



Article

Morphological and Molecular Characterization of *Nothotylenchus medians* and *N. similis* (Nematoda: Anguinidae) from Southern Alberta, Canada

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Abstract: The nematode family, Anguinidae, is a diverse group of polyphagous nematodes, generally known as fungal feeders or parasites of aerial plant parts. Here, we present the morphological and molecular characterization of adult females of two *Nothotylenchus* species, *N. medians* and *N. similis*, along with host association and geographical distribution data of the genus. Both species are recorded as new reports from Canada and designated as reference populations for future studies. Morphological or morphometrical variation was not observed in the Canadian population of *N. medians* and *N. similis*, in comparison with the original description. Phylogenetic analyses based on 18S and D2–D3 of 28S genes placed both species within Anguinidae. Since the biology of the genus *Nothotylenchus* has not been rigorously characterized, the habitat and distribution information presented in this study will shed some light on the ecology of these nematodes. Notably, the detection of *N. medians* and *N. similis* in our nematode inventory survey indicates that considerable *Nothotylenchus* diversity is hidden in these soils. Consequently, increased surveys and more in-depth research are needed to explore the full diversity of anguinids inhabiting these cultivated areas.

Keywords: morphology; morphometrics; nematode management programs; new record; soil health; plant-parasitic nematodes; taxonomy



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1. Introduction

Family Anguinidae Nicoll [1] is a diverse group of stylet-bearing nematodes generally known as fungal feeders or parasites of the aerial parts of plants [2]. The family contains about 15 genera, however only *Ditylenchus* Filipjev [3] and *Anguina* Scopoli [4] species have received significant attention due to their parasitic potential and quarantine regulations [5]. Among other genera of Anguinidae, *Nothotylenchus* Thorne [6] was formulated for those *Ditylenchus*-like species that do not possess a valvular median pharyngeal bulb [2,6]. Based on similarity, *Nothotylenchus* has been synonymized with *Ditylenchus* in several studies [7–9]. According to the new classification system described by Decraemer and Hunt [5], *Nothotylenchus* is regarded as a valid genus. Recently, Hashemi and Karegar [10] reviewed the entire genus and improved the species identification by providing a compendium of *Nothotylenchus* nominal species.

The genus *Nothotylenchus* is composed of ectoparasitic nematodes that have adapted to a diverse range of ecological niches [2,11–13]. This genus is the second-largest of the family Anguinidae after *Ditylenchus*, and currently comprises 41 nominal species [10,14]. These species have been reported from both agricultural and natural systems, but no significant plant damage has been associated with any *Nothotylenchus* spp., except for *N. acris*, which was reported to cause stunting and deformation of strawberry plants [15]. Biogeographically, the majority of *Nothotylenchus* species originate from Asian countries,

whereas only *N. acris* Thorne, [6], *N. acutus* Khan, [16], and *N. attenuatus* Mulvey [17] are known to occur in Canada [17–22].

Recently, several studies reported the presence of endo- and ectoparasitic nematodes from agricultural areas of southern Alberta, Canada [23–27]. Therefore, we performed detailed samplings in cultivated areas near Barnwell in southern Alberta, with the goal of characterizing the existing biodiversity of plant-parasitic nematodes (PPNs). In our recent nematode biodiversity survey, we detected high densities (~50–60 individuals in 250 g fresh soil) of two anguinid species. Given the potential economic importance of this group, the species were immediately processed for morphological and molecular characterization. Preliminary light microscopy analysis revealed the presence of lateral fields with six lateral lines, flattened lip regions, delicate stylets, and a fusiform median pharyngeal bulb without valves. Based on morphological characteristics, the species were identified as members of *Nothotylenchus*. Further, detailed morphometrical characterization of these populations confirmed their close resemblance to *N. medians* Thorne and Malek [28] and *N. similis* Thorne and Malek [28]. Since *Nothotylenchus* spp. have never been detected in southern Alberta field surveys, the present study was conducted with the main objectives of: (1) morphometrical and molecular characterization of *N. medians* and *N. similis* as newly reported species from Canada, and (2) to establish detailed molecular phylogeny, host association, and biogeography of the genus *Nothotylenchus*.

In practice, it is almost impossible to identify all nematode species inhabiting each soil sample. Having advanced knowledge of existing nematode biodiversity will aid researchers in rapid nematode diagnostics. Moreover, recognition and accurate identification of recovered species are important to detect nematodes that may pose a potential threat and implement effective regulatory measures.

2. Materials and Methods

2.1. Isolation and Morphological Studies

The nematode inventory survey was conducted near village of Barnwell (Alberta, Canada) to examine the wide spectrum of PPNs associated with cultivated plants. We collected several soil and root samples from different headland areas of a cultivated field, and maintained them in the cold storage (4 °C) at the University of Lethbridge (Alberta, Canada) until processing. Nematodes were extracted from the soil samples using the modified Cobb sieving and flotation-centrifugation method [29]. Individual anguinid taxa were collected from the mixture of soil nematodes and assigned the population numbers 42A and 42B. For preliminary examination, fresh adults of each species were transferred to a drop of distilled water, heat relaxed, and observed under a Zeiss Axioskope 40 microscope. For morphometric studies, the nematodes were fixed, and permanent slides were prepared according to the methods of Seinhorst [30] and De Grisse [31]. Images of each specimen were acquired using a Zeiss Axioskope 40 microscope equipped with a Zeiss AxioCam 208 camera (Carl Zeiss, Jena, Germany). Measurements from the images were performed using ZEN blue 3.1 imaging software (Carl Zeiss).

2.2. DNA Extraction, PCR, and Sequencing

After preliminary microscopic examination, a single nematode of each taxon was transferred to a 0.2 mL PCR tube, and the DNA was extracted as described in Maria et al. [32]. Briefly, the single nematodes were crushed in 6 µL Milli-Q ultrapure water using a sterile pipette tip. The tubes containing crushed nematodes were briefly spun and immediately incubated at –80 °C for at least 30 min. Then, the samples were heated to 85 °C for 2 min, briefly spun, followed by the addition of 2 µL proteinase K and 2 µL 10× PCR buffer. The tubes were incubated at 56 °C for 2 to 3 h, followed by a 10-min period at 95 °C. These tubes, containing crude nematode DNA, were cooled to 4 °C and used in the subsequent PCR analyses. Three sets of DNA primers (Integrated DNA Technologies, Coralville, IA, USA) were used to amplify the 18S, 28S, and ITS1 of the ribosomal RNA (rRNA) genes. The partial 18S rRNA gene was amplified with 1813F and 2646R primers [33]. The 28S rRNA

gene was amplified using D2A and D3B primers [34] and the ITS1 gene was amplified using F194 [35] and AB28-R primers [36]. For the 18S, 28S, and ITS1 genes, the PCR conditions were as described in Holterman et al. [33], De Ley et al. [34], and Ferris et al. [35], respectively. Amplified PCR products were resolved by electrophoresis in 1% agarose gels and visualized by staining with GelRed (Biotium, Fremont, CA, USA). PCR products containing amplified DNA fragments of interest were sent to Azenta Life Sciences for DNA sequencing (South Plainfield, NJ, USA).

2.3. Phylogenetic Analyses

In the present study, we obtained DNA sequences for the 28S rRNA (D2–D3 domains), ITS1 rRNA, and 18S rRNA genes of both *Nothotylenchus* populations. These sequences and additional anguinid taxa DNA sequences present in GenBank were used for phylogenetic analysis. The selection of outgroup taxa for each dataset was based on previously published studies [13,14,37]. Multiple nucleotide sequence alignments for the different genes were performed using the heuristics progressive method FFT-NS-2 algorithm of MAFFT v.7.450 [38]. The BioEdit v7.2.5 program [39] was used for sequence alignment visualization. For alignment editing, we used Gblocks v0.91b [40] on the Castresana Laboratory server (available online: http://molevol.cmima.csic.es/castresana/Gblocks_server.html (accessed on 10 November 2021) with options for a less stringent selection (minimum number of sequences for a conserved or a flanking position: 50% of the number of sequences +1; maximum number of contiguous non-conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half)). Phylogenetic analyses were performed using Bayesian inference (BI) in MrBayes v3.1.2 [41]. The best-fit model of DNA evolution was achieved using JModelTest v2.1.7 [42] with the Akaike information criterion (AIC). Accordingly, the selected models were the general time-reversible model with invariable sites and a gamma-shaped distribution (GTR + I + G), and GTR + G for the D2–D3 segments of the 28S rRNA and partial 18S, respectively. The best-fit model, base frequency, proportion of invariable sites, gamma distribution shape parameters, and substitution rates in the AIC were then used in MrBayes for the phylogenetic analyses, which run with four chains for 4×10^6 generations in all datasets. A combined analysis of the three ribosomal genes was not undertaken due to several sequences not being available for all species. The sampling for Markov chains was carried out at intervals of 100 generations. For each analysis, two runs were conducted. After discarding 30% of the samples for burn-in and evaluating convergence, the remaining samples were retained for more in-depth analyses. The topologies were used to generate a 50% majority-rule consensus tree. On each appropriate clade, posterior probabilities (PP) were calculated. FigTree software v1.42 [43] was used for the visualization of phylogenetic trees from all analyses.

3. Results

3.1. Description of *Nothotylenchus medians*

Female: Body ventrally arcuate after heat relaxed, general appearance cylindroid except at extremities (Figure 1). Cuticle finely annulated, lateral field with six equidistant incisures. Lip region low, anteriorly flattened, with rounded margins, continuous with the body contours, having two or three fine annuli. Labial framework slightly sclerotized, outer margin of the basal plate extending into two to three annuli inside the body. Stylet delicate, conus 35–40% of the total stylet length (Table 1). Stylet knobs small, rounded, anteriorly sloping. Dorsal pharyngeal gland orifice (DGO) situated close to the stylet knobs. Median pharyngeal bulb valveless, indiscernible, and fusiform in shape. Isthmus slender, encircled with nerve ring.

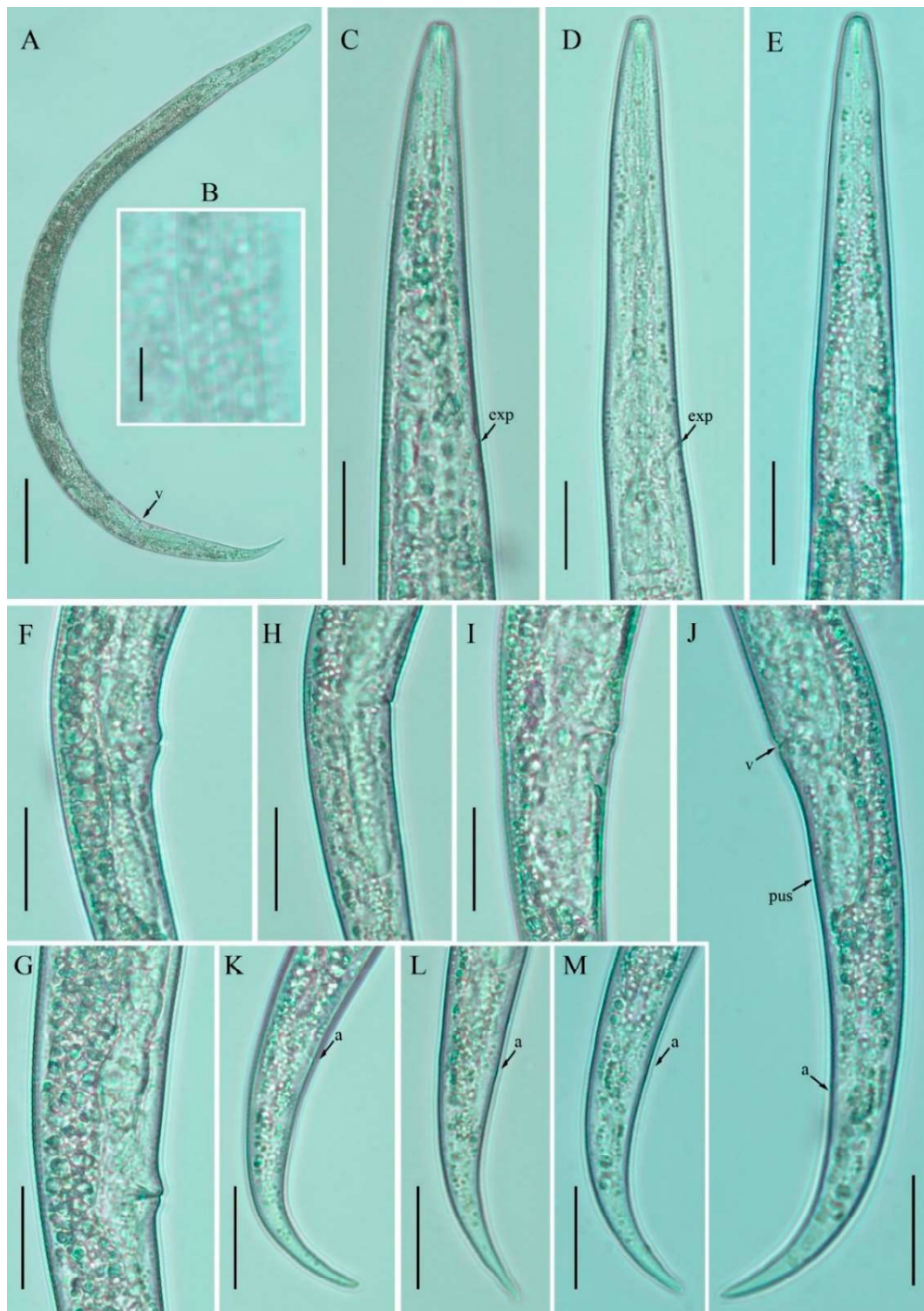


Figure 1. Photomicrographs of female *Nothotylenchus medians*. (A) Entire body; (B) lateral lines; (C–E) pharyngeal regions; (F–I) vulval regions; (J) posterior body to tail terminus; (K–M) tail regions. Scale bars: (A) 50 μm ; (C–M) 20 μm ; (B) 5 μm . Arrowheads: (a) anus; (exp) excretory pore; (pus) post-vulval uterine sac; (v) vulva.

Table 1. Morphometrics of female *Nothotylenchus medians* examined in this study and from the original and other published descriptions. All measurements are in μm and in the form: the mean \pm standard deviation and/or the range.

Characters	This Study	Thorne and Malek [28]	Brzeski [8]	
Locality	Canadian population	USA	Poland	Syria
Host	<i>Artiplex</i> sp.	Cultivated field	Unknown	
n	16	–	13	3
Body length	598.3 \pm 48.9 (536.0–740.0)	660	734 (660–803)	612–624
a	25.5 \pm 2.6 (21.3–32.2)	32	31 (25–36)	30–32
b	6.1 \pm 0.3 (5.5–6.6)	6.3	5.6 (5.0–6.0)	4.7–5.2
c	11.0 \pm 0.9 (9.4–12.0)	14	12.5 (11.4–13.2)	3.2–3.5
c'	4.6 \pm 0.4 (3.8–5.3)	2.8–5.8	4.1 (3.7–4.6)	13.8 (12.6–14.5)
MB	42.0 \pm 2.1 (35.3–45.0)	–	39 (35–42)	38–42
V	79.2 \pm 1.0 (77.1–81.0)	76	82 (79–83)	–
Lip height	2.2 \pm 0.1 (2.0–2.4)	–	–	–
Lip width	5.3 \pm 0.3 (5.0–5.8)	–	–	–
Stylet length	8.1 \pm 0.6 (7.3–9.5)	8.0	7.3 (6.5–8.0)	6.8 (6.0–7.5)
Anterior end to excretory pore	82.6 \pm 4.7 (79.0–98.0)	–	99 (88–105)	91–96
Pharynx length	98.0 \pm 5.7 (88.0–116.0)	–	131 (111–144)	124 (119–130)
Maximum body width	23.6 \pm 2.4 (19.3–28.0)	–	–	–
Vulva body width	21.4 \pm 1.9 (18.3–25.0)	–	–	–
Post-vulval uterine sac (PUS) length	30.9 \pm 6.9 (20.0–44.0)	–	–	–
Distance from vulva to anus	69.0 \pm 6.7 (60.0–88.0)	–	–	–
Distance from vulva to tail terminus	124.5 \pm 10.5 (110.0–150.0)	–	–	–
Anal body width	12.2 \pm 1.0 (10.0–13.6)	–	–	–
Tail length	56.0 \pm 5.8 (46.0–66.0)	–	59 (52–63)	42–64

Abbreviations: n, number of specimens on which the measurements are based; a, body length/greatest body diameter; b, body length/distance from anterior end to pharyngo-intestinal junction; c, body length/tail length; c', tail length/tail diameter at anus; MB, distance between the anterior end of the body and center of the median pharyngeal bulb expressed as the percentage (%) of the pharynx length; V, distance from the body anterior end to the vulva expressed as a percentage (%) of the body length.

Hemizonid streak-like two to three annuli anterior to the excretory pore. Excretory pore located slightly anterior to or in range of the anterior level of the pharyngeal basal bulb. Basal pharyngeal bulb pyriform to slightly elongated, abutting intestine. Ovary outstretched with oocytes in a single row, spermatheca and crustaformeria well developed, the columnar arrangement of crustaformeria well discernable. Spermatheca elongate, vulva a transverse slit occupying less than half of the corresponding body width. Vulval lips prominent, sometimes protruding slightly. Post-vulval uterine sac pouch-like, present along the ventral body wall extending halfway to the anus. Anus a minute slit. Tail conical tapering uniformly to a finely rounded to pointed terminus.

Male: Not found.

Juveniles: Present but not studied.

Remarks: This species was first described by Thorne and Malek [28] from cultivated fields of South Dakota, USA. Brzeski [8] described two more populations of the same species from Poland and Syria. Except for the shorter pharynx length, the morphology and morphometry of the Canadian population are consistent with the original description of *N. medians* and the subsequent populations. The populations from Poland and Syria showed longer pharynx lengths (131 (111–144) μm and 124 (119–130) μm , respectively, vs. 98.0 (88.0–116.0) μm) compared with the Canadian population. In terms of habitat, the USA and Canadian populations of *N. medians* are similar; both populations were found in cultivated areas. Specifically, the Canadian population of *N. medians* was recovered in the rhizosphere of *Artiplex* sp. growing on the headland of a cultivated potato field. The

presence of males was reported in the original description; however, we did not observe any males in the Canadian population of *N. medians*.

3.2. Description of *Nothotylenchus similis*

Female: Body C-shaped after heat relaxed, general appearance cylindroid except at extremities (Figure 2). Cuticle finely annulated, lateral field with six incisures, inner lines are weaker than the outer ones. Lip region low, anteriorly flattened, with rounded margins, continuous with the body contours having two or three fine annuli. Labial framework slightly sclerotized, outer margin of basal plate extending into two to three annuli inside the body. Stylet delicate, conus 25–30% of total stylet length (Table 2). Stylet knobs small, rounded, anteriorly sloping. Dorsal pharyngeal gland orifice (DGO) situated close to the stylet knobs. Median pharyngeal bulb valveless, fusiform in shape. Isthmus slender encircled with nerve ring. Hemizonid streak-like, two to three annuli anterior to the excretory pore. Excretory pore located at the anterior level of the pharyngeal basal bulb. Basal pharyngeal bulb cylindroid to slightly elongate abutting the intestine. Ovary outstretched with oocytes in a single row, spermatheca and crustaformeria well developed, the columnar arrangement of crustaformeria well discernable. Spermatheca elongate, irregular-shaped, vulva a transverse slit occupying less than half of the corresponding body width. Vulval lips simple, not protruding. Post-vulval uterine sac small along the ventral body wall, same size as maximum body width. Anus, a minute slit. Tail cylindrical, wider at the anal region tapering uniformly to a finely rounded to slightly pointed terminus.

Male: Not found.

Juveniles: Present but not studied.

Remarks: This species was first described by Thorne and Malek [28] from South Dakota, USA, in the rhizosphere of pasture grass. Here, we found *N. similis* in the rhizosphere of undetermined grasses growing on the headland of a cultivated potato field. After the formal description, this species was never reported outside its type locality. The general appearance and dimensions of the Canadian population of *N. similis* are consistent with the original description. Both the USA and Canadian populations of *N. similis* have been found from grasses, indicating their host preference in the family *Poaceae* Barnhart. The authors of the original description likely provided the measurements of holotype female, which is why we cannot determine intraspecific variation range. Consequently, we refer to the Canadian population of *N. similis* as the reference population for future studies, until topotypes of this species can be sequenced. Moreover, the male was described in the original description; however, we did not observe any male in the Canadian population of *N. similis*.

3.3. Host Association and Distribution of the Species in Genus *Nothotylenchus*

Genus *Nothotylenchus* is recognized as the second-largest genus of the family Anguinidae with 41 nominal species [2,10]. The majority of *Nothotylenchus* species were described decades ago, with some original descriptions in languages other than English and often difficult to access. To study the host associations and distribution of *Nothotylenchus* species, we collected all original descriptions from web resources and through personal communication. Through detailed literature review, we found that the food preference of *Nothotylenchus* species is very diverse; the species were either reported from the rhizosphere of cultivated crops (alfalfa, rice), vegetables (cabbage, eggplant, pea, potato, and sugar beet), fruit trees (apple, citrus, grapevine, mango, palm, and pear), grasses (bermudagrass, *Sesleria* sp., and turfgrasses), and some very unusual hosts (mosses, dead and decaying wood, insect frass, periphytons, and deciduous shrubs; Table 3). Regarding distribution, of 41 nominal species 28 were reported from Asian countries (e.g., India (14 spp.), Iran (8 spp.), Russia (1 sp.), Japan (1 sp.), and Pakistan (1 sp.)), 8 from USA, and 10 from European countries including Russian regions (Table 3). Since the feeding preference and biology of *Nothotylenchus* species are poorly understood, here we present a brief review of host

associations and their place of discovery with the hope that this information will be useful to shed some light on the biology and biogeography of *Nothotylenchus* species.

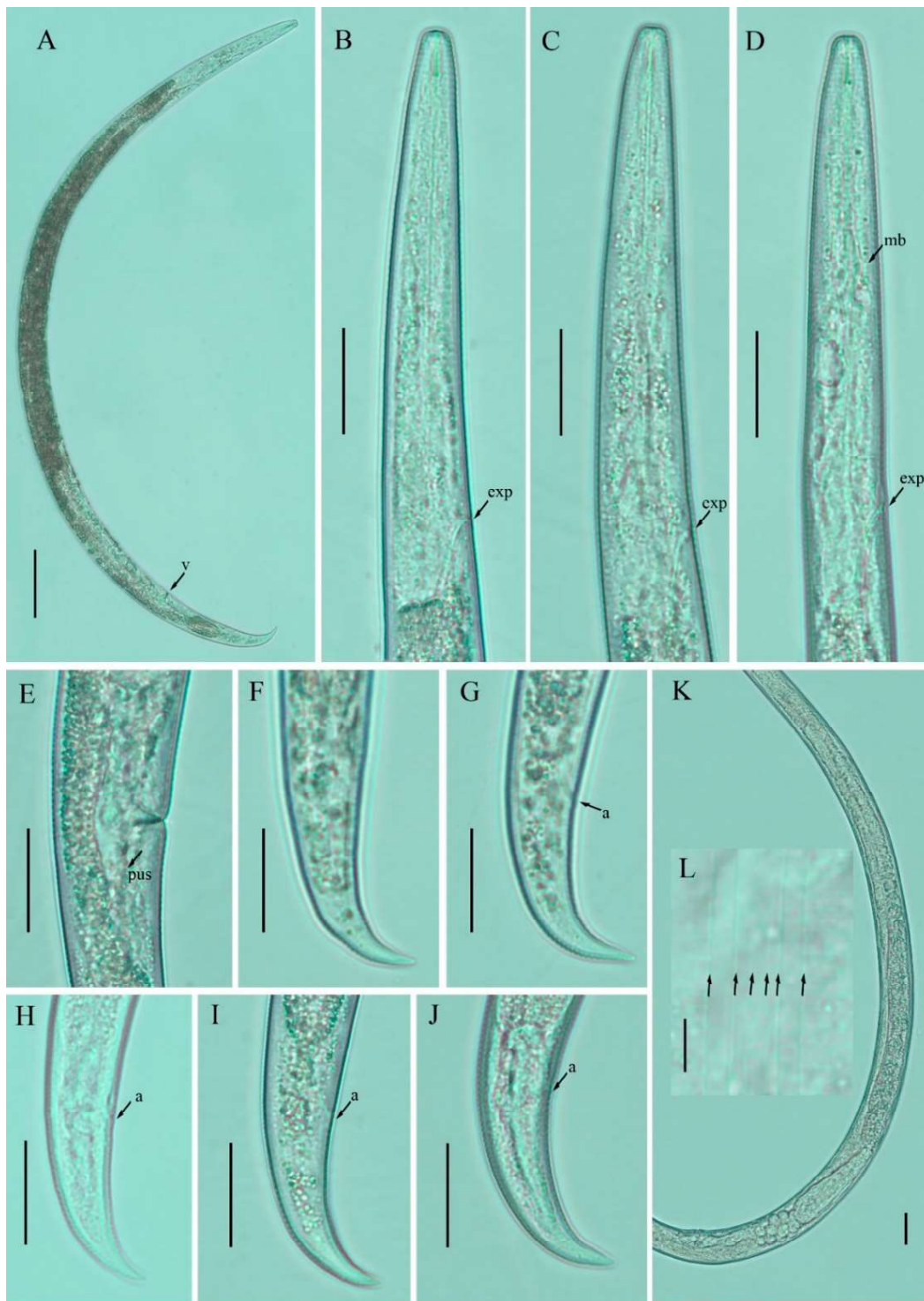


Figure 2. Photomicrographs of *Nothotylenchus similis* female. (A) Entire body; (B–D) pharyngeal regions; (E) vulval region; (F–J) tail regions; (K) posterior body to tail terminus; (L) lateral lines (arrowheads indicating the number of lines). Scale bars: (A) 50 μm ; (B–K) 20 μm ; (L) 5 μm . Arrowheads: (a) anus; (exp) excretory pore; (mb) median bulb; (pus) post-vulval uterine sac; (v) vulva.

Table 2. Morphometrics of female *Nothotylenchus similis* from Canada and from the original description. All measurements are in μm and in the form: the mean \pm standard deviation and/or the range.

Characters	Canadian Population	Thorne and Malek [28]
Host	Grasses	Pasture grass
n	16	–
Body length	783.0 \pm 48.5 (693.0–885.0)	900
a	30.0 \pm 3.4 (25.0–38.4)	24
b	6.4 \pm 0.4 (5.8–7.3)	4.3
c	16.5 \pm 1.1 (14.5–18.5)	17
c'	2.9 \pm 0.2 (2.4–3.3)	2.9
MB	43.0 \pm 2.0 (38.5–46.4)	–
V	84.1 \pm 0.9 (82.5–85.5)	84
Lip height	2.7 \pm 0.2 (2.3–2.9)	–
Lip width	6.9 \pm 0.5 (6.4–7.5)	–
Stylet length	9.8 \pm 0.5 (8.6–10.6)	10
Anterior end to excretory pore	108.1 \pm 5.5 (100.0–116.0)	–
Pharynx length	123.1 \pm 7.0 (112.0–136.0)	–
Maximum body width	26.4 \pm 2.8 (22.0–31.0)	–
Vulva body width	23.3 \pm 2.4 (20.0–27.0)	–
Post-vulval uterine sac (PUS) length	25.7 \pm 2.7 (21.0–31.0)	–
Distance from vulva to anus	77.0 \pm 6.1 (70.0–88.0)	–
Distance from vulva to tail terminus	124.6 \pm 6.9 (115.0–137.0)	–
Anal body width	16.2 \pm 0.9 (15.0–17.6)	–
Tail length	47.6 \pm 2.9 (40.0–52.0)	–

Abbreviations: n, number of specimens on which measurements are based; a, body length/greatest body diameter; b, body length/distance from anterior end to pharyngo-intestinal junction; c, body length/tail length; c', tail length/tail diameter at anus; MB, distance between the anterior end of the body and center of the median pharyngeal bulb expressed as percentage (%) of the pharynx length; V, distance from the body anterior end to the vulva expressed as a percentage (%) of the body length.

Table 3. Host association and distribution of nominal *Nothotylenchus* species.

Species Name	Host	Location	Reference	
1	<i>N. acris</i>	Rhizosphere of <i>Alfalfa</i> crowns, red clover roots, and sugar beets.	USA	Thorne [6]
		Native prairie sod.	USA	Thorne and Malek [28]
		Grass roots growing in a dry pond bed.	Canada	Mulvey [17]
2	<i>N. acutus</i>	Rhizosphere of deciduous shrub (<i>Plumeria acutifolia</i>).	India	Khan [16]
		Rhizosphere of wild hay.	Canada	Mulvey [17]
		Cultivated sandy soil.	Poland	Brzeski [8]
		Roots around dwarf shrub (<i>Thuja orientalis</i> L.).	Korea	Lee et al. [44]
3	<i>N. adasi</i>	Loamy sand around roots of Sugar beet (<i>Beta vulgaris</i>).	England	Sykes [45]
		Wet sandy low peat soil.	Poland	Brzeski [8]
		Pulse fields.	Iran	Ahmadi et al. [46]
4	<i>N. affinis</i>	Dying <i>Alfalfa</i> crowns.	USA	Thorne [6]
		–	Iran	Kheiri [18]
5	<i>N. andrassy</i>	Moss samples (<i>Sphagnum</i> sp.).	Iran	Jalalinasab, Nassaj-Hosseini, and Heydari [14]
6	<i>N. antricolus</i>	Wet and rotten pieces of wood, that wood was found in the Kólyok cave located in Mánfa Southern Hungary.	Hungary	Andrássy [47]
7	<i>N. attenuatus</i>	Marshy area, wet grass, and moss, near Skeleton Lake.	Canada	Mulvey [17]
8	<i>N. basiri</i>	The soil around the roots of the mango tree (<i>Mangifera indica</i> L.).	India	Khan [16]

Table 3. Cont.

	Species Name	Host	Location	Reference
9	<i>N. brzeskii</i>	Rhizosphere of <i>Alfafa</i> sp.	Iran	Hashemi and Karegar [10]
10	<i>N. bhatnagari</i>	Rhizosphere of sorghum (<i>Sorghum vulgare</i>).	India	Tikyani and Khera [48]
11	<i>N. bhattii</i>	Rhizosphere of Bermuda grass (<i>Cynodon dactylon</i> L.).	India	(Das and Bajaj) Hashemi and Karegar [10,21]
12	<i>N. boroki</i>	Pine and Birchwood dead and rotten material.	Central Russia	Gagarin [12]
13	<i>N. citri</i>	Rhizosphere of <i>Citrus</i> sp.	India	(Varaprasad, Khan, and Lal) Siddiqi [49,50]
14	<i>N. clavatus</i>	Roots of paddy (<i>Oryza sativa</i> L.).	India	Dhanachand and Gambhir [51]
15	<i>N. cylindricollis</i>	Soil and organic debris at the base of <i>Ananas</i> sp.	Paraguay	Thorne [6]
16	<i>N. cylindricus</i>	Rhizosphere of cabbage (<i>Brassica oleracea</i> L.) and potato (<i>Solanum tuberosum</i> L.).	India	Khan and Siddiqi [52]
17	<i>N. danubialis</i>	<i>Periphyton</i> found in the Danube River.	Hungary	Andrássy [53]
18	<i>N. drymocolus</i>	Isolated from decaying organic matter of bark beetles (<i>Orthotomicus suturalis</i> ; <i>Hylaster ater</i> , <i>H. attenuatus</i>) residing in spruce and pine trees, respectively.	Germany	Rühm [54]
19	<i>N. fotedari</i>	Fallow field soil, previously cultivated with Mung Bean (<i>Phaseolus aureus</i>).	India	Mahajan [19]
20	<i>N. geraerti</i>	Rhizosphere of potato (<i>Solanum tuberosum</i> L.) and wheat (<i>Triticum aestivum</i> L.).	Iran	Kheiri [18]
21	<i>N. goldeni</i>	–	Iran	Hashemi and Karegar [10]
22	<i>N. hexaglyphus</i>	Rhizosphere of apple (<i>Pyrus malus</i>).	Pakistan	Maqbool [20]
		Rhizosphere of potato (<i>Solanum tuberosum</i> L.).	India	Khan and Siddiqi [52]
		Soil near a water canal.	Poland	Brzeski [8]
		Rhizosphere of palm.	Iran	Esmaeili and Heydari [22]
23	<i>N. loksai</i>	–	Iran	Hashemi and Karegar [10]
24	<i>N. medians</i>	Grass rhizosphere (<i>Sesleria</i> sp.).	Romania	Andrássy [55]
		Cultivated field.	USA	Thorne and Malek [28]
		<i>Artiplax</i> sp.	Poland, Syria	Brzeski [8]
25	<i>N. oryzae</i>	Roots of paddy (<i>Oryza sativa</i> L.).	Canada	This study
26	<i>N. persicus</i>	Rhizosphere of grapevine (<i>Vitis vinifera</i> sp.).	India	(Mathur, Khan, and Prasad) Siddiqi [50,56]
27	<i>N. petilus</i>	Associated with <i>Dendroctomis terehrans</i> in loblolly pine. (<i>Pinus taeda</i> L.).	Iran	Esmaeili, Heydari, Castillo, and Palomares-Rius [37]
28	<i>N. phoenixae</i>	Rhizosphere of palm trees (<i>Phoenix dactylifera</i> L.).	USA	Massey [11]
29	<i>N. siddiqii</i>	Rhizosphere of <i>Alfalfa</i> .	Iran	Esmaeili, Heydari, and Ye [13]
30	<i>N. similis</i>	Nature pasture grass.	Iran	Hashemi and Karegar [10]
		Grasses	USA	Thorne and Malek [28]
			Canada	This study
31	<i>N. singhi</i>	Rhizosphere of Cabbage (<i>Brassica olearacea</i> L.).	India	Das and Shivaswamy [57]
32	<i>N. solani</i>	Rhizosphere of eggplant (<i>Solanum melongena</i> L.).	India	(Varaprasad, Khan, and Lal) Siddiqi [49,50]
33	<i>N. taylori</i>	Rhizosphere of potato (<i>Solanum tuberosum</i> L.).	India	Husain and Khan [58]
		Rhizosphere of <i>Eucalyptus</i> sp.	Sudan	Zeidan and Geraert [59]
34	<i>N. tenuis</i>	Pinewood dead and rotten material.	Central Russia	Gagarin [12]
35	<i>N. thornei</i>	Moss collected from the forest.	Germany	Andrássy [60]

Table 3. Cont.

	Species Name	Host	Location	Reference
36	<i>N. truncatus</i>	Rhizosphere of apple and pear trees.	Georgia	Eliashvili and Vacheishvili [61]
37	<i>N. tuberosus</i>	Rhizosphere of potatoes (<i>Solanum tuberosum</i> L.).	Iran	Kheiri [18]
38	<i>N. turfus</i>	Rhizosphere of grasses.	Japan	(Yokoo) Siddiqi [50,62]
39	<i>N. uniformis</i>	Thin branches of a large tree.	Far Eastern Russia	Truskova and Eroshenko [63]
40	<i>N. utschini</i>	Wild rice (<i>Zizania latifolia</i> Griseb) growing on the bank of a water reservoir.	Central Russia	Gagarin [64]
41	<i>N. websteri</i>	Rhizosphere of pea (<i>Pisum sativum</i> L.).	India	Kumar [65]

3.4. Molecular Characterization and Phylogenetic Relationships of *Nothotylenchus medians* and *Nothotylenchus similis* with Related Anguinid Species

We used partial 18S, 28S, and ITS1 sequencing to characterize the two anguinid species recovered in this study. The DNA sequence fragment lengths of D2–D3 of 28S and ITS1 genes for *N. medians* are 718 bp and 743 bp, respectively, whereas the sequence fragment lengths of 18S, D2–D3 of 28S and ITS1 genes for *N. similis* are 549 bp, 435–625 bp and 743 bp, respectively. The newly obtained sequences were submitted to NCBI under the following accession numbers: partial 18S (OL622072 for *N. similis*); D2–D3 of 28S (OL622064–OL622066 for *N. medians*; and OL622061–OL622063 for *N. similis*); ITS1 (OL622071 for *N. medians*; OL622068–OL622070 for *N. similis*).

For *Nothotylenchus* species very limited DNA sequence-based information is available in NCBI (10 sequences only). Therefore, the 18S and D2–D3 of the 28S trees were constructed with the closest anguinid species sequences obtained through a BlastN search. Figure 3 presents an 18S Bayesian phylogenetic tree constructed with the most highly matched anguinid species and the sequence of *N. similis*. Here, the Canadian population of *N. similis* grouped with *N. adasi*, *Anguina pacificae* Cid del Prado Vera and Maggenti [66], and an unidentified *Ditylenchus* sp. from the Netherlands. This clade is further grouped with *D. gigas* Vovlas, Troccoli, Palomares-Rius, De Luca, Liébanas, Landa, Subbotin, and Castillo [67], *Subanguina radicularis* (Greeff) Paramonov [68,69], *A. agrostis* (Steinbuch) Filipjev [3,70], and *A. tritici*. (Steinbuch) Chitwood [70,71]. The sequence identity of *N. similis* with aforementioned species is 96–99% with 7–23 nucleotide difference. The other two *Nothotylenchus* species, namely *N. andrassy* and *N. phoenixae*, occupied the middle position in the tree. Another unidentified *Nothotylenchus* sp. from Iran [72] grouped further away from known *Nothotylenchus* species.

Figure 4 presents D2–D3 of 28S Bayesian phylogenetic tree constructed with the highly matched anguinid species and the sequences of the Canadian populations of both *Nothotylenchus* species. In this tree, *N. medians* and *N. similis* formed a clade with a few species of *Ditylenchus* and an identified Tylenchidae sp.1 HMM2018 from Mexico City [73]. This clade further shares a branch with *N. persicus* and *N. phoenixae*. The sequence identity of *N. medians* with related *Ditylenchus* and *Nothotylenchus* species is 83–97% with 23–140 nucleotide difference. Whereas the sequence identity of *N. similis* with related *Ditylenchus* and *Nothotylenchus* species is 80–92% with 22–129 nucleotide difference. The ITS phylogenetic tree was not carried out because of the low sequence identity and poor coverage with other anguinids in the NCBI database. However, to highlight the relevance of these sequences, we searched the BlastN homology tool for the *N. medians* ITS sequence, which found *Ditylenchus persicus* and *D. askenasyi* to have 88–89% identity and 47–95% sequence coverage. Similarly, the BlastN search for the *N. similis* ITS sequence found several populations of *Ditylenchus destructor* to have 85% identity and 50% sequence coverage.

The results of both phylogenetic trees support the inclusion of *Nothotylenchus* species in the family Anguinidae and indicate that *Nothotylenchus* species are not monophyletic. However, at this point, the phylogenetic relationships of *Nothotylenchus* species are unresolved due to the lack of enough available molecular data.

4. Discussion

Understanding the existing biodiversity of PPNs is important because of their wide range of survival adaptations and dispersal routes [24,25,27]. In our prior nematode inventory surveys, *Nothotylenchus* species were never detected because some field areas (headland vegetation) had not been surveyed for nematode infestation. The detection of *Nothotylenchus* species in the headland areas of cultivated fields indicated that weeds or field vegetation on headlands are potential reservoirs and sources of harboring PPNs.

Our genus-wide literature review highlighted that *Nothotylenchus* species occur in a wide variety of habitats, mostly in the rhizospheres of plants and less frequently in mosses, periphyton, and decaying wood materials, indicating that the species in this genus are quite generalized and could survive in a multitude of environments and plant hosts. Unlike the closest relative, *Ditylenchus*, *Nothotylenchus* species do not have a cosmopolitan distribution; so far, only *N. acris*, *N. acutus*, *N. affinis*, *N. adasi*, *N. hexaglyphus*, *N. medians*, and *N. similis* have been found outside of their type locality [8,10,17,18,28].

The majority of *Nothotylenchus* species are amphimictic (i.e., male and female are required for reproduction); however, there are 8 species (*N. antricolus*, *N. attenuatus*, *N. clavatus*, *N. hexaglyphus*, *N. loksai*, *N. thornei*, *N. truncatus*, and *N. tuberosus*) that were described without males. Among these species, some were reported with empty spermathecae, and some with or without sperms. It appears that *Nothotylenchus* males are important in reproduction; however, during our survey, no *Nothotylenchus* males were encountered in the examined collections, suggesting that they are not very common for these species.

Integrative taxonomic identification covering morphological and molecular aspects of a species offers adequate data to properly assign species identification to the relevant taxa [74]. Regarding *Nothotylenchus*, only recently described species were characterized with molecular markers. Of 41 nominal species, the molecular characterization is only available for *N. andrassyi*, *N. persicus*, and *N. phoenixae*. The lack of molecular information prohibited us from constructing a more complete and conclusive phylogenetic relationship of *Nothotylenchus* species. Our findings are in line with several researchers who reported that insufficient molecular data is the limiting factor in studying *Nothotylenchus* species' relationships with each other and with related anguinids [13,14,37]. Based on the available phylogeny results, it may be concluded that *Nothotylenchus* species are composed of divergent lineages. This has been reflected in their feeding habits as well, e.g., *N. antricolus* was found in the rotten pieces of decaying wood in a cave [47], *N. boroki* and *N. tenuis*, in the deadwood materials of pine and Birch [12], and *N. drymocolus* and *N. petilus*, in insect frass [11,54]. Considering this, we anticipate that a genus-wide phylogenetic analysis would shed more light on the ecology and biology of these nematodes.

Taxonomic records presently list *N. acris*, *N. acutus*, and *N. attenuatus* from Canada, indicating there are gaps in our knowledge of this complex group that ought to be studied in more detail [17]. The discovery of *N. medians* and *N. similis* from cultivated areas in southern Alberta illustrate that considerable anguinid diversity is hidden within these soils. Consequently, increased surveys and more in-depth research are needed in order to further our understanding of the full diversity of anguinids inhabiting Canada's cultivated soils.

5. Conclusions

Our study aimed to update and summarize the current state of knowledge regarding the distribution and host associations of *Nothotylenchus* species. Understanding the occurrence and distribution of plant-parasitic nematodes is crucial—some are economical pests, and some are important to soil function. In the present study, we describe two anguinid species, namely *N. medians* and *N. similis*, as the first such records from Canada.

The presence of *Nothotylenchus* species in our cultivated areas does not indicate that yield losses are inevitable—not all nematode species are destined to harm or significantly reduce crop production. However, we assert that the recognition and accurate identification of detected species are important to assess if these nematodes can pose any potential threat in the future and to predict if these species may eventually require appropriate control strategies and regulatory measures. Importantly, we found that the headland areas of cultivated fields are the main sources of nematode infestation. Finally, our discovery of PPNs hitherto unreported from a cultivated region indicates that exploration of the full diversity of inhabiting anguinids warrants increased research attention.

Accession numbers obtained in this study: *Nothotylenchus medians*; D2–D3 of 28S, OL622064–OL622066 and ITS1, OL622071. *Nothotylenchus similis*: partial 18S, OL622072; D2–D3 of 28S, OL622061–OL622063; ITS1 OL622068–OL622070.

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