

Article

Morphological and Molecular Characterization of *Hoplolaimus seinhorsti* Luc, 1958 with Its First Report on Turfgrass

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Abstract: In the 2023 summer season, a *Hoplolaimus* population collected from the rhizosphere of bermudagrass collected in Shenzhen, China was morphologically and molecularly identified as *H. seinhorsti*. As far as we are aware, this is the first documentation of *H. seinhorsti* from turfgrass. The average body length of females in this population is 1465 μm . Lip region is set off from body by a deep constriction, with 4 annules, basal annule of lip region has 9–10 longitudinal striations. A single incisure is faint in lateral field. Excretory pore (EP) is located at level of esophageal glands. Hemizonid is situated 2 annules posterior to EP. Stylet is strong, with large, tulip-shaped basal knobs, 33 to 43 μm long. Esophageal glands dorsally overlap the intestine, with six gland nuclei. Intestine does not overlap rectum. Vulval opening is situated at 53–59% of total body length from the anterior end, posterior epiptygma is visible. Reproductive system didelphic with two equally developed outreached ovaries, no spermatheca observed. Tail shorter, 0.8–1.4 anal body diameter long, with rounded tip, with 10–16 annules. Phylogenetic analysis using partial sequences of 18S and the expansion fragment D2-D3 of 28S rDNA genes suggested that studied *H. seinhorsti* is clustered in a monophyletic clade with high support with the other populations of *H. seinhorsti*, revealed closer relationship of this species to *H. columbus*, *H. indicus*, *H. dubius* and *H. pararobustus* than to *H. galeatus*, *H. stephanus*, *H. concaudajuvencus* and *H. magnistylus*, and supported that the present *H. seinhorsti* is a separate species from *H. columbus*, *H. galeatus*, *H. concaudajuvencus* and *H. stephanus* found on turfgrasses.

Keywords: *Hoplolaimus*; *H. seinhorsti*; lance nematode; turfgrass; morphology; 18S rRNA; D2-D3 of 28S rRNA; ribosomal DNA (rDNA); phylogeny

1. Introduction

Hoplolaimus von Daday 1905 in the family Hoplolaimidae Filipjev 1934 is a genus of nematodes known commonly as lance nematodes, thus far comprising 37 nominal species [1–7]. Traditionally species identification of the Hoplolaimidae Filipjev 1934 relies on morphological features and morphometric characters. However, in some cases, nematode species determination based upon morphology encounters difficulties or potential misidentification due to interspecific variation of phenotypic traits or overlapping of key descriptive morphological and morphometric characters [8,9]. rDNA-based approaches on the small subunit (SSU), D2-D3 expansion segment of the large subunit (LSU) and ITS rRNA genes have been employed to identify the species and to analyze phylogenetic relationships among species within Hoplolaimidae [10–14].



Out of described 37 *Hoplolaimus* species, three species are considered as pests of agricultural crops, i.e., *H. columbus* Sher, 1963, *H. galeatus* (Cobb, 1913), Thorne, 1935 and *H. magnistylus* Robbins, 1982 [15]. *H. columbus* can infect cotton [16], soybean [17], and corn [18]. *H. magnistylus* can affect crops such as cotton etc. [19]. *H. galeatus* can be found in many crops, as well as many species of grasses [20–22]. On turfgrasses, lance nematodes (*Hoplolaimus* spp.) are one of the most common plant parasitic nematodes. Four species of the genus are associated with turfgrasses, i.e., *H. galeatus* with different types of turfgrasses [22], *H. columbus* with bermudagrass [23], *H. concaudajuvencus* Golden and Minton, 1970 with bermudagrass [24], and *H. stephanus* Sher, 1963 with creeping bentgrass [25]. But *H. galeatus* is the predominant species on turfgrasses including bermudagrass, St. Augustine, zoysia, and centipede grass, and cause problems with turfgrasses. Lance nematodes include endo-, ecto- and semi-endoparasitic nematodes, they move completely inside a root, some feed on epidermal and cortical cells externally in the roots, causing a reduction in root hair number as well as water and nutrient uptake, root rot, and finally destroy the root system of plants, leading to yellowing and dying patches on the aboveground. Besides harming plants directly, the nematodes open wounds while feeding roots, through which microorganisms in the soil can enter and damage roots, speeding up the root to rot of grasses.

Hoplolaimus seinhorsti Luc 1958 was first described by Luc from the roots of cotton in Madagascar and assigned to the genus *Hoplolaimus* [26]. It has been reported from some countries and regions in Africa, Asia, Central America and Caribbean, South America, Oceania [27]. It is associated with many plant species such as *Capsicum annuum* L., *Cicer arietinum* L., *Cleome aculeata* L., *Cleome rutidosperma* DC, *Cocos nucifera* L., *Commelina diffusa* Burn.f., *Dieffenbachia sequine* Schott., *Echinochloa colona* (L.) Link., *Eleusine indica* Gaertn., *Eragrostis pilosa* Beauv., *Euphorbia hirta* L., *Gossypium hirsutum* L., *Mimosa pudica* L., *Musa* sp. L., *Oryza sativa* L., *Passiflora* sp. L., *Phenax sonneratii* Wedd., *Pilea microphylla* (L.) Liebm., *Setaria barbata* Kunth, *Solanum lycopersicum* L., *Solanum melongena* L., *Vigna mungo* Hepper., *Vigna unguiculata* Van Eselt. ex Verdc., *Vigna sinensis*, *Zea mays* L. [17,28–31]. However, thus far no related report of *H. seinhorsti* on turfgrasses. Recent plant parasitic nematode surveys conducted on turfgrasses in Shenzhen, China revealed one nematode population associated with bermudagrass (*Cynodon dactylon* (L.) Pers.). Preliminary morphological observations indicated that the population appeared to be related to *H. seinhorsti*. The objectives of this paper were to: (i) characterise *H. seinhorsti* from the rhizosphere of bermudagrass in Shenzhen, China based upon integrative taxonomic approaches including morphological, morphometric and molecular data (partial 18S rRNA and D2-D3 expansion segment of 28S rRNA sequences); and (ii) determine the molecular phylogenetic affinities of *H. seinhorsti* with closely related populations based on molecular data.

2. Materials and Methods

2.1. Soil Sampling

In 2023, soil samples for extracting nematodes were collected from the root zone of bermudagrass (*C. dactylon*) on a golf course at Shenzhen, China (22.5261° N, 113.9590° E). Each sample has 12 plugs (1.5 cm diam. × 20 cm deep) collected at roughly equal intervals in a zig-zag pattern across an area of 500 m² or less. The samples were combined and placed in sealed plastic bags and then stored at 4 °C for further nematode species analysis.

2.2. Morphological Characterization

Nematodes in soil samples were extracted using the rapid centrifugal-flotation method [32]. After heat-killing nematode specimens were treated using the formalin-glycerin method [33]. Measurements were carried out with the aid of a camera lucida and a stage micrometer. The morphometrics process was conducted using Ye's method [34]. Photomicrographs were taken with a camera (Nikon DS-Fi1) attached via a C-mount Adapter fitted on a microscopy (Nikon ECLIPSE 80i) and treated using Adobe Photoshop CS6.

2.3. Molecular Characterization

We picked one male or female nematode under microscope and placed it into 50 µL of worm lysis buffer that contains Proteinase K to extract DNA as a PCR template [35] and put the DNA in refrigerator at –20 °C until they are used.

We used the following primers for DNA amplification and DNA sequencing: the forward primer 18S965 (5'-GGCGATCAGATACCGCCCTAGTT-3') and reverse primer 18S1573R (5'-TACAAAGGGCAGGGACGTAAT-3') [36] for SSU 18S, the forward primer D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and reverse primer D3B (5'-TCGGAAGGAACCACTACTA-3') [37] for LSU 28S.

We used Dream Taq Green PCR Master Mix DNA polymerase (Thermo Fisher Scientific [China] Co. Ltd., Shanghai, China) for PCR. The total 25 μ L reaction contains 1 μ L DNA, 1 μ L forward primer, 1 μ L reverse primer, 9.5 μ L DNA-free water and 12.5 μ L Taq Master Mix DNA polymerase. The reaction program [38] was as follows: denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 2 min. A final extension was performed at 72 °C for 10 min. We used ExoSap-IT (Affymetrix Inc., Santa Clara, CA, USA) for cleaning of PCR products following the manufacturer's protocol. Guangzhou Tianyihuiyuan Gene Science & Technology Co., Ltd., Guangzhou, China conducted sequencing of PCR products by using an ABI PRISM 3730 sequencing system.

The sequences of rDNA SSU and LSU in this project were deposited in the GenBank database under the accession numbers PQ014655 and PQ014654, respectively. The DNA sequences were compared with other nematode species in GenBank using the BLAST homology search program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome, accessed on 15 July 2024). The most similar sequences were downloaded for phylogenetic analysis. DNA sequences were aligned using Mega5.05 [39]. The base substitution models in the SSU and LSU sets were evaluated using MODELTEST 3.06 [40], and then Akaike information criterion (AIC) was generated. The Akaike-supported model, the base frequencies, the rates of the base substitution, the proportion of invariable sites and the gamma distribution shape parameters in AIC were used in phylogenetic analysis [41]. Bayesian analysis was conducted to confirm the tree topology for each gene separately using MrBayes 3.1.0 [42] running the chain for 1×10^6 generations and setting the 'burn-in' at 1000 [41]. The Markov chain Monte Carlo methods [43] within a Bayesian framework were used to estimate the posterior probabilities (pp) of the phylogenetic trees using the 50% majority-rule.

3. Results

3.1. Morphological Description

Female: Vermiform cylindrical body slightly tapering towards front end and curved ventrally into open C-shape heat killed. Cuticle with distinct annulation, annules 2 μ m wide at mid-body. Subcuticular striation prominent, 2 striae for each annule. Lip region hemispherical, set off from body by a deep constriction, with 4 annules. 9–10 longitudinal striations around basal annule of lip region. Labial framework prominent and sclerotized. A single incisure indistinct in lateral fields. Phasmids large, about 4 μ m in diameter, anterior one at about 30%, posterior one at about 80% of body length from anterior end. Excretory pore (EP) 126 to 168 μ m from anterior end, located at esophageal gland level. Hemizonid situated 2 annules posterior to EP. Hemizonion not seen. Stylet massive, large, strong, with large, tulip-shaped basal knobs, 33 to 43 μ m long. Orifice of dorsal esophageal gland about 5 μ m behind stylet base. Metacarpus oval, well-developed, with sclerotized valve, occupying about half of the body diameter. Esophageal glands dorsally overlapping the intestine with six gland nuclei (Dorsal esophageal gland with 4 nuclei). Intestine does not overlap rectum. Vulval opening oval, slightly posteriorly located at 53–59% of total body length, vagina slightly directed anteriorly, about 1/2 vulval body diameter long, vulval lips not protruding, posterior epiptygma visible. No spermatheca observed. Reproductive system didelphic with two equally developed outreached ovaries, no spermatheca observed. Tail short, 0.8–1.4 anal body diameter long, with rounded tip, with 10–16 annules (Figure 1).

Male: Not observed.

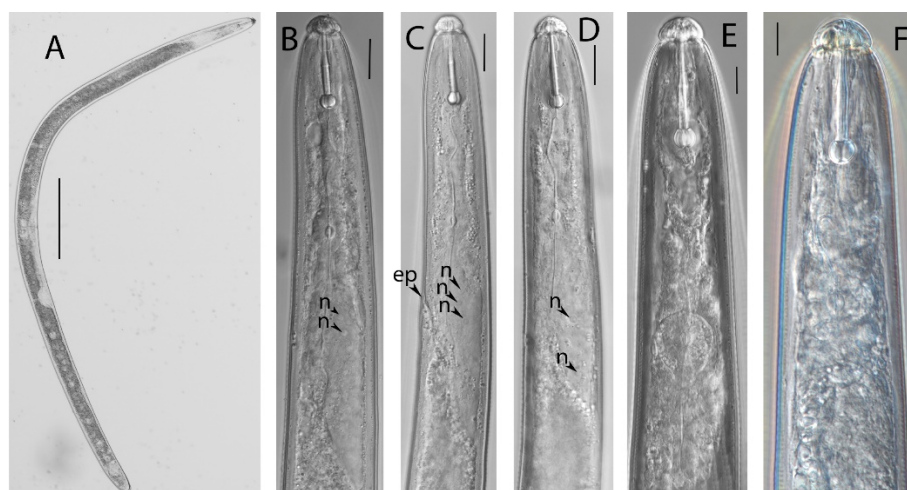


Figure 1. Cont.

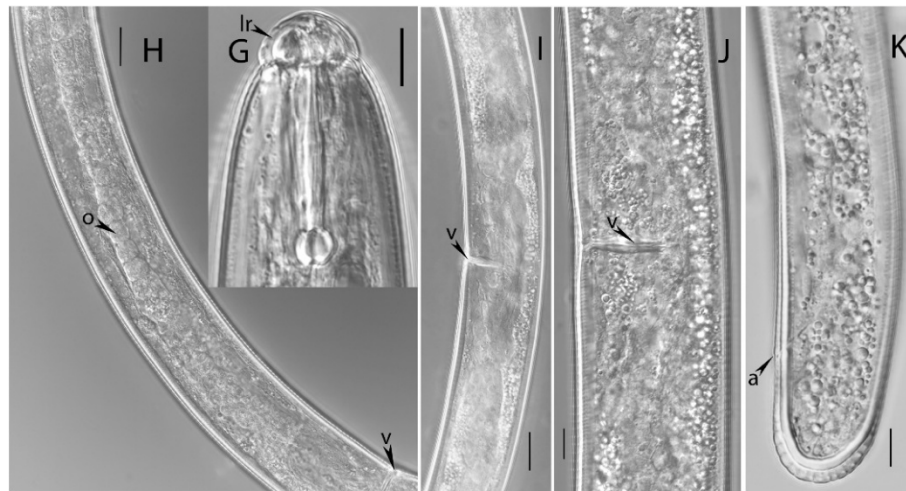


Figure 1. Adult female of *Hoplolaimus seinhorsti* in lateral and subventral view. (A): Entire body; (B–F): Anterior body (ep arrow refers to excretory pore; n arrow refers to nucleus); (G): Lip and stylet (lr arrow refers to lip ring); (H): Posterior ovary (o arrow refers to ovary); (I, J): Vulva (v arrow); (K): Tail (a arrow refers to anus). (Scale bars: A = 200 μ m; B, E, F, G, J, K, G = 10 μ m; C, D, H, I = 20 μ m).

Morphometrics: See Table 1.

Table 1. Morphometrics of females of *Hoplolaimus seinhorsti* populations mounted in formalin-glycerin in this study compared to those reported by Sher [1963]. All measurements in μ m and in the format: mean \pm s.d. (Range).

Character	Present Study	Sher, 1963
<i>n</i>	10	20
L	1465.1 \pm 69.4 (1311.1–1572.8)	1060–1560
a	31.5 \pm 3.2 (25.8–35.9)	25–34
b'	6.7 \pm 0.6 (5.9–7.6)	6.0–10.1
c	46.3 \pm 7.4 (37.8–59.7)	38–74
c'	1.1 \pm 0.2 (0.8–1.4)	-
V	56.1 \pm 1.9 (53.2–58.7)	52–60
Stylet length	40.5 \pm 3.0 (33.9–43.3)	40–49
Stylet conus length	19.5 \pm 2.4 (16.9–24.4)	-
DGO	5.2 \pm 0.4 (4.5–5.9)	-
Pharynx length	218.6 \pm 14.4 (208.0–220.7)	-
Lip region width	16.9 \pm 0.7 (15.6–18.0)	-
Lip region height	8.0 \pm 0.5 (7.4–9.1)	-
Body width	46.8 \pm 4.4 (42.6–57.1)	-
Anal body width	30.7 \pm 2.5 (27.4–35.5)	-
Tail length	32.4 \pm 5.1 (24.5–38.8)	-
Annulus width	2.1 \pm 0.2 (1.9–2.3)	-
Excretory pore from anterior end	148.8 \pm 13.8 (126.5–168.3)	-

Table 1. Cont.

Character	Present Study	Sher, 1963
Median bulb (Metacarpus) diameter	20.1 ± 1.2 (18.5–22.7)	-
Tail annuli	13.2 ± 1.7 (10–16)	10–15
Head–vulva	821.2 ± 32.3 (741.2–857.5)	-
Anterior phasmid	31.2 ± 1.9 (27.4–40.5)	31–44
Posterior phasmid	80.5 ± 4.2 (74.8–87.3)	74–83

Note: n = number of specimens on which measurements are based; L = overall body length; V = % distance of vulva from anterior relative to body length; a = body length/greatest body diameter; b' = body length/pharynx length; c = body length/tail length; c' = tail length/tail diameter at anus.

3.2. Molecular Characterization and Phylogenetic Relationships

A 724-bp 18S rDNA and a 754-bp 28S rDNA D2-D3 of *H. seinhorsti* in the present study was amplified and sequenced, respectively. A blastn search for this species has good matches with its corresponding species. From 18S sequence, the studied *H. seinhorsti* and a population of *H. seinhorsti* (OR288226) from GenBank yielded 724 total characters with 100% identity, intraspecific sequence variation (ISV) for *H. seinhorsti* was 0% (0 nucleotide, nt). The studied *H. seinhorsti* shared 680 (680/724 = 93.92%) and 723 (723/724 = 99.86%) identical nucleotides with two populations of *H. columbus* (KJ934149 and KJ934150), and 615 (615/621 = 99.03%), 715 (715/724 = 98.76%), 690 (690/700 = 98.57%) and 690 (690/700 = 98.57%) identical nucleotides with four populations of *H. galeatus* (AY912053, KJ934131, PP425960 and OR125668), respectively. From the 28S sequence, alignment of the studied *H. seinhorsti* with other two populations of *H. seinhorsti* (MN462842 and KX446969) from GenBank showed 99.72% and 100% identities, ISV for *H. seinhorsti* were 0.28% (2 nt) and 0% (0 nt), respectively. The studied *H. seinhorsti* shared respective 684 (684/685 = 99.85%), 671 (671/690 = 97.25%), 672 (672/674 = 99.70%) and 659 (659/660 = 99.85%) identical nucleotides with four populations of *H. columbus* (EU554665, HQ678715, EU626788 and OR578553); 627 (627/629 = 99.68%) with *H. dubius* (MF421901); 627 (627/686 = 91.40%), 627 (627/686 = 91.40%), 593 (593/705 = 84.11%), 571 (571/693 = 82.40%), 618 (618/707 = 87.41%), 589 (589/701 = 84.02%) and 626 (626/687 = 91.27%) with seven populations of *H. galeatus* (EU626784, EU626788, HQ678709, HQ678710, HQ678711, HQ678712 and OL813748); 627 (627/630 = 99.52%), 661 (661/663 = 99.70%) and 675 (675/677 = 99.70%) with three populations of *H. indicus* (MF421900, MW361276 and OM218728); 544 (544/605 = 89.92%) with *H. magnistylus* (MF767410); 657 (657/686 = 95.77%), 656 (656/686 = 95.63%), 657 (657/686 = 95.77%), 657 (657/687 = 95.63%) and 672 (672/702 = 95.73%) with five populations of *H. pararobustus* (OP459420, OP459421, OP459422, OP459423 and MT302643); and 619 (619/684 = 90.50%), 586 (586/682 = 85.92%), 661 (661/736 = 89.81%), 668 (668/734 = 91.01%) identical nucleotides with four populations of *H. stephanus* (HQ678717, HQ678719, HQ678721 and KX347887), and 623 (623/689 = 90.42%) with *H. concaudajuvencus* (EU626792).

We conducted on phylogenetic analyses of the partial 18S and 28S D2-D3 for examining the relationships among the most common species from the present study and relevant species from Genbank. The phylogenetic tree inferred from 18S rDNA sequences (Figure 2) using *Belonolaimus longicaudatus* Rau, 1958 and *Filenchus vulgaris* (Brzesi, 1961) Lownsbery & Lownsbery, 1985 as outgroups demonstrates: (i) the 43 selected species/populations of *Hoplolaimina* together with the present study population are in a highly-supported (100% pp) monophyletic clade; (ii) the eight selected *Hoplolaimus* species/populations (KJ934131, KJ934149, KJ934150, MK292131, AY912053, OR125668, OR28822 and PP425960) together with the present study population *H. seinhorsti* are grouped in monophyletic clade with 89% pp.; (iii) the present study population *H. seinhorsti* is clustered in monophyletic clade with another *H. seinhorsti* population (OR288226) with 85% pp.

The phylogenetic tree inferred from 28S rDNA D2-D3 expansion segments (Figure 3) using *Heterodera glycines* Ichinohe, 1952 as an outgroup demonstrates: (i) the present study population *H. seinhorsti* and the selected total 28 species/populations of the genus *Hoplolaimus* are distinctly clustered into two monophyletic clades with 100% and 92% pp, respectively, i.e., one clade containing the present study population *H. seinhorsti*, the other two populations of *H. seinhorsti* (KX446969 and MN462842), four populations of *H. columbus* (EU554665, HQ678715, HQ678716, OR578533), three populations of *H. indicus* (MF421900, MW361276 and OM218728) and one population of *H. dubius* (MF421901), and the other clade including five populations of *H. pararobustus*

(OP459420, OP459421, OP459422, OP459423 and MT302643), four populations of *H. Stephanus* (HQ678711, HQ678719, HQ678721 and KX347887), seven populations of *H. galeatus* (EU626784, EU626788, HQ678709, HQ678710, HQ678711, HQ678712 and OL813748), one population of *H. concaudajuvencus* (EU626792) and one population of *H. magnistylus* (MF767410); (ii) the present study population *H. seinhorsti* is a putative sister to the other two populations of *H. seinhorsti* (KX446969 and MN462842) with 90% pp; (iii) the present study population *H. seinhorsti* appears to be closer to *H. columbus*, *H. indicus*, *H. dubius* and *H. pararobustus* than to *H. concaudajuvencus*, *H. galeatus*, *H. magnistylus* and *H. Stephanus*.

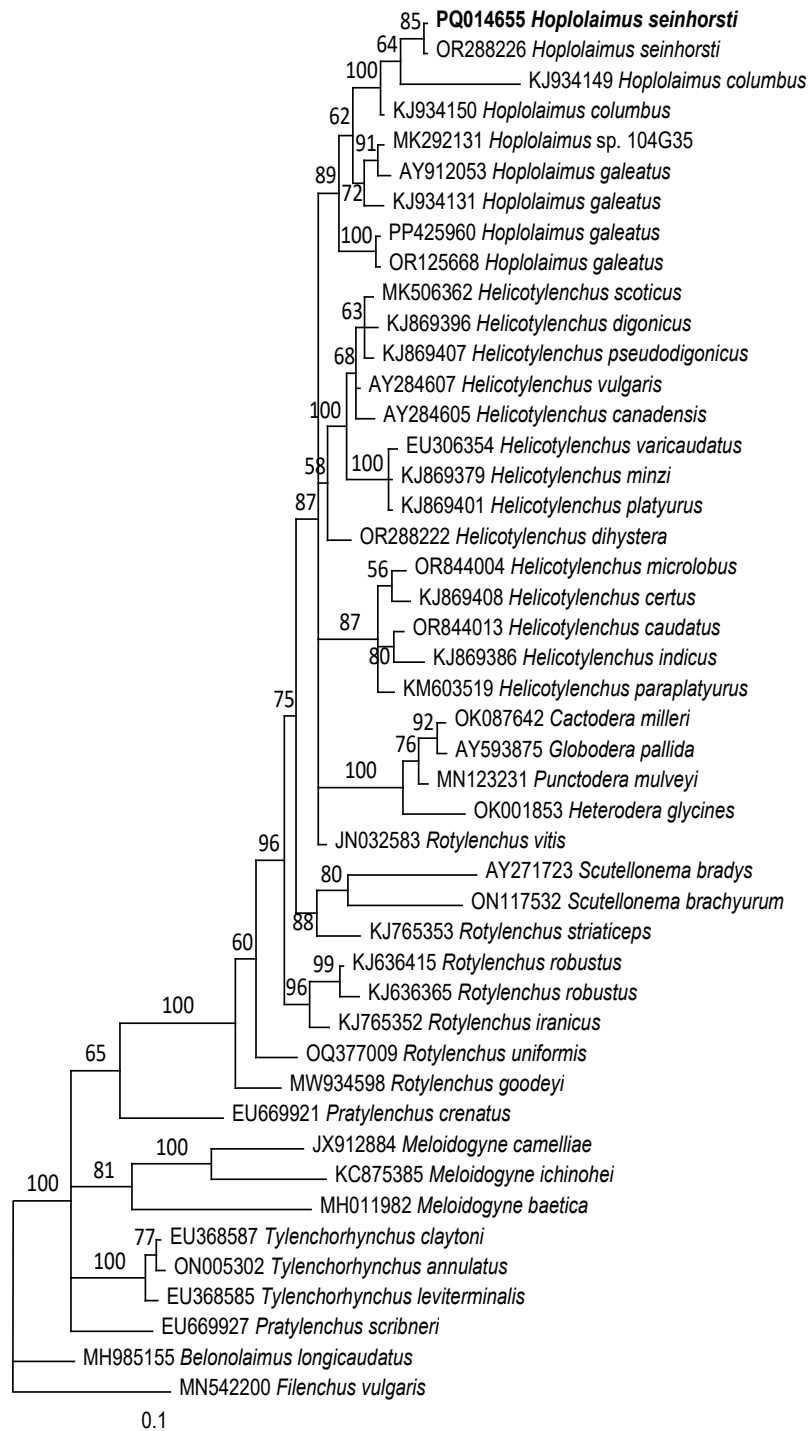


Figure 2. The 10001st Bayesian tree inferred from 18S under the TIM + I + G model (lnL = 3286.1877; freqA = 0.2145; freqC = 0.2329; freqG = 0.2841; freqT = 0.2685; R(a) = 1; R(b) = 4.6299; R(c) = 1.7344; R(d) = 1.7344; R(e) = 8.8190; R(f) = 1; Pinvar = 0.6602; Shape = 0.4282). Posterior probability values exceeding 50% are given on appropriate clades. The species in the present study is showed in bold font.

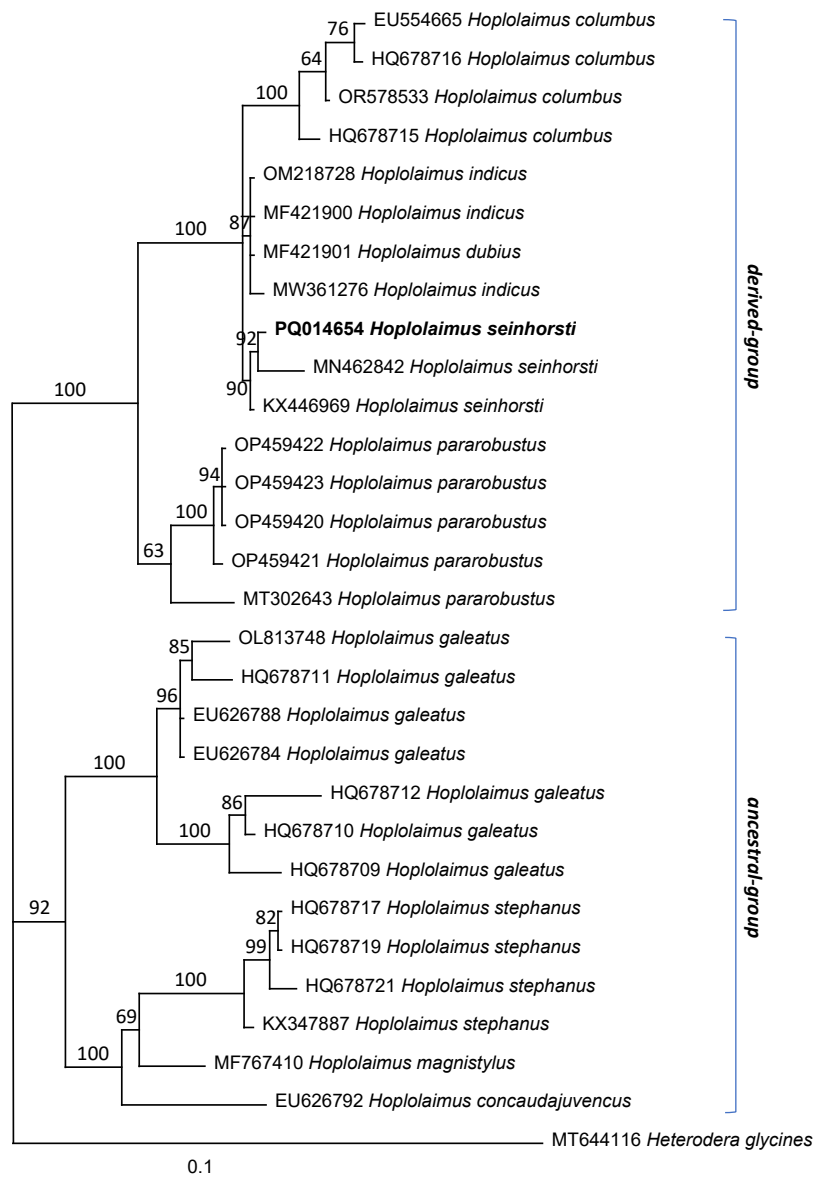


Figure 3. The 10001st Bayesian tree inferred from D2-D3 under the GTR+G model (lnL = 3046.0740; freqA = 0.2067; freqC = 0.2239; freqG = 0.3294; freqT = 0.2400; R(a) = 0.7573; R(b) = 2.0011; R(c) = 1.0206; R(d) = 0.4185; R(e) = 3.6698; R(f) = 1; Pinvar = 0; Shape = 0.5345). Posterior probability values exceeding 50% are given on appropriate clades. The species in the present study is showed in bold font.

4. Discussion

Hoplolaimus seinhorsti was reported from many crops including cotton, sugarcane, cowpea, sorghum, rice, corn, melon, many types of vegetables, coconut, and banana [7,17,28–31]. *Hoplolaimus seinhorsti* in this study was found in bermudagrass (*C. dactylon*) on a golf course in Shenzhen, China. From our perspective this is the first report of *H. seinhorsti* from turfgrass. Previous research indicated that the *Hoplolaimus* species associated with turfgrasses have four species, i.e., *H. columbus*, *H. concaudajuvencus*, *H. galeatus* and *H. stephanus* [22–25]. Of which *H. columbus* and *H. concaudajuvencus* were found in bermudagrass, *H. stephanus* was from bentgrass (*Agrostis stolonifera* L.), and *H. galeatus* was documented to occur in multiple turfgrasses such as bermudagrass as well as St. Augustine (*Sternotaphrum secundatum*), zoysiagrass (*Zoysia* spp.), and centipede grass (*Eremochloa ophiuroides*). *H. galeatus* is the most predominant species on turfgrasses, which is widely distributed and found with high prevalence on golf courses in Carolinas, USA [44], and is also considered as an economically important pest of turfgrasses [45,46]. Several previous research work have revealed a broad diversity of nematodes associated with turfgrasses in many countries in the world [41,44,47,48]. In the present study *H. seinhorsti* found on turfgrass was characterized morphologically and molecularly, which not only enriches diversity of nematode species but also aids with diagnosis of nematodes on turfgrasses.

Morphologically Fortuner [49] divided the *Hoplolaimus* into ancestral and derived groups according to several phenotypic traits including the number of gland nuclei (three vs. six nuclei), the lateral line number (four vs. less than four), the excretory pore position (below vs. above the hemizonid), and the presence of either regular or irregular striae on the basal lip annulus. *H. seinhorsti* in the present study has derived characters. Previous research indicated that *H. concaudajuvencus*, *H. galeatus*, *H. magnistylus* and *H. stephanus* have ancestral morphological characters, while *H. columbus*, *H. indicus* and *H. seinhorsti* belong to derived group [15,50]. Our phylogenetic analysis based upon 28S D2-D3 data set showed that the studied *H. seinhorsti* is clustered in a monophyletic clade with 100% pp with the other *H. seinhorsti* populations, *H. columbus*, *H. dubius*, *H. indicus* and *H. pararobustus*, while *H. concaudajuvencus*, *H. galeatus*, *H. magnistylus* and *H. stephanus* are grouped in another monophyletic clade with 92% pp (Figure 3). The phylogenetic inference based upon 18S rDNA data set also revealed the placement of the present *H. seinhorsti* which is grouped in a highly supported (100% pp) with the other *H. seinhorsti* population and *H. columbus* (Figure 2). These results agree with previous studies [15,50], suggesting that the morphological classification scheme proposed by Fortuner [49] is highly consistent with molecular phylogenetic analysis results, and is well supported by molecular data.

Thus far, *H. columbus*, *H. concaudajuvencus*, *H. galeatus* and *H. stephanus* were found on turfgrasses. However, morphologically the studied *H. seinhorsti* differs from *H. columbus* by possessing different annules of lip region (4 vs. 3), tail annules (10–15 vs. 16–22), longitudinal striations of basal annule of lip region (9–10 vs. 10–15), and digestive system morphology (intestine does not overlap rectum vs. overlap) [17], from *H. galeatus* by possessing different annules of lip region (4 vs. 5), nuclei of esophageal glands (6 vs. 3), lateral incisures (1 vs. 4), longitudinal striations of basal annule of lip region (9–10 vs. 32–36), and digestive system morphology (intestine does not overlap rectum vs. overlap) [51], from *H. stephanus* by possessing different nuclei of esophageal glands (6 vs. 3), longitudinal striations of basal annule of lip region (9–10 vs. 24–28), lateral incisures (1 vs. 4), and a shorter stylet (33.9–43.3 vs. 43.0–50.0 μm in females) [17], from *H. concaudajuvencus* by possessing different annules of lip region (4 vs. 5–6), longitudinal striations of basal annule of lip region (9–10 vs. 36), lateral incisures (1 vs. 4), nuclei of esophageal glands (6 vs. 3), and a shorter stylet (33.9–43.3 vs. 50.4–55.6 μm in females) [24]. Sequence comparative analysis of the 18S and D2-D3 of 28S rDNA, and molecular phylogenetic inference based upon D2-D3 of 28S data set supported that the studied *H. seinhorsti* is a separate species from *H. columbus*, *H. concaudajuvencus*, *H. galeatus* and *H. stephanus*.

Author Contributions

Y.Z.: Conceptualization; data curation and analysis; investigation; methodology; original draft preparation; revised and edited draft; J.A.R.: Conceptualization; data curation; methodology; supervision; revised and edited draft. Both authors have read the versions of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

All data generated or analyzed during this study are included in this published article. All data supporting the findings of this study will be made available to qualified researchers upon publication.

Conflicts of Interest

The authors declare no conflict of interest.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

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