show deviations were: average body length (376–472 vs 323-486 µm), ratio a (13.6–16.9vs 12.2–18.7), ratio b (4.6–5.6 vs 3.88–5.93), telenchium length (9–11.8 vs 5.1–10.8 µm), stylet (50–58 vs 52-61 µm) and annulus width (3.6–5.2 vs 2.9–3.8 µm) (Table 1).

Hemicriconemoides rosae HCN females were differentiated from the *H. communis* Edward and Misra (1964) in terms of number of sheath annuli, anus position, anterior secretory to excretory position and the presence of males. *H. communis* was found to have more annuli than *H. rosae* (102–115 vs 110–130), smaller VL/VB (0.9–2.0 vs 1.3–2.0). The position of anus is uncertain in *H. rosae*; however, anus is clearly visible in *H. communis*. Males are present in *H. communis*, whereas in *H. rosae* no males were observed (Table 2).

Based on morphological characterization, *H. rosae* HCN was separated from the *H. wessoni* Chitwood and Birchfield (1957) in terms of ratio a, c and other

Table 1 Morphometrics of *Hemicriconemoides rosae* isolate HCN from the mustard (present population), rose population (Rathour et al., 2003) and sugarcane population (Khan et al., 2019). All measurements are in µm (except ratio and percentage) and in the form of mean ± standard deviation (range)

Character	Mustard population isolate HCN	Rose population (Rathour et al., 2003)	Sugarcane population (Khan et al., 2019)
n	15	6	32
L	443 ± 25 (376–472)	490 (470–510)	398 (323–486)
a (Length/Diameter at mid-body)	15.3 ± 0.9 (13.6–16.9)	16 (16–17.6)	15.4 (12.2–18.7)
b (Length/Pharynx length)	5.1 ± 0.3 (4.6–5.6)	5.2 (4.9–5.4)	4.8 (3.9–4.9)
c (Length/Tail)	16.4 ± 1.7 (14.1–20)	–	–
c' (Tail/diameter at anus)	22 ± 1.8 (19.4–25)	–	–
V (anterior to vulval/total body length) × 100	92 ± 1.4 (90–95)	93.2 (92–94.5)	91.5 (89.9–92.7)
M (ratio between the length of prorhabdion and stylet in %)	85.6 ± 2.5 (81.2–91.5)	85.3*	85.6 (82.2–90.4)
EP (Excretory pore to anterior end)	116 ± 5.5 (102–123)	–	–
WEP (Diameter at excretory pore)	28 ± 0.9 (25–29)	–	–
NR (Nerve ring)	71 ± 2.4 (64–74)	–	–
PL (Pharynx length)	87 ± 5.4 (72–94)	102*	84 (71–103)
EBL (oesophageal bulb length)	15.8 ± 2.2 (11.5–18.7)	–	–
EBW (oesophageal bulb width)	12 ± 1.8 (8.8–15)	–	–
Tail	27 ± 2.7 (23–31)	–	–
Diam. at anus	19.8 ± 1.3 (18–23)	–	–
Diam. at mid body	29 ± 2.0 (26–33)	26.5*	25.9 (23.4–29.6)
V' (Distance from the anterior end to vulva)	409 ± 26 (340–444)	–	–
Stylet	54 ± 2.4 (50–58)	52 (50–55)	55 (52–61)
Stylet Knob height	2.8 ± 0.5 (2.4–4.0)	2.6*	2.7 (2.3–3.8)
Stylet Knob width	5.7 ± 0.7 (4.8–6.9)	6.9* (4.8–6.9)	6.2 (4.8–7.4)
Metenchium length	44 ± 2.3 (40–49)	44.8*	47 (44–51)
Telenchium length	10.3 ± 0.8 (9–11.8)	7.7*	8.0 (5.1–10.8)
R (Total number of body annules)	108 ± 4.1 (102–115)	116*	116 (109–124)
Rst (Annules from anterior extremity to base of stylet)	13.9 ± 2.0 (12–20)	13 (12–14)	16.6 (15–18)
Roes (Annules from anterior extremity to base of oesophagus)	21 ± 0.8 (20–22)	23.5 (21–26)	25.9 (21–30)
Ran (Annules from anus to tail terminus)	6.9 ± 0.6 (6–8)	–	–
Rex (Annules from anterior extremity to excretory pore)	28 ± 2.0 (25–33)	–	28 (22–30)
Rv (Annules from vulva to tail terminus)	9.6 ± 1.5 (8–13)	7.5 (6–9)	7.5 (7–9)
MBL (Median bulb length)	14.7 ± 1.6 (11.5–16.4)	–	–
MBW (Median bulb width)	12.8 ± 1.3 (10.9–15.5)	–	–
Lip Length	5.9 ± 0.2 (5.2–6.6)	5.7*	5.8 (5.0–6.5)
Lip Width	5.1 ± 0.5 (4.3–5.9)	5.1*	5.4 (4.5–6.0)
D % (excretory pore/pharynx length × 100)	134 ± 10.9 (115–163)	–	–
E % (excretory pore/Tail) × 100	430 ± 49 (365–505)	–	–
VL (distance from vulva to terminus)	35 ± 6.1 (23–27)	34.7*	33.7 (28.6–39.5)
VB (body diameter at vulva)	24 ± 1.8 (21–28)	21.6*	18.8 (15.0–21.6)
VL/VB (distance from vulva to terminus/body diameter at vulva)	1.5 ± 0.3 (0.9–2.0)	1.6*	1.8 (1.3–2.2)
Sty%L (Stylet length/body length × 100)	12.3 ± 1.0 (10.7–14.8)	10.7*	14.0 (12.0–16.2)
First lip diam	8.6 ± 0.5 (8.2–9.9)	8.5*	8.8 (8.0–10.6)
Second lip diam	12.1 ± 0.7 (10.1–14.2)	12.3*	11.7 (10.0–13.3)
First body annulus diam	14.6 ± 0.4 (12.3–15.2)	14.8*	14.2 (12.1–15.8)
Second body annulus diam	16.9 ± 0.9 (14.5–19.9)	17.2*	15.7 (13.5–18.8)
Third body annulus diam	18.1 ± 1.2 (16.2–21.3)	18.9*	17.2 (15.2–21.0)
Annulus width	4.4 ± 0.4 (3.6–5.2)	4.3*	3.4 (2.9–3.8)

* Values were determined in holotype specimens

Table 2 Comparative morphometrics of adult female isolate HCN with original *Hemicriconemoides rosae* population and other species of the *Hemicriconemoides* genus

Characters	Authors	L	a	b	V	PL	Stylet	R	Rst	Roes	Rv	Ran	Sty%L	Annulus W
H. rosae	Present isolate HCN, Mustard	443 (376–472)	15.3 (13.6–16.9)	5.1 (4.6–5.6)	92 (90–95)	87 (72–94)	54 (50–58)	108 (102–115)	13.9 (12–20)	21 (20–22)	9.6 (8–13)	6.9 (6–8)	12.3 (10.7–14.8)	4.4 (3.6–5.2)
H. communis	Edward and Misra (1964)	480 (340–500)	16 (12–25)	4.5 (4–5)	93 (89–96)	109*	54* (51–58)	115 (110–130)	16*	26*	8*	7*	10.8*	3.5*
H. wessoni	Chitwood and Birchfield (1957)	429.2 (382.5–499.8)	12.7 (10.7–15)	4.6 (4.0–5.5)	92 (89–93.5)	–	54 (50–60)	(76–83)	–	–	9*	5*	15.9*	(4–5)
H. californianus	Pinochet and Raski (1975)	440 (410–460)	17 (16–19)	4.1 (3.4–4.6)	91 (90–92)	–	80 (77–83)	119 (112–127)	–	–	11 (10–12)	5 (4–6)	–	–
H. macrodorus	Volvas et al. (2000)	691 (548–761)	17 (15–21)	4.4 (3.8–5.2)	95 (94–96)	152 (140–171)	101 (90–110)	137 (127–148)	23 (18–27)	–	13 (2–14)	8 (7–9)	15 (13–18)	5.8*
H. chitwoodi	Esser (1960)	540 (480–590)	15.5 (13.4–17)	3.8 (3.5–4.1)	90.1 (88.1–91.0)	–	91 (85–95)	124 (116–133)	–	–	(12–16)	(8–11)	–	–
H. parataiwanensis	Decraemer and Geraert (1992)	499 (440–535)	15.2 (13.8–16)	4.2 (3.9–4.5)	90 (88.7–91)	120.2 (107–129)	79.4 (77–82)	138.1 (129–145)	25.4 (23–29)	–	9.1 (8–10)	5 (4–10)	15.9*	3.5*
H. paracamelliae	Maria et al. (2018)	563 (519–604)	16.9 (15.3–18.5)	4.4 (4.1–5.0)	88.9 (87.7–90.1)	127 (119–135)	83 (80–85)	132 (124–140)	23.1 (22–26)	34.5 (31–37)	14.8 (13–16)	8 (5.0–10.0)	14.7 (13.7–16.3)	–
H. kanayaensis	Nakasono and Ichinohe (1961)	571 (500–631)	21.5 (18.7–24.4)	4.8 (3.3–5.6)	88.9 (87.5–91.5)	–	74 (66–79)	150 (142–164)	–	–	18 (16–21)	12 (11–15)	12.9*	4.6*
H. phoenicis	Van den Berg et al. (2015)	630 (549–699)	21.6 (16.7–24.3)	4.7 (4–5.7)	46 (27–62)	133 (117–149)	88 (81–97)	130 (123–137)	23 (21–25)	32 (28–36)	14 (13–19)	10 (9–15)	14 (12.6–16.3)	5.5 (4.5–6.5)
H. litchi	Edward and Misra (1964)	480 (450–500)	15 (13–18)	4.6 (3–5)	92 (91–93)	107*	63 (60–65)	130 (128–133)	12*	25*	12*	7*	13.1*	3.5*
H. strictathecatus	Esser (1960)	560 (510–590)	–	–	–	–	79 (73–83)	138 (127–152)	18.8*	27.3*	(11–13)	–	–	–
H. cocophilus	Dasgupta et al. (1969)	(460–500)	(14–15)	(4.0–5.0)	(91–92)	–	(50–57)	(108–143)	–	–	(9–10)	(8–9)	–	(3–5)
H. brachyurus	Dasgupta et al. (1969)	(400–540)	(13–17)	(4.3–5.2)	(93–95)	96*	(56–64)	(98–119)	–	–	(7–9)	(6–7)	–	–

All measurements are in µm (except ratio and percentage) and in the form of mean ± standard deviation (range)

* Values were determined in holotype specimens

L = Body length, PL = Pharynx length, a(L/BD), b(L/PS), V% (anterior to vulval/total body length) × 100, Stylet = Stylet length, Stylet knob W = Stylet knob width, R = Total number of body annules, Rst = Annules from anterior extremity to base of stylet, Roes. = Annules from anterior extremity to base of oesophagus, Rv = Annules from vulva to tail terminus, Ran = Annules from anus to tail terminus, Annulus W = Annulus width, Sty%L = Stylet length/body length*100

parameters. The ratio a of *H. wessoni* (12.3–14.8) was found to be smaller when compared with *H. rosae* (13.6–16.9). The ratio c of *H. wessoni* (16.2–22.9) is larger when compared with *H. rosae* (14.1–20). Besides this, other characteristics of the *H. rosae* isolate HCN showed differences with the *H. wessoni* such as tail shape and labial disc. The labial disc in case of *H. rosae* was found to be elevated, whereas in case of *H. wessoni*, slight or no elevation was observed. The pharyngeal bulb in *H. rosae* showed no overlapping; however, in *H. wessoni* dorsal overlapping was present. The shape of the tail in *H. rosae* was found to be conoid and the membranous sheath enclosed the tail tip dorsally, whereas in *H. wessoni* the tail shape was found to be conoid but membranous sheath may or may not enclose the tail tip (Table 2).

The *H. rosae* isolate HCN showed differences from the *H. minutus* Esser (1960) in terms of de Manian indices, i.e. a and b, stylet and pharyngeal bulb. The ratios a (13.6–16.9 vs 10.3–16.4) and b (4.6–5.6 vs 3.0–3.8) of *H. rosae* were found to be higher than *H. minutus*. The stylet length of *H. rosae* was found to be smaller than *H. minutus* (50–58 vs 70.5–80.5 µm). The pharyngeal bulb of *H. rosae* showed no overlapping, while in case of *H. minutus* overlapping was present.

Principal component analysis

Principal component analysis was done using morphometric data of the nematode isolates, i.e. *H. communis* (Edward and Misra 1964), *H. wessoni* (Chitwood & Birchfield, 1957), *H. californianus* (Pinochet & Raski, 1975), *H. macrodorus* (Volvas et al., 2000), *H. chitwoodi* (Esser, 1960), *H. parataiwanensis* (Decraemer & Geraert, 1992), *H. paracamelliae* (Maria et al., 2018), *H. kanayaensis* (Nakasono & Ichinohe, 1961), *H. phoenicis* (Van den Berg et al., 2015), *H. litchi* (Edward & Misra, 1964), *H. strictathecatus* (Esser, 1960), *H. cocophilus* (Dasgupta et al., 1969), *H. brachyurus* (Dasgupta et al., 1969) isolated from different geographical areas and the studied isolate HCN.

The results of the PCA showed that there are variations in the morphometry between the *Hemicriconemoides rosae* isolate HCN recovered from mustard in the present investigation and the *Hemicriconemoides rosae* (Khan et al., 2019) isolated from the sugarcane and with the other species of *Hemicriconemoides species* (Dasgupta et al., 1969). However, the analysed parameters of morphometry showed less variations between the present isolate HCN with the originally described *Hemicriconemoides rosae* (Rathour et al.,

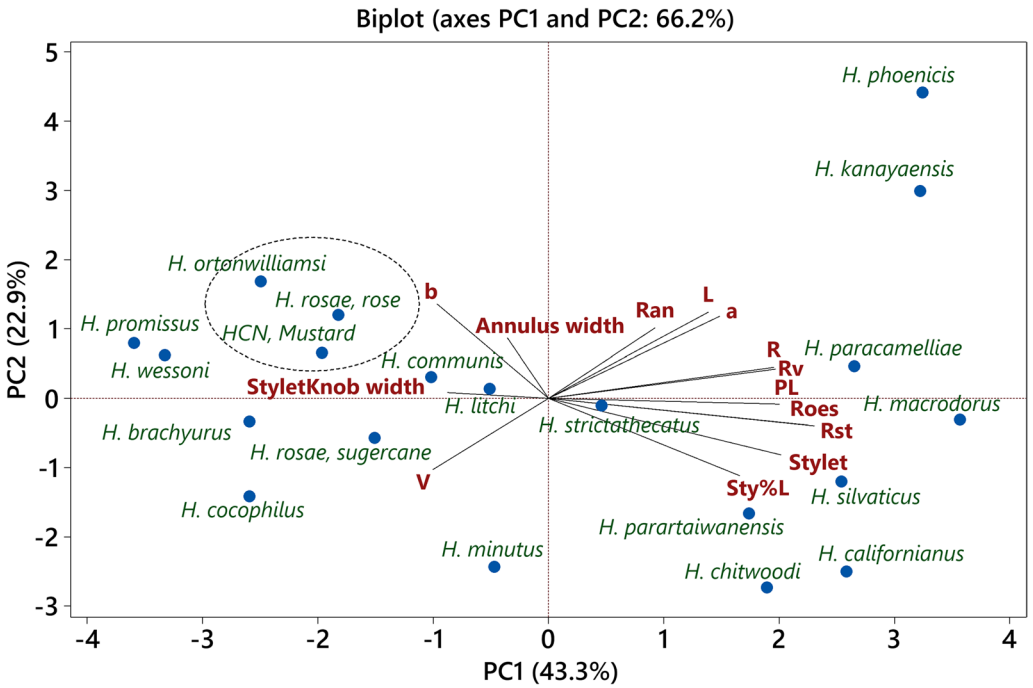

Fig. 3 The principal component analysis (PCA) of different populations of *Hemicriconemoides* species based on female specimens with different body parameters as factors. The biplot was based on two major principal components; PC1 and PC2. L = Body length, PL = Pharynx length, a (L/BD), b(L/PS), V% (anterior to vulval/total body length) × 100, Stylet = Stylet length, Stylet knob W = Stylet knob width, R = Total number of body annules, Rst = Annules from anterior extremity to base of stylet, Roes. = Annules from anterior extremity to base of oesophagus, Rv = Annules from vulva to tail terminus, Ran = Annules from anus to tail terminus, Annules W = Annules width, Sty%L = Stylet length/body length*100. The ellipses represent the group of nematodes/isolates coming together showing fewer variations

2003) (Fig. 3). Besides this, the isolates showed differences from the other *Hemicriconemoides* species (Fig. 3).

The PCA based on the morphometry of females showed an accumulated variability of 66.2% (Fig. 3). The contribution of PC1 and PC2 was found out to be 43.3% and 22.9%, respectively (Fig. 3). Four parameters, annulus width (r = -0.06), b (r = -0.16), stylet knob width (r = -0.15) and V% (r = -0.17) were found to be negatively correlated across nematode/species in PC1. Ten characters out of fourteen characters were found to be positively correlated across isolates, and the remaining characters were negatively correlated considering PC1 (Fig. 3). The highest coefficient of correlation was observed in annuli from anus to tail terminus (r = 0.38) and annuli from anterior extremity to base of stylet (r = 0.37) in PC1. Considering PC2, nine characters out of fourteen were found to be positively correlated and the remaining were negatively correlated (Fig. 3, Table 3). The highest coefficient of correlation was observed in b (r = 0.43) PC2.

Table 3 Loading scores of the variables and factor score of the observations for females of Genus *Hemicriconemoides* spp

Characters	Loading scores of the variables		Species	Factor score of the observations	
	Females			Females	
Variable	PCA1	PCA2		PCA1	PCA2
L	0.23	0.39	HCN, Mustard	− 1.96	0.65
a	0.25	0.37	*H. rosae*, rose	− 1.82	1.20
b	− 0.16	0.43	*H. rosae*, sugarcane	− 1.51	− 0.57
V	− 0.17	− 0.32	*H. promissus*	− 3.59	0.80
PL	0.33	− 0.03	*H. litchi*	− 0.51	0.14
Stylet	0.33	− 0.26	*H. silvaticus*	2.54	− 1.20
R	0.32	0.14	*H. strictathecatus*	0.46	− 0.11
Rst	0.37	− 0.12	*H. brachyurus*	− 2.59	− 0.34
Roes	0.38	− 0.13	*H. californianus*	2.58	− 2.50
Rv	0.33	0.13	*H. macrodorus*	3.57	− 0.31
Ran	0.15	0.32	*H. chitwoodi*	1.89	− 2.73
Stylet Knob width	− 0.15	0.03	*H. wessoni*	− 3.33	0.62
Sty%L	0.27	− 0.35	*H. minutus*	− 0.47	− 2.44
Annulus width	− 0.06	0.27	*H. ortonwilliamsi*	− 2.49	1.68
			H. communis	− 1.02	0.31
			H. cocophilus	− 2.59	− 1.41
			H. parataiwanensis	1.74	− 1.66
			H. paracamelliae	2.65	0.46
			H. kanayaensis	3.22	2.99
			H. phoenicis	3.24	4.41

Molecular analysis

The *Hemicriconemoides* species isolate HCN examined in present study has been characterized molecularly by the sequences of two genes, i.e. ITS rDNA (695 bp) and D2D3 fragments of 28S rDNA (708 bp). The ITS and D2D3 sequences of *Hemicriconemoides rosae* isolate HCN were deposited in NCBI GenBank with accession number ON844213 and ON844212, respectively.

Pairwise distances (Kimura 2-parameter) based on the ITS regions revealed the genetic distances between the *Hemicriconemoides* species. The present isolate HCN (ON844213) genetic distance with previously described *Hemicriconemoides rosae* (MK371815, MK371814, and MK371816) was found to be 0.0000. The present isolate *Hemicriconemoides rosae* HCN (ON844213) showed greatest genetic distance (0.1915) from *H. kanayaensis* (MG029568). The average overall genetic distance between in-group species was 0.1675 ranging from 0.0000 to 0.3055 between *H. rosae* (MK371815, MK371814, MK371816) and *H. kanayaensis* (MG029568) (Additional file 1: S1).

The average overall genetic distance (Kimura 2-parameter) based on D2D3 regions between in-group species was 0.1336 ranging from 0.0000 between *H. rosae* (MK371813) and *H. rosae* (MK371811) to 0.2326 between *H. minutus* (KF856516) and *H. parataiwanensis* (MG029573) (Additional file 2: S2).

Phylogenetic analysis

ITS and D2D3 regions are well-conserved regions in the nematodes and are used to ascertain the relationship between closely and distantly related species. The phylogenetic relatedness of the *Hemicriconemoides rosae* with other species of *Hemicriconemoides* was established from the comparison of concatenated sequences (Fig. 4, Additional file 3: S3). There are two major clades in the rDNA phylogenetic tree of *Hemicriconemoides*. It is interesting to note that the phylogenetic analysis was in agreement with the morphology and molecular analysis. The first cluster includes the *Hemicriconemoides brachyurus*, *H. cocophilus*, *H. ortonwilliamsi*, *H. minutus*, *H wessoni*, *H. macrodorus* and *H. promissus*. The second cluster includes the *Hemicriconemoides rosae* HCN, *Hemicriconemoides rosae* and *Hemicriconemoides* species. A thorough analysis of sequences of the 28S and ITS rDNA gene of the HCN isolate described in this study reveals clear relatedness with 18 other *Hemicriconemoides* species. Bayesian inference phylogenetic analyses based on the concatenated sequences (ITS and 28S rDNA) showed a clear monophyly of the group formed by the isolate HCN and already described *Hemicriconemoides rosae* Khan et al. (2019) (Fig. 4, Additional file 3: S3).

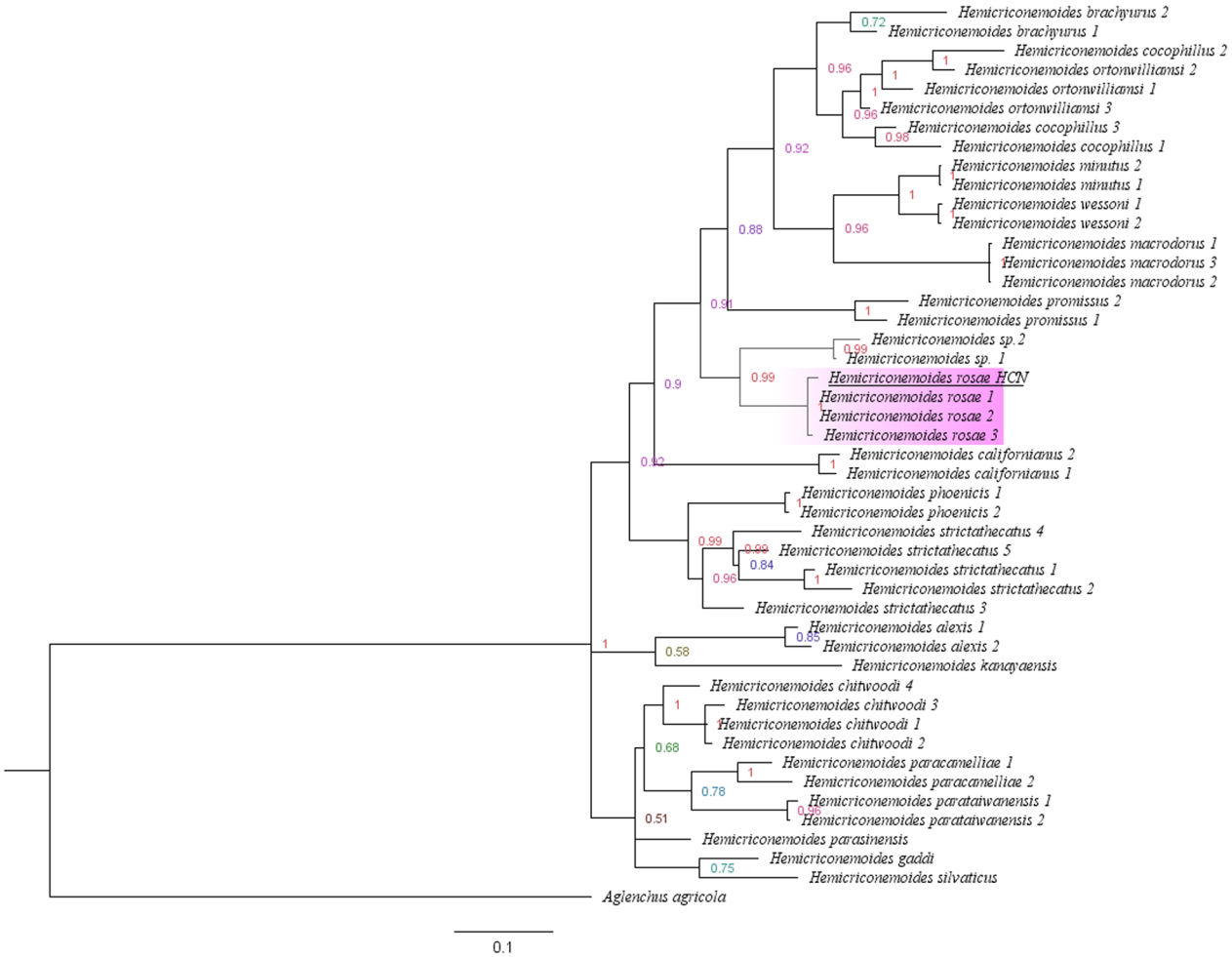

Fig. 4 Phylogenetic relationships within populations and species of *Hemicriconemoides* as inferred from Bayesian analysis based on the concatenated sequences of ITS and 28S rRNA. Numbers at the nodes indicate Bayesian posterior probability. Newly obtained sequences of *H. rosae* underlined. The scale bar represents 0.01 substitutions per nucleotide position

Relationship between various morphometric parameters

The results obtained from the two tailed Pearson's correlation analysis revealed that some parameters of *Hemicriconemoides rosae* isolate HCN showed significant correlation with other morphometric parameters (Table 4). The morphometric parameters which were focused in the study to understand the relation between various parameters were body length, excretory pore, tail, pharynx stylet, de Manian indices a, b, c and c', nerve ring, vulva width and other parameters. In this, 15 females of the isolate HCN were analysed to figure out the correlation between the morphometric data. Each morphometric parameter was replicated 3 times to get the final results.

The results based on the two-tailed Pearson's correlation suggested that body length has highest correlation in comparison with others with the morphometric data. Though body length showed correlation with the analysed parameters, no correlation has been observed with the de Man indices except a ratio of female which showed positive correlation ($r=0.6978$, $p=0.0038$) (Fig. 5, Table 4). The index a also showed positive correlation with other parameters, i.e. excretory pore ($r=0.7389$, $p=0.0017$, Table 4), oesophageal bulb ($r=0.6953$, $p=0.0040$, Table 4), vulva-anterior end ($r=0.7538$, $p=0.0012$, Table 4). However, index a showed no correlation with other indices (Table 4). The index b showed positive correlation with the index c ($r=0.5689$, $p=0.0269$, Table 4) and negative correlation with pharynx ($r=-0.5443$, $p=0.0359$, Table 4). Among all the parameters, the c' showed positive correlation with the tail ($r=0.5966$, $p=0.0189$, Table 4) and negative correlation with index c ($r=-0.6779$, $p=0.0055$, Table 4). These observations suggested that body length, vulva position, de Manian index 'a' are important for the evaluation of the females of *Hemicriconemoides rosae*.

Table 4 Correlation of morphometric data of *Hemicriconemoides rosae* isolate HCN was determined by two tailed Pearson's correlation

	L	a	b	c	c′	V
L	1	**0.6978****	0.3446	0.3492	0.1044	0.3261
a	**0.6978****	1	0.2484	0.3704	−0.01959	0.3505
b	0.3446	0.2484	1	**0.5689***	−0.4858	0.1374
c	0.3492	0.3704	**0.5689***	1	−**0.6779****	**0.5414***
c′	0.1044	−0.01959	−0.4858	−**0.6779****	1	−0.08350
V	0.3261	0.3505	0.1374	**0.5414***	−0.08350	1
EP	**0.9651*****	**0.7389****	0.3727	0.3506	0.1033	0.3744
NR	**0.5820***	0.4458	−0.3041	−0.05316	0.4115	**0.5198***
PL	**0.5990***	0.4690	−**0.5443***	−0.1700	0.4952	0.1685
EBL	**0.6816****	**0.6953****	0.1143	0.4009	−0.2365	0.1240
T	**0.5458***	0.2028	0.05948	−0.3330	**0.5966***	−0.04380
Body diameter: anus	**0.5670***	0.4774	−0.07373	−0.1201	0.09106	0.1515
Body diameter: mid-body	**0.5379***	0.4317	0.2305	−0.01556	0.009445	−0.1235
VDesh	**0.9715*****	**0.7538****	0.3453	0.4479	0.07187	**0.5407***
Stylet	**0.5498***	0.1846	−0.1253	−0.1770	0.1626	−0.1398
MBL	**0.5448***	0.4334	−0.02157	0.09194	0.1999	**0.5293***
WV	**0.5872***	0.4855	0.1949	0.1943	0.06458	0.1955

Bold and asterisk (*) values represent the statistical difference. $p < 0.05$ was considered a statistically significant difference

* Shows statistical significant; Pearson's correlation

* Shows $p < 0.05$, ** shows $p < 0.01$, *** shows $p < 0.001$

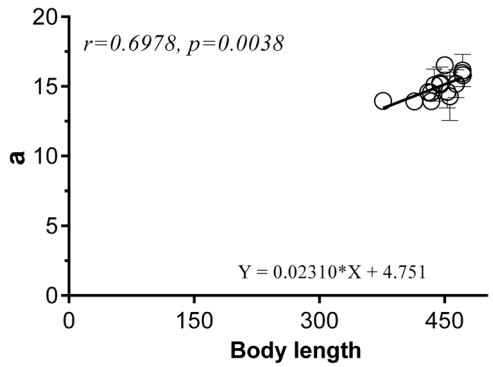

Fig. 5 Relationship between the body length with ratio 'a' as determined by regression analysis of females of *Hemicriconemoides* isolate HCN. The symbol represents the values of ratio 'a' at specific body length. The solid line denotes linear regressions between the body length and ratio 'a'.

Discussion

Hemicriconemoides was first described by Chitwood & Birchfield, 1957 and further redescribed by Dasgupta et al., 1969. These nematodes are commonly referred to as sheathoid nematodes due to the body cuticle of female covered by an outer accessory layer/sheath with smooth annules which is missing in juveniles. They are mostly distributed in temperate areas of the world mainly in Africa, America, Australia and South Asia and South Europe. This genus contains 54 valid species (Geraert, 2010; Khan et al., 2019) and is associated with many crops.

The *H. rosae* (Rathour et al., 2003) possessed very less morphological and morphometric characters which were used for the comparison with the studied isolate HCN. The morphological data of the HCN isolate showed much resemblance with the original description of the *H. rosae* (Rathour et al., 2003); however, it showed more deviation from the sugarcane population of *H. rosae* (Khan et al., 2019) (Table 1).

The various morphometric characters are not sufficient for the delineation of the species. Molecular characterization is essential in order to validate the taxonomic position as well as the authenticity and phylogenetic relationships among the species of a particular genus and between different nematode orders. The molecular analysis based on the conserved regions ITS and D2D3 revealed the present isolate HCN to be *H. rosae* as it showed very less genetic distance from the already described *H. rosae* (ITS:0.0000, D2D3: 0.0014, 0.0129) (Additional file 1: S1 and Additional file 2: S2).

The phylogenetic analysis was found to be consistent with the molecular data which revealed that the present isolate to be *H. rosae* (Fig. 4). The phylogenetic analysis based on the concatenated sequences (ITS and D2D3) region revealed that mustard population of *H. rosae* HCN formed a monophyletic clade with the sugarcane population of *H. rosae* with a Bayesian posterior probability 1.

The phylogenetic tree revealed that *H. rosae* formed a separate branch without including any other *Hemicriconemoides* species, however, the position of *H. rosae* in the phylogenetic tree was close to *H. promissus*, *H. macrodorus*, *H. wessoni* and *H. minutus* which found to be consistent with the previous observations (Khan et al., 2019).

The PCA showed large intraspecific and interspecific variations. These variations were found to be independent of the collection localities of soil samples. It was evident from the previous studies that there were large intraspecific variations present among the nematodes which was also observed among the present isolate HCN (mustard population) and *Hemicriconemoides rosae* (sugarcane population) isolates of the present investigation (Khan et al., 2019). However, less intraspecific variations were observed between present isolate HCN and originally described *Hemicriconemoides rosae* (Rathour et al., 2003) (Fig. 3, Table 3).

The results obtained from the analysis of morphometric data based on two tailed Pearson's correlation revealed that in case of females of *H. rosae*, de Manian index a is very important for the evaluation as it showed positive correlation with body length (Fig. 5, Table 4). Similar results were observed in the genus *Eucephalobus* and *Acrobeles* (Amirzadi et al., 2011, 2013). The results of Fortuner (1990) also exhibited the highest correlation between the de Manian indices especially a and b and the length. Furthermore, the other ratios, i.e. V also important along with the de Manian indices, i.e. a, c and c' which is also consistent with the previous findings (Fortuner, 1984). It was found from the previous studies that a, c and c' de Manian indices are essential for the genus *Helicotylenchus* Steiner, 1945. Similar findings were obtained by Amirzadi et al. (2013) for genus *Acrobeles* von Linstow (1877) where correlation between de Manian indices (b and c') and body length was found positive. The present study on the females of genus *Hemicriconemoides* also revealed the positive correlation between the body length and ratio a (Fig. 5, Table 4). The reason behind that some morphological characters showed highest degree of correlation; however, some of them showed least might be due to control of those characters by a specific gene cluster (Amirzadi et al., 2013). It can be concluded from the results that there are some characters which showed high correlation with body length which results in the increase or decrease in the characters with the body length (Fig. 5, Table 4).

Conclusions

The present isolate HCN were identified as *H. rosae* based on the morphological, molecular and phylogenetic analysis. The infection of *H. rosae* in the sugarcane and rose fields is very high in the western Uttar Pradesh, India (Khan et al., 2019; Rathour et al., 2003). The present investigation was carried out in the fields of mustard which is an alarming sign as mustard is one of the cash crops of India having economic importance. From the studies, it is clear that species of genus *Hemicriconemoides* possess certain characteristics which cause potent damage to the crops, ultimately affecting the yield (Inserra et al., 2014; Maria et al., 2018). The information revealed from the present investigation can be exploited by taxonomists for the comparison, identification and correlation between the morphometric parameters of the species.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41936-023-00338-6.

Additional file 1: S1. Pairwise distances (Kimura 2-parameter) between species of *Hemicriconemoides* based on ITS (Internal transcribed spacer) regions. Out-groups were not included.

Additional file 2: S2. Pairwise distances (Kimura 2-parameter) of the between *Hemicriconemoides* species based on D2D3 (Large ribosomal subunit, 28S) regions. Out-groups were not included.

Additional file 3: S3. List of GenBank accession numbers of the *Hemicriconemoides* species used in the phylogenetic tree.

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Author contributions

The study was designed by HS and AKC. Interpretation of the data was done by HS. Manuscript writing was done by all authors. Tables and reference settings were done by HS. Errors and grammatical mistakes in manuscript were removed and edited by AKC. Then, final manuscript was read and approved by both authors.

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Availability of data and materials

The data and material of this manuscript are available from corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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