

## Morphological and Molecular Characteristics of *Pratylenchus haiduongensis* sp. n., a New Species of Root–Lesion Nematodes Associated with Carrot in Vietnam

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**Abstract:** *Pratylenchus haiduongensis* sp. n. is described as associated with carrot (*Daucus carota* subsp. sativus (Hoffm.) Schübl. & G. Martens) in Hai Duong Province, Vietnam. *P. haiduongensis* sp. n. is characterized by the lip region with three annuli and slightly separated from the body. Stylet knobs are rounded (never indented anteriorly). The lateral field includes four incisures, bearing areolation at the pharynx region and tail region and occasionally appears in the vulval region. Sometimes the appearances of oblique broken striae divide the lateral field into five or six incisures. The ovary is distinct with one row of oocytes. Spermatheca is oval in shape with round central cavity, without sperm or reduced in some specimens. The postvulval uterine sac is long surpassing the vulva body diameter by 2 to 2.5 times (PUS = 31 to 65 µm). High vulva position with V = 66 to 75%. The tail shape can be subhemispherical with a smooth, slightly indented, broadly smooth, or cleft terminus observed in some specimens. The matrix code of *P. haiduongensis* sp. n. is: A2, B1, C4, D(1,3), E1, F(5,6), G(1,2), H(1,4); I(1,2,3,4), J1, K(1,2) according to Castillo and Vovlas (2007). The LSU–D2D3 segment and the ITS–rDNA region of this species were amplified and sequenced. The morphological characters and molecular phylogenetic analyses confirmed that this is a new species of the genus *Pratylenchus* in Vietnam.

**Key words:** carrot, molecular, morphology, new species, root–lesion nematode, SEM, Vietnam.

The nematodes belong to the genus *Pratylenchus* and have a wide host range and appear around the world. They are widely distributed throughout the tropics, subtropics, and temperate regions (Jatala and Bridge, 1990). They caused lesions on the roots that affect the growth and development of crops and lead to significant losses of yield (Castillo and Vovlas, 2007). The genus *Pratylenchus* ranked second among the top of nematodes which caused damage to the crops. Currently, nearly 70 species of genus *Pratylenchus* have been recorded all over the world (Castillo and Vovlas, 2007). In Vietnam, 12 species of the genus *Pratylenchus* were recorded. These species parasitize on many important crops, such as pineapples, sugar cane, maize, tobacco, coffee, carrots, etc. (Nguyen and Nguyen, 2000).

Carrots are one of the main crops with high nutrient and economical value that are commonly grown in Hai Duong Province (Nguyen et al., 2016). One of the main reasons that caused the disease on carrots is the lesions nematodes *Pratylenchus* (Nguyen et al., 2016). In Vietnam, five species of the genus *Pratylenchus* were found on carrots namely: *Pratylenchus thornei*, *Pratylenchus zaeae*, *Pratylenchus coffeae*, *Pratylenchus penetrans*, and *Pratylenchus*.

*pratensis* (Nguyen and Nguyen, 2000; Nguyen et al., 2016).

Very recently, a survey of plant–parasitic nematodes reported one *Pratylenchus* sp. population from a damaged carrot field in Hai Duong Province that has similarities in morphology with *Pratylenchus parazeae*. However, the molecular studies of this population and the scanning electron microscopy (SEM) pictures revealed that they are separated species from *P. parazeae*. The new species is herein described as *P. haiduongensis* n. sp. through extensive morphological and molecular studies on D2D3 expansion domains of a large subunit (LSU–D2D3) and ITS.

### MATERIALS AND METHODS

#### *Nematode population sampling*

Soil and root samples were collected at carrot growing areas in Nam Sach District (population 3,655, and population 3,658) and Cam Giang District (population 4,728), Hai Duong Province in May 2015. Nematodes were extracted from soil and roots by the method described by Nguyen (2003). A single female was picked out and transferred to carrot discs and kept at 25°C to culture for 8 wk. After that, purified nematodes reared on carrot discs were used for morphological and molecular analysis (Coyle et al., 2014). Then, the worms were killed by heat, fixed in the TAF solution, processed to anhydrous glycerol according with Seinhorst (1959) technique, and mounted on permanent glass slides for permanent preservation and observation under light microscope.

#### *Morphological studies*

**Light microscopy:** For morphometric and morphological examination, nematodes were observed through the Carl Zeiss Axio Lab. A1 light microscope. Measurements, line drawings, and pictures were taken using the ZEN lite

Received for publication May 9, 2017.

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The authors thank the farmer in Hai Duong Province for allowing us to collect soil and carrot root samples and acknowledge the assistance of Research Technical Services of University of Jaén (Spain) for the scanning electron microscopy study. This research was supported by the National Foundation for Science and Technology Development (NAFOSTED) project (code: 106–NN.03–2013.56) and Basic Foundation project from the Institute of Ecology and Biological Resource (IEBR) (code: IEBR.DT.04/16–17).

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This paper was edited by Zafar A. Handoo.

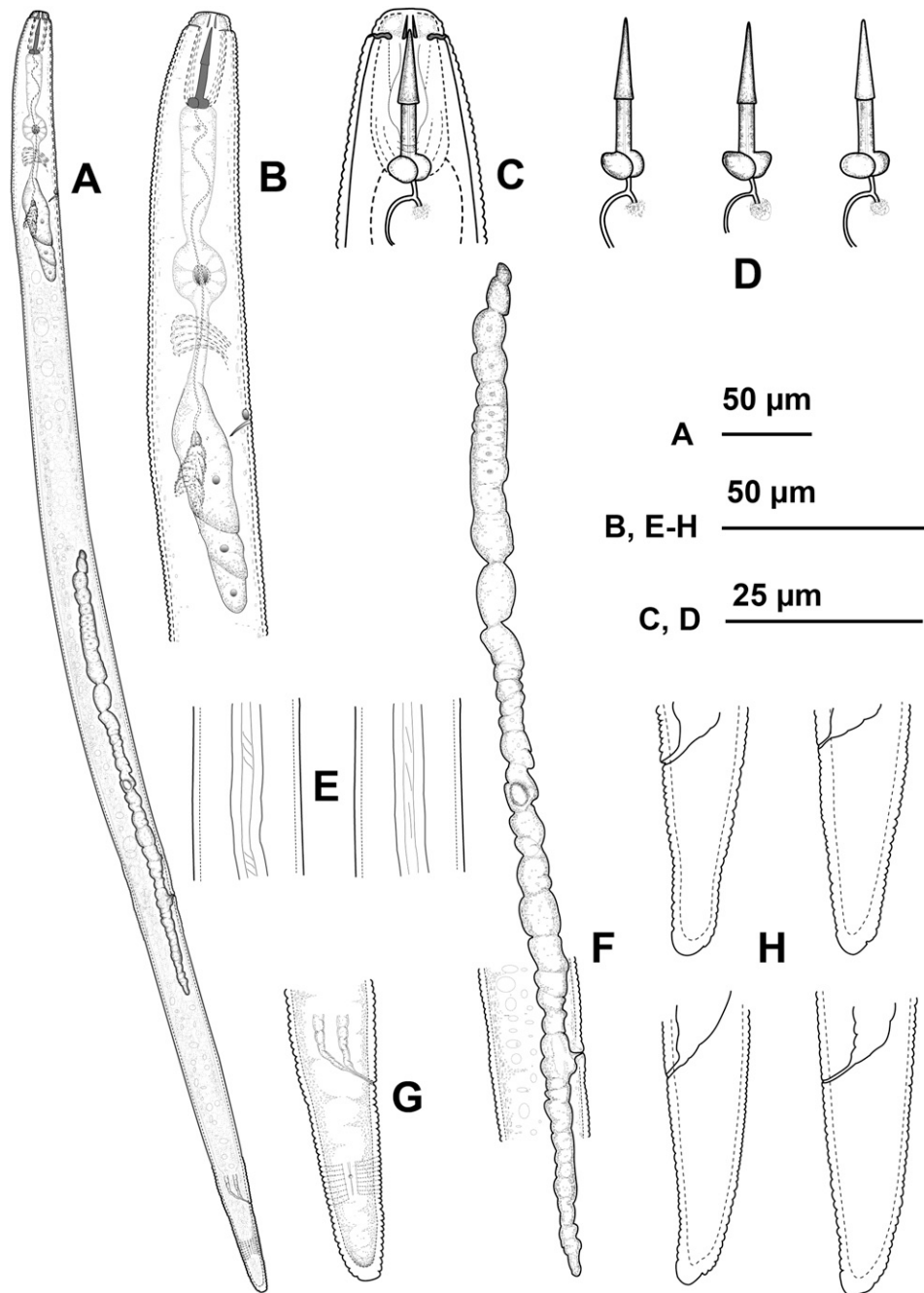


FIG. 1. *Pratylenchus haiduongensis* n sp. n. (♀). A. Entire body. B. Anterior region. C. Anterior end. D. Stylet shape. E. Lateral field. F. Anterior branch of female reproductive system. G. Tail region. H. Variation in the tail shape.

software on ZEISS Axiocam ERc5s digital camera. Raw photographs were edited using Adobe® Illustrator® CS.

**Scanning electron microscopy (SEM):** After the examination and identification, one specimen in good condition was selected to its observation under SEM following the protocol by Abolafia (2015). The nematode was hydrated in distilled water, dehydrated in a graded ethanol and acetone series, critical point dried, coated with gold, and observed with a Zeiss Merlin Scanning Electron Microscope.

**DNA extraction, PCR, and sequencing:** DNA was extracted from a single individual nematode following Waeyenberge et al. (2000). The nematode was transferred to a 0.5 ml

ependorf tube containing 18 µl of Worm Lysis Buffer (50 mM KCL, 10 mM Tris pH 8.3, 2.5 mM MgCl<sub>2</sub>, 0.45% NP 40, and 0.45% Tween 20) and 2 µl of proteinase K (600 µg ml<sup>-1</sup>) (Thermo Scientific). The tubes were incubated at 65°C (1 hr) and then at 95°C (15 min). PCR and sequence protocols were described in detail by De Ley et al. (1999). The D2D3 fragment (28S-rDNA) and ITS-rDNA region was cloned by primers D2D3 and V2F/V2R with PCR reaction components. Primers for LSU D2D3 amplification were D2A (5'-ACAAGTACCGTGGGG AAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGC TAC TA-3') (Subbotin et al., 2006). Primers for the

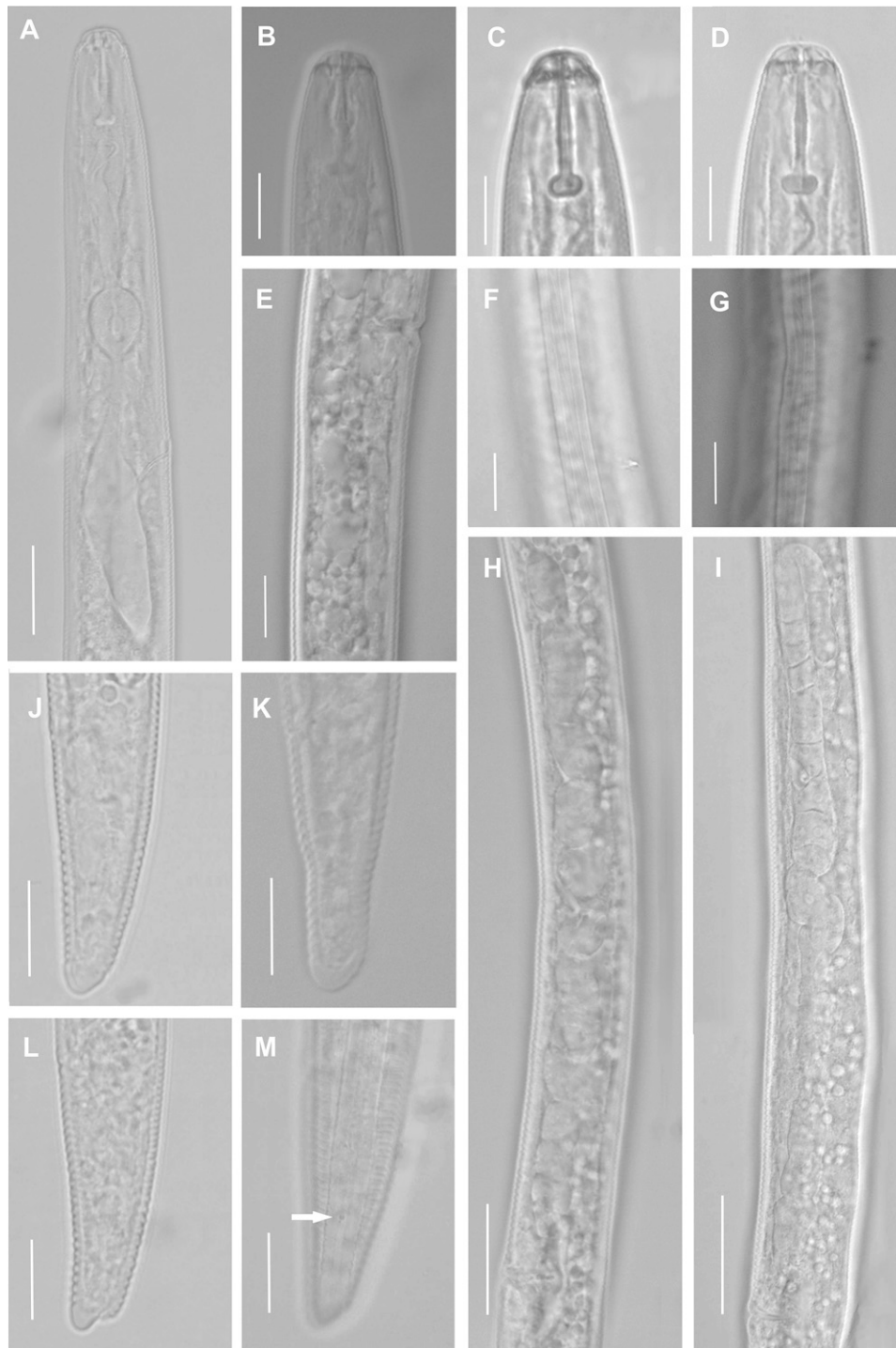


FIG. 2. *Pratylenchus haiduongensis* n. sp. n. (♀, light microscopy). A. Anterior region. B to D. Anterior region with stylet. E. Vulva in ventral view. F, G. Lateral lines near midbody. H, I. Anterior female genital system. J to L. Variation morphology in tail. M. Phasmid (arrow shows phasmid) (Scale bar: A, H, I = 20  $\mu$ m; B to G = 10  $\mu$ m.)

ITS from Vrain et al. (1992) were modified: VRAIN 2F (5'-CTTTGTACACACCGCCCGTCGCT-3') and VRAIN 2R (5'-TTTCACTCGCCGTTACTAAGGGAATC-3').

*Phylogenetic analyses:* DNA sequences were analyzed using the BLAST homology search program of nematode sequence in the GenBank. Nematode sequences with the highest *e* values for the BLAST similarity were aligned by the ClustalW software (Thompson et al., 1994). Sequence

alignments were manually edited using ChromasPro software (ChromasPro 1.7.4; Technelysium Pty Ltd, Tewantin, QLD, Australia). The sequence dataset was analyzed using the Maximum Likelihood (ML) by the MEGA 6 program (Tamura et al., 2013). The best fit model of DNA evolution for the Bayesian interferred was obtained using the Modeltest in MEGA 6 which was implemented under the best-fitting evolutionary model (TN93 + G) with the

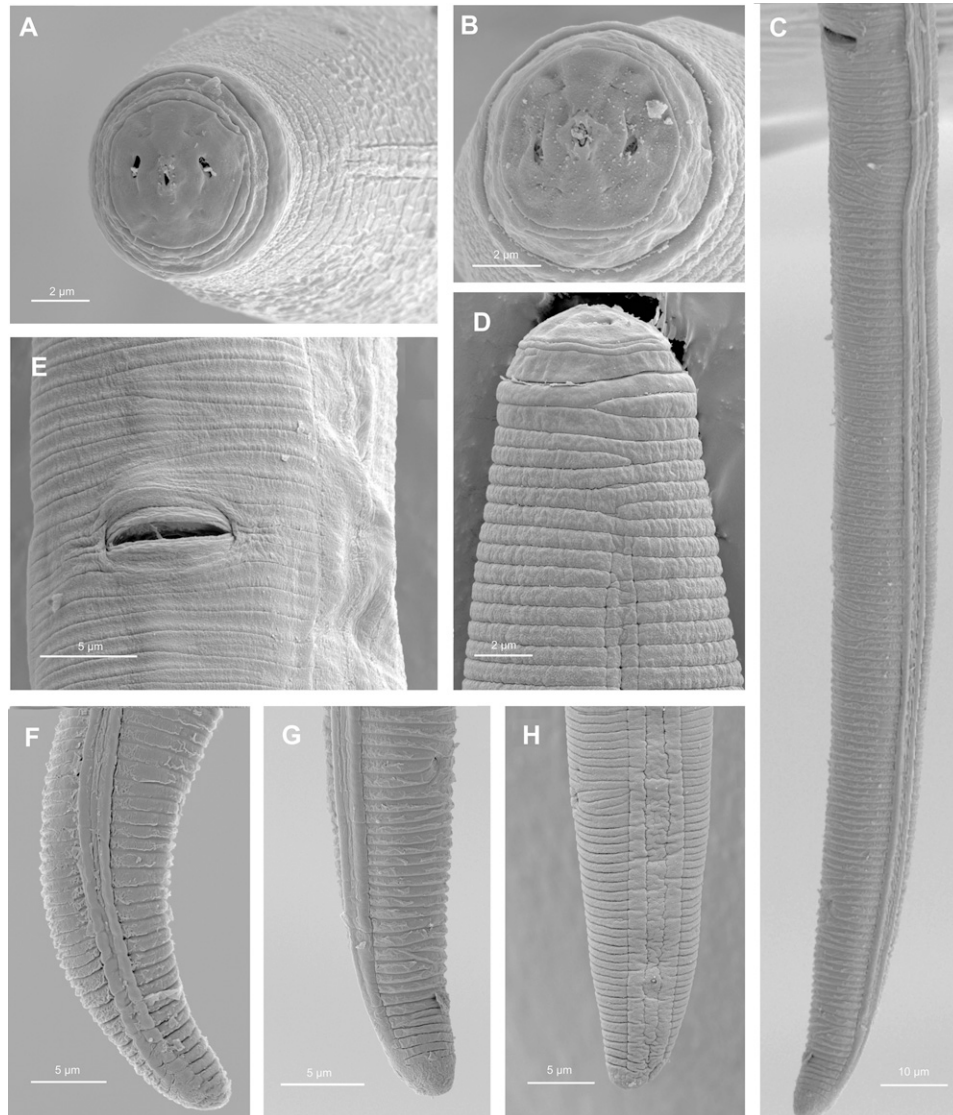


FIG. 3. *Pratylenchus haiduongensis* sp. n. (♀, SEM). A, B, D. Anterior region. C. Posterior region with vulva position and lateral view. E. Vulva position. F to H. Variation in the tail shape and phasmid position.

D2D3 and the model (GTR + G) with ITS-rDNA sequence; 1,000 bootstrap replications were executed. Out-group taxa were chosen according to the results of previous published data (Subbotin *et al.*, 2008; De Luca *et al.*, 2011). The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities are given on appropriate clades. The trees were visualized with the program FigTree v1.4.0 and drawn with Adobe Acrobat XI Pro 11.0.1.

#### RESULTS AND DISCUSSION SYSTEMATICS

*P. haiduongensis* n. sp.  
(Figs. 1–7, Table 1).

#### Description

The description of the root-lesion nematode *P. haiduongensis* n. sp.: The measurements of holotype and 90

paratype females were listed in Table 1. Illustrations and photos were shown in Figs. 1–3.

*Female*: The body straight or slightly ventrally curved after heat-killing. Body annuli *ca* 1.0 µm wide at midbody. The head region with three cuticle rings, slightly separated from the body (Fig. 3A,B). On the *en face* view showed by the SEM, lateral margins of oral disc were distinct and prominent, subdorsal and subventral segments slightly separated from the labial disc by two grooves, four short lines marked in the ventral and dorsal segment (Fig. 3B), amphidial apertures oval incomplete, and occasionally initiation of a fourth lip annulus visible on one side of the head region in some specimens (Fig. 3A,B, D). Stylet is robust and strongly sclerotized. Stylet conus *ca* 50% of the entire stylet length. Stylet shaft tubular, basal knobs are rounded or slightly anteriorly flattened (rounded = 72%, anteriorly flattened = 28%, respectively) (Fig. 2C,D). Pharynx with an elliptical median bulb and

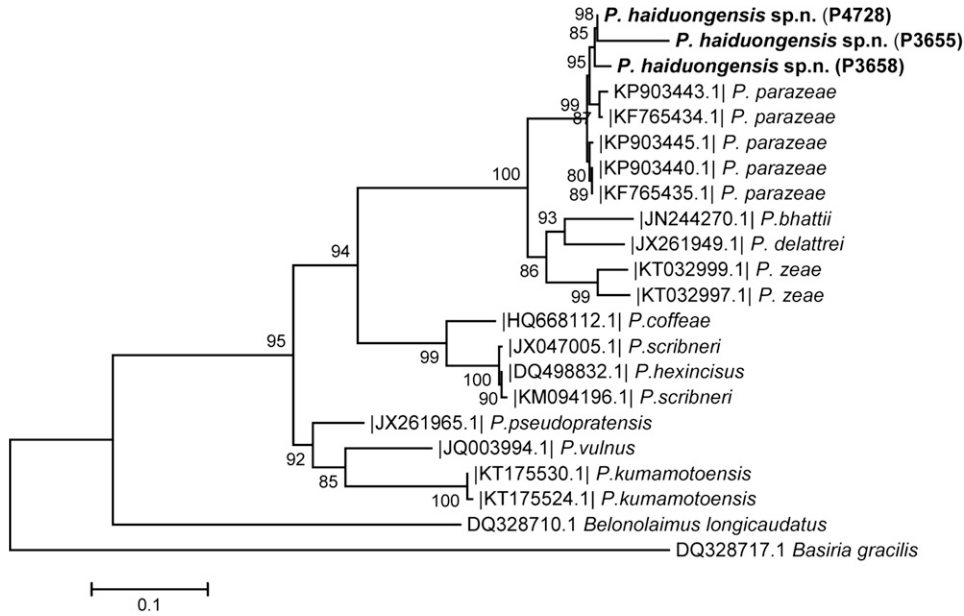


FIG. 4. Phylogenetic relationships of *Pratylenchus haiduongensis* sp. n. with related *Pratylenchus* species, based on the LSU–D2D3 (28S rDNA) sequences from Maximum Likelihood analysis. The TN93 + G model was run with 1,000 bootstrap replicates (BIC = 8,583.9; AICc = 8,168.0; lnL = -4,028.7; G = 0.466; R = 1.87). Support values are given above branches. Sequences generated in this work are indicated in bold.

rather long glandular lobe that overlaps the intestine over the developed pharyngeal–intestinal junction ventrally; isthmus is slender and encircling by nerve ring (Fig. 2A, B). The dorsal gland nucleus is located behind the pharyngeal–intestinal junction. Nuclei of ventrosublateral glands located in third of pharyngeal lobe. Hemizonid located just above the excretory pore at the level of the pharyngeal–intestinal junction. Hemizonion was not seen. Lateral field beginning at level of the stylet, with

four lateral lines at midbody, occupy about one-third of the corresponding body diameter, central band sometimes illustrated with oblique striae. Lateral field bearing areolation at the pharynx region and tail region and sometimes appears in the vulval region. Reproductive tract monoprodelfic, germinative zone outstretched (two rows of oocytes and reflexion reproductive tract are observed in some specimens). Spermatheca oval in shape with round central cavity, without sperm or reduced in

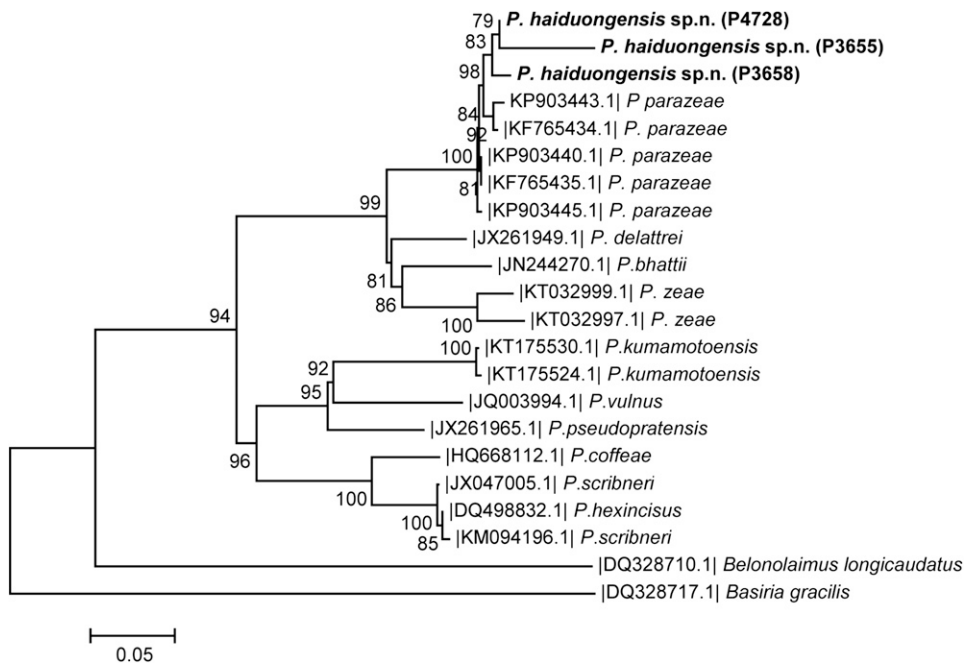


FIG. 5. Phylogenetic relationships of *Pratylenchus haiduongensis* sp. n. from LSU D2D3 (28S rDNA) with related *Pratylenchus* species based on Maximum Evolution analysis. Support values are given above branches. Sequences generated in this work are indicated in bold.

some specimens. Postuterine branch is long, *ca* from one-third to two-fifths of the vulva–anus distance. Under SEM, the vulval region is flat or slightly protruded with short vulva lips (Fig. 3E); the vulva position is high ( $V = 66\%$  to  $75\%$ ). The tail shape can be subhemispherical with a smooth terminus, slightly indented, broadly smooth, or a cleft terminus observed in some specimens (Fig. 2J–M). Phasmids are pore-like and located at one-third from the tail tip (Fig. 3F–H).

*Male*: Not found.

*Type host and locality*: Holotype and paratypes from a population were extracted from roots and rhizosphere of carrot collected in Nam Sach District, Hai Duong Provinces (GPS coordinates:  $20^{\circ}59'04''$  N and  $106^{\circ}17'12''$ E), Vietnam. The other locality is in Cam Giang District, Hai Duong Provinces with GPS coordinates:  $20^{\circ}56'12''$  N and  $106^{\circ}11'17''$  E.

*Etymology*: The species name refers to the locality, Hai Duong Province, where the nematodes were collected.

*Type material*: Holotype and paratypes are deposited in the Nematode Collection of the Institute of Ecology and Biological Resources (IEBR), Vietnamese Academy of Science and Technology, 18 Hoang Quoc Viet Road,

Hanoi, Vietnam, and ten female paratypes are deposited in the nematode collection of the Zoology Museum, Ghent University, K. L. Ledeganckstraat 35, Ghent, Belgium. The D2D3 and ITS sequences are deposited in the GenBank with accession numbers MF429811 to MF429813 and MF429808 to MF429810, respectively.

*Diagnosis*: The new species *P. haiduongensis* sp. n. females are characterized by a combination of the following morphological features: The lip region with three annuli and slightly separated from the body. Stylet knobs are rounded (never indented anteriorly). The lateral field includes four incisures, bearing areolation at the pharynx region and tail region, occasionally appears in the vulval region. Sometimes, the appearance of oblique broken striae divides the lateral field into five or six lines. The ovary is distinct with one row of oocytes. Spermatheca is oval in shape with round central cavity, without sperm or reduced in some specimens. Long postvulval uterine sac surpasses the vulva body diameter by 2 to 2.5 times ( $PUS = 31$  to  $65\ \mu\text{m}$ ). High vulva with  $V = 66\%$  to  $75\%$ . The tail shape subhemispherical or with a smooth terminus. The matrix code of *P. haiduongensis* sp. n. is: A2, B1, C4, D(1,3), E1,

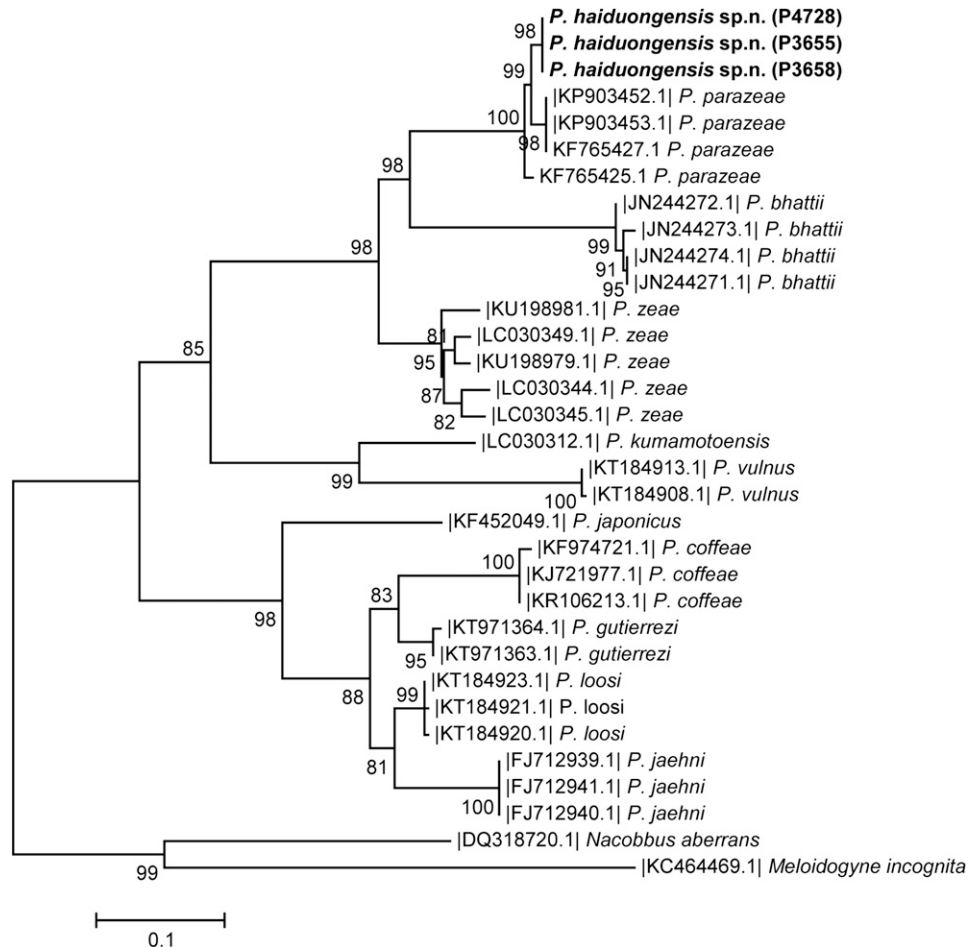


FIG. 6. Phylogenetic relationships of *Pratylenchus haiduongensis* sp. n. with related *Pratylenchus* species based on ITS rDNA sequences from Maximum Likelihood analysis. The GTR + G model analysis was chosen with GTR + G (BIC = 6,523.0; AICc = 6,081.5; lnL = -2,978.3; G = 0.26; R = 1.19). Support values are given above branches. Sequences generated in this work are indicated in bold.

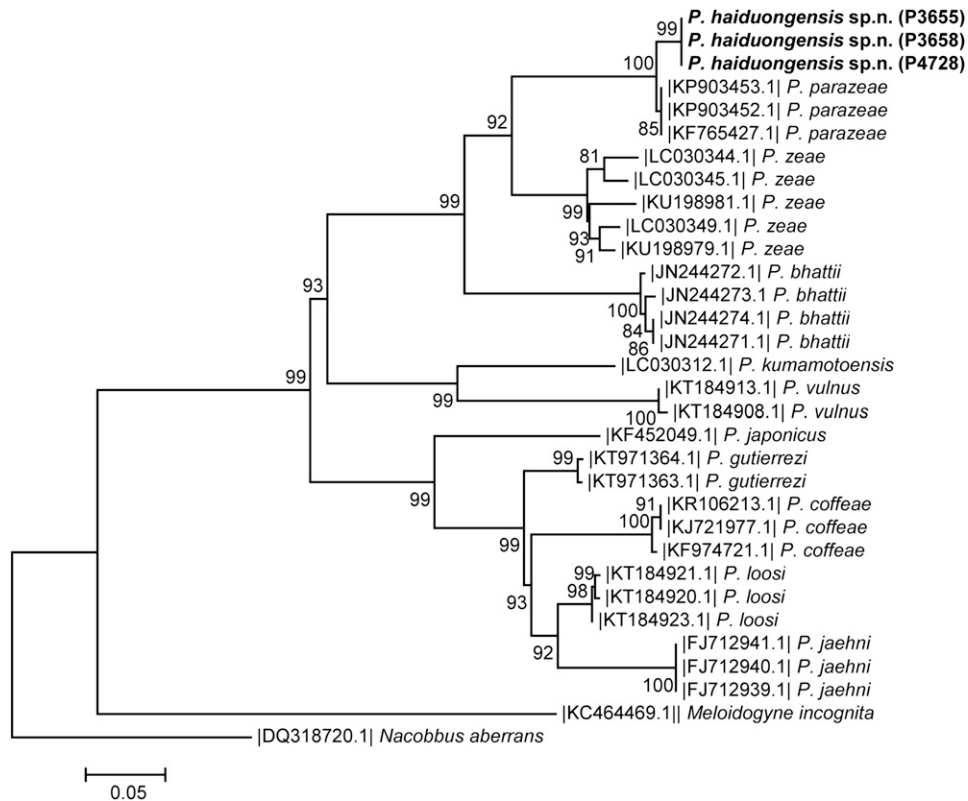


FIG. 7. Phylogenetic relationships of *Pratylenchus haiduongensis* sp. n. with related *Pratylenchus* species based on ITS rDNA sequences from of *Pratylenchus* spp. from Maximum Evolution analyses. Support values are given above branches. Sequences generated in this work are indicated in bold.

F(5,6), G(1,2), H(1,4); I(1,2,3,4), J1, K(1,2) according to Castillo and Vovlas (2007).

**Relationships:** *P. haiduongensis* sp. n. is most similar to *P. parazeae* (Wang et al., 2015), *Pratylenchus yassini* (Zeidan and Geraert, 1991), *Pratylenchus curvicauda* (Siddiqi et al., 1991), *Pratylenchus cruciferus* (Bajaj and Bhatti, 1984), *P. zaeae* (Roman and Hirschmann, 1969), and *Pratylenchus subranjani* (Mizukubo et al., 1990).

*P. haiduongensis* sp. n. differs from *P. parazeae* (Wang et al., 2015) by having smaller body length (460 to 679  $\mu\text{m}$  vs. 530 to 680  $\mu\text{m}$ ); slightly separated lip region with slightly separated labial disc vs. continuous lip region with plain and smooth labial disc; *en face* view showed by SEM, lateral margins of oral disc were distinct and prominent, subdorsal and subventral segments are slightly separated from the labial disc by two grooves, and four short lines marked in ventral and dorsal segment vs. smooth and undivided; smaller lip region width (7.3 to 8.3  $\mu\text{m}$  vs. 7.8 to 9.2  $\mu\text{m}$ ); longer stylet (18 to 20  $\mu\text{m}$  vs. 16.7 to 19.2  $\mu\text{m}$ ); stylet knobs are rounded or slightly anteriorly flattened, never indented anteriorly vs. rounded to indented anteriorly knobs, and the lateral field having prominent ridges vs. lateral field having narrow incisures.

*P. haiduongensis* sp. n. differs from *P. yassini* (Zeidan and Geraert, 1991) by having longer body length (460 to 679 vs. 430 to 600); slightly separated lip region with slightly separated labial disc vs. offset by a fine but deep

constriction lip region with undivided front plate amalgamated; lateral field with oblique striae vs. diagonally interrupted lines in central band; longer PUS (31 to 65  $\mu\text{m}$  vs. 20 to 42  $\mu\text{m}$ ), longer vulval body diameter (14 to 26  $\mu\text{m}$  vs. 17 to 20  $\mu\text{m}$ ), and shorter pharyngeal gland (29 to 62 vs. 85 to 129); tail smooth at terminus (50% smooth, 30% ventrally pronounced, and 20% with a cleft) vs. tail annulated.

*P. haiduongensis* sp. n. differs from *P. curvicauda* (Siddiqi et al., 1991) by having larger body length (460 to 679  $\mu\text{m}$  vs. 450 to 550  $\mu\text{m}$ ); the head region with three cuticle rings slightly separate from the body vs. cephalic region broadly rounded to truncate, continuous with the body; longer stylet (18 to 20  $\mu\text{m}$  vs. 15 to 16.5  $\mu\text{m}$ ); shorter pharyngeal gland overlap (26 to 62  $\mu\text{m}$  vs. 47 to 80  $\mu\text{m}$ ); lateral field with areolation vs. without areolation; longer PUS (31 to 65  $\mu\text{m}$  vs. 19 to 37  $\mu\text{m}$ ).

*P. haiduongensis* sp. n. differs from *P. cruciferus* (Bajaj and Bhatti, 1984) by having shorter body length (460 to 679 vs. 648 to 793); the head region with three cuticle rings slightly separate from the body vs. the head region flat, continuous with the body; longer stylet (18 to 20  $\mu\text{m}$  vs. 15 to 16  $\mu\text{m}$ ); PUS longer (31 to 65  $\mu\text{m}$  vs. 18 to 30  $\mu\text{m}$ ); lateral field with areolation at the pharynx and tail regions vs. without areolation; and more anterior vulval position (V = 66 to 75 vs. 76 to 81).

*P. haiduongensis* sp. n. differs from *P. subranjani* (Mizukubo et al., 1990) by having slightly longer body





(460 to 679  $\mu\text{m}$  vs. 386 to 572  $\mu\text{m}$ ); the shape of stylet knobs (72% rounded, 28% anteriorly flattened vs. mostly in indented condition and by no means rounded); round or oval and empty spermatheca vs. rarely observed, lower V% value (66 to 75 vs. 73 to 77); longer vulva-anus distance (92 to 189  $\mu\text{m}$  vs. 77 to 118  $\mu\text{m}$ ); tail with smooth terminus (50% of individuals with smooth tail tip, 30% with ventrally pronounced tail tip, and 20% with a tail tip having a cleft vs. 53% bluntly pointed, 30% subhemispherical, 15% subdigitate, and 3% truncate, respectively).

*P. haiduongensis* sp. n. differs from *P. zae* (Roman and Hirschmann, 1969) by the relatively longer body (460 to 679  $\mu\text{m}$  vs. 463 to 657  $\mu\text{m}$ ) and longer stylet (18 to 20  $\mu\text{m}$  vs. 13.6 to 16.6  $\mu\text{m}$ ); lateral field (inner band sometimes with oblique striae; areolation at the pharynx region and tail region; sometimes appears in the vulval region vs. the inner band showing a slight irregularity in midbody region without areolation); tail shape (subcylindrical vs. conoid); tail terminus rounded to bluntly pointed vs. generally almost pointed, narrowly rounded to subacute.

**Molecular characteristics:** PCR amplification of the LSU D2D3 region of three populations of *P. haiduongensis* sp. n. yielded a single product with a length of 670 bp. Sequence diversity reached varied from 1% to 2% (7 to 12 nucleotides) within *P. haiduongensis* sp. n. and 4% to 11.2% for the closely related species (*P. parazeae* and *P. zae*) to *P. haiduongensis* sp. n. Phylogenetic relationships within *Pratylenchus* species based on the LSU D2D3 sequences were generated by ML and Minimum Evolution (Figs. 4,5). Both ML and ME trees showed that *P. haiduongensis* clustered in a subgroup standing apart and related to the *P. parazeae*, *P. zae*, *Pratylenchus delattrei*, and *Pratylenchus bhattii* with genetic distances from 0.02 to 0.2. The value of intraspecific genetic distance from 0.1 to 0.37 was significant difference. The clade composed by *P. haiduongensis* populations is together with *P. parazeae*. However, *P. haiduongensis* populations are grouped in a clade separate from the one composed of *P. parazeae* with high support (bootstrap of ML = 85 and ME = 83).

The ITS sequences alignments of *P. haiduongensis* sp. n. is 910 bp. The sequence diversity within *P. haiduongensis* sp. n. populations varied from 1 to 5 nucleotides (0.1% to 0.5%), and the sequence diversity within *P. haiduongensis* sp. n., *P. parazeae*, *P. zae*, and *P. bhattii* varied from 16 to 220 nucleotides (1.7% to 24%). Intraspecific variations of *P. haiduongensis* sp. n. with *P. parazeae* were 2%. The phylogenetic tree constructed based on ITS sequences were generated by ML and Minimum Evolution is given (Figs. 6,7). The new species clustered in a subgroup standing apart and related to the *P. parazeae*, *P. zae*, and *P. bhattii* with genetic distances from 0.02 to 0.26. *P. haiduongensis* sp. n. is highly supported to the group in a monophyletic clade with *P. parazeae* with significant divergence.

The PCR was not specific for *P. haiduongensis* sp. n. using specific primer for *P. parazeae* following Wang et al. (2015) (data not shown).

This study provided clear evidence of the difficulty of discriminating *P. haiduongensis* sp. n. and *P. parazeae* based only on morphological characters. *P. haiduongensis* sp. n. belongs to “*pratensis group*” which cannot be separated conveniently on the basis of biometrical measurements because of overlapping ranges (Frederick and Tarjan, 1989). There are only rare distinct morphological characters that can separate species in “*pratensis group*” (Frederick and Tarjan, 1989). *En face* in *Pratylenchus* which can be seen only in SEM, this feature is an important taxonomic character (Corbett and Clark, 1983). So far, *P. coffeae*, *Pratylenchus loosi*, and *Pratylenchus jaehni* or *P. zae* and *Pratylenchus bolivianus* are clearly separated base on *enface* structures (Corbett and Clark, 1983; Duncan et al., 1999; Inserra et al., 2001; Troccoli et al., 2016).

Molecular diagnostics should be low-cost, user-friendly, and reliable in distinguishing nematode species (Barker and Davis, 1996; Jones et al., 1997). Some new species difficult to distinguish morphologically from other species have been identified using molecular characterization. This was realized for species level identification such as *P. jaehni*, *P. hippeastri*, *P. lentis*, and *P. bolivianus* (Inserra et al., 2001, 2007; Troccoli et al., 2008, 2016). Intraspecific ITS variation has been observed in *Pratylenchus* (Handoo et al., 2001; De Luca et al., 2011; Wang et al., 2015; Troccoli et al., 2016). Subbotin et al. (2008) analyzed the phylogeny and separated *Pratylenchus* species generally congruent with those defined by characters derived from lip patterns, numbers of lip annules, and spermatheca shape. Morphological results suggest the need for sophisticated character discovery and analysis for morphology based phylogenetics in nematodes.

Morphological and molecular data provide evidence that *P. haiduongensis* sp. n. are distinctly described as *Pratylenchus* species and indicate that this is a new species of this genus. The genetic similarity between *P. haiduongensis* sp. n. and *P. parazeae* is reflected in their morphological similarities. *P. haiduongensis* sp. n. and *P. parazeae* share important morphological characters, such as the flat and head region with three cuticle rings, an empty spermatheca, and an anterior vulva position.

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