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Beech leaf disease symptoms caused by newly recognized nematode subspecies *Litylenchus crenatae mccannii* (Anguinata) described from *Fagus grandifolia* in North America

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Abstract

Symptoms of beech leaf disease (BLD), first reported in Ohio in 2012, include interveinal greening, thickening and often chlorosis in leaves, canopy thinning and mortality. Nematodes from diseased leaves of American beech (*Fagus grandifolia*) sent by the Ohio Department of Agriculture to the USDA, Beltsville, MD in autumn 2017 were identified as the first recorded North American population of *Litylenchus crenatae* (*Nematology*, 21, 2019, 5), originally described from Japan. This and other populations from Ohio, Pennsylvania and the neighbouring province of Ontario, Canada showed some differences in morphometric averages among females compared to the Japanese population. Ribosomal DNA marker sequences were nearly identical to the population from Japan. A sequence for the COI marker was also generated, although it was not available from the Japanese population. The nematode was not encountered in *Fagus crenata* (its host in Japan) living among nematode-infested *Fagus grandifolia* in the Holden Arboretum, nor has *L. crenatae* been reported in American beech in Japan. The morphological and host range differences in North American populations are nomenclaturally distinguished as *L. crenatae mccannii* ssp. n. from the population in Japan. Low-temperature scanning electron microscopy (LT-SEM) demonstrated five lip annules and a highly flexible cuticle. Females, juveniles and eggs were imaged within buds with a Hirox Digital microscope and an LT-SEM. Nematodes swarmed to the tips of freshly cut beech buds, but explants could not be maintained. Inoculation of fresh nematodes from infested leaves or buds to buds or leaves of *F. grandifolia* seedlings resulted in BLD leaf symptoms. Injuring dormant buds prior to nematode application, in fall or spring, promoted the most reliable symptom expression. The biogeography and physiology of anguinid nematode leaf galling, and potential co-factors and transmission are discussed.

KEYWORDS

Anguinidae, digital and scanning electron microscopy, foliar nematode, host range, molecular identification, morphometrics, new continent detection, new subspecies, symptom transmission, taxonomy

1 | INTRODUCTION

Symptoms of beach leaf disease (BLD) were first detected in northern Ohio, USA in 2012 and have since been found in northern Pennsylvania, New York, Ontario, Canada (Ewing, Hausman, Pogacnik, Slot, & Bonello, 2018) and Connecticut. Some American beech trees, *Fagus grandifolia* Ehrh. and European beech trees, *Fagus sylvatica* L. from Perry, Ohio showed symptoms of disease in the summer of 2017. Specimens were sent to the Nematology Department at Ohio State University, and the Ohio Department of Agriculture, Reynoldsburg, Ohio. The beech leaf disease symptoms included interveinal darkening, some puckering, crinkling and irregularly thickened leaves (Figure 1). Mature forest trees exhibited thinned crowns and branch dieback. The interveinal darkening was similar to eriophyid mite damage, but those bud mites were not noticed in association with infested leaves. Nearly angular leaf spots, upon close inspection with a stereo-microscope, showed very small angular lesions within the darkened areas. The appearance was similar to foliar nematode damage that appears as larger angular lesions. BLD is associated with tree mortality within 7 years of detection.

Morphologically, these nematodess had a relatively large, slender body length, short stylet length, more posterior vulva and higher c′ value (tail length/anal body width) than most *Aphelenchoides* (Shahina, 1996) and *Bursaphelenchus taphrorychi* (Tomalak, Malewski, Gu, & Qiang, 2017) from European beech. Unlike *Aphelenchoides* or *Bursaphelenchus*, females also had a small, narrow, weak median bulb and 6 lateral incisures. Upon further inspection, it was determined to be the first population of *Litylenchus crenatae* Kanzaki et al. (2019) from the western hemisphere, herein designated a new subspecies. Other populations from Ohio, Pennsylvania and Ontario, Canada were also characterized with molecular markers and microscopic analysis of their morphology. Nematodes stages within buds and leaves were examined and imaged. In order to fulfil Koch's postulates, *L. crenatae mccannii* ssp. n. was used to inoculate otherwise healthy American beech seedlings in Ohio, USA and Ontario, Canada greenhouses to test whether BLD symptoms would result.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Infested leaves were collected in September, 2017 from Perry, Ohio (Lake County), USA by an Ohio Department of Agriculture nursery inspector from ailing American beech trees *Fagus grandifolia* Ehrh. and European beech trees, *Fagus sylvatica* L. Other leaf specimens in 2018 were sent from Kirtland, Ohio (Lake County), Potter County, Pennsylvania, USA, Crawford County, Pennsylvania, and Elgin and Norfolk Counties, Ontario, Canada for morphological and molecular confirmation using 28S and ITS 1,2 rDNA markers. Some specimens were dissected from leaves in water, measured and imaged using a microscope. Some leaves were dissected and stained with acid fuchsin (Byrd, Kirkpatrick, & Barker, 1983) for 5–10 days at room temperature to reveal nematodes in leaves.

2.2 | Microscopy

Nematodes from leaves were imaged on an Olympus BX51 microscope equipped with polarization optics and with a DP73 camera (Olympus America Inc.). Measurements in micrometres were taken with the calibrated measuring tool in the imaging program cellSens ver 1.6 (Olympus America Inc.). Fixed specimens were processed for permanent slides with the formalin-glycerine method (Golden, 1990) and imaged with a Q-Imaging Retiga EXi Color Digital Camera (Q-Imaging) attached to a Leica Wild MPS48 Leitz DMRB compound microscope (Leica Microsystems). Measurements and morphometrics were calculated on an Excel spreadsheet. The LT-SEM protocol of Carta, Bauchan, Hsu, and Yuceer (2010) was used employing a Hitachi S-4700 field emission SEM (Hitachi High Technologies America, Inc.) with a Quorum CryoPrep PP2000 (Quorum Technologies Ltd.) cryotransfer system to observe nematodes isolated from leaves and within buds. A Hirox MXB-504RZ digital microscope was also used to observe nematodes in buds (Hirox USA, Inc.).

2.3 | DNA Isolation, amplification, sequencing, alignment

2.3.1 | DNA extraction

DNA extraction was performed by freeze-thaw lysis with a single live nematode in a 0.2 ml PCR tube containing 25 µl of extraction buffer (10 mM Tris pH 8.2, 2.5 mM MgCl2, 50 mM KCl, 0.45% TWEEN 20 and 0.05% gelatin) (Carta & Li, 2019).

Fifteen specimens from BLD leaves collected November, 2017 in Perry, Ohio were processed for individual DNA extraction, and 10 of them, prior to being transferred to each PCR tube, were imaged as vouchers for morphological and morphometrical analysis.

PCR amplification and DNA Sequencing: The 3.5 kb ribosomal DNA (rDNA), ranging from near-full length 18S, internal transcribed spacer (ITS), to 28S (D1-D3) was amplified and sequenced from each of the imaged specimens using recently modified procedures of Carta and Li (2019). Cytochrome c oxidase I (COI) was amplified by PCR with the primer sets, COI-F1 and COI-R2 (Kanzaki & Futai, 2002). All primers used for amplification and sequencing are listed in Table 1. Each 25 µl PCR reaction was prepared with 2 µl of the extract and 23 µl of the PCR master mix containing 0.625 U DreamTaq™ Hot Start DNA Polymerase (Thermo Fisher Scientific) per the manufacturer's protocol. The PCR conditions for the COI were 94°C for 1 min, 5 cycles of 94°C for 40 s, 45°C 45 s, 72°C 1 min, 35 cycles of 94°C for 40 s, 51°C 45 s, 72°C 1 min and final extension at 72°C for 5 min. PCR products were visualized with the Lonza FlashGel™ DNA system (VWR International) and then treated with ExoSAP-IT reagent (Affymetrix, Inc) according to the manufacturer's protocol. Direct DNA sequencing for the COI

FIGURE 1 Leaf symptoms include darkened green bands, chlorosis and necrosis, Perry, OH Fall 2017 (a) American beech *Fagus grandifolia;* (b) European beech, *Fagus sylvatica* images of David McCann

TABLE 1 Primers used for PCR and sequencing

Primers	Direction	Sequence (5′–3′)	Loci	PCR	Sequence	Reference
18S-CL-F3	F	CTTGTCTCAAAGATTAAGCCATGCAT	18S	√	√	Carta and Wick (2018)
18S-530R	R	GCGGCTGCTGGCACCACACTT	18S		√	Thomas (2011)
18S-530F	F	AAGTCTGGTGCCAGCAGCCGC	18S		√	Thomas (2011)
18S-CL-F2	F	CTGTGATGCCCTTAGATGTCC	18S		√	Carta and Wick (2018)
18S-CL-R7	R	ACCTTGTTACGACTTTTGCCCGGTTCA	18S		√	This study
ITS-CL-F2	F	ATTACGTCCCTGCCCTTTGTA	18S		√	Carta and Wick (2018)
rDNA1.58S	R	ACGAGCCGAGTGATCCACCG	5.8S		√	Cherry, Szalanski, Todd and Powers (1997)
AB28	R	ATATGCTTAAGTTCAGCGGGT	28S		√	Vrain, Wakarchuk, Levesque and Hamilton (1992)
28S-CL-F3	F	AAGAGAGAGTTAAAGAGGACGTGAA	28S		√	This study
28S-CL-R1	R	ACTCCTTGGTCCGTGTTTCAAG	28S		√	This study
28S-CL-F2	F	CGACCCGTCTTGAAACAC	28S		√	This study
28S-CL-R	R	CAGCTACTAGATGGTTCGATTAGTC	28S	√	√	This study
COI-R2	R	GTAGCAGCAGTAAAATAAGCACG	COI	√	√	Kanzaki and Futai (2002)
COI-F1	F	CCTACTATGATTGGTGGTTTTGGTAATTG	COI	√	√	Kanzaki and Futai (2002)

was performed bidirectionally with an ABI BigDye Terminator v3.1 kit and in an ABI 3730xl DNA Analyzer (Applied Biosystems) owned by the USDA Systematic Entomology Lab. The marker sequences derived from *Litylenchus* sp. specimens, 104H78 and 104H82 were deposited to GenBank with accession numbers for rDNA (MK292137, MK292138) and COI (MN524968, and MN524969).

Sequences were compared with Clustal W in Genius (ver 11.1.5) with those from the type population from Morioka, Iwate Pref., Japan for GenBank accession numbers LC383723 for SSU, LC383724 for D2-D3 LSU and LC383725 for ITS rDNA.

2.4 | Nematode plant inoculations

2.4.1 | Nematode collection and quantification

Nematodes were isolated from leaves collected with severe BLD symptoms, and a modification of the "water soaking" isolation method (Zhen, Agudelo, & Gerard, 2012) was employed. In short, 5 leaves with symptoms of BLD were cut into 1-cm^2 pieces and placed in a petri dish containing 4% potato dextrose agar. Leaves were than soaked overnight in sterile water at 22°C. After soaking, the liquid containing

nematodes was carefully collected with a pipettor and then centrifuged at 1252*g* for 2 min to concentrate the nematodes. Water was then removed through pipetting and nematodes resuspended into 1-ml total volume of sterile water. Approximately, 200-µl of the nematode suspension was removed and 200-µl of 100% ethanol was added to fix the nematodes prior to quantification. Nematodes were counted using a Sedgewick rafter at a magnification of 4X, on an Olympus BH-2 dissecting microscope. Quantification was made to standardize the number of nematodes used for leaf and bud inoculation. The nematodes remaining in the sterile water were used for leaf and bud inoculation as described below. Some nematodes treated with ethanol were also retained for morphological identification and DNA sequencing. Nematodes used for autumn tree inoculation were collected on 3 October 2018, and those used for spring bud inoculation were collected on 22 April 2019.

2.4.2 | Tree leaf and bud inoculation

Three different types of plant inoculations were conducted; (a) new leaf inoculation, (b) dormant bud inoculation prior to winter dormancy and (c) dormant bud inoculation just prior to leaf out in spring. For new leaf inoculation, mature, 1-m tall American beech trees (*Fagus grandifolia*) that had been kept dormant in cold storage were placed in a warm greenhouse 7 September 2018. Trees broke dormancy and began to grow after 2 weeks and newly emerged, fully expanded leaves were available for inoculation in early October 2018. Four treatments were conducted on each of four trees: uninjured leaf, injured leaf, injured leaf that was inoculated with 100 µl of a water suspension containing 400 nematodes (4,000/ml), and an injured leaf that was inoculated with 100 µl of a water suspension containing 800 nematodes (8,000/ml). Leaves were injured using a sterile dissecting needle by making small holes in the leaf tissue and by scraping the needle across the underside and upper side of each leaf. Leaves were injured as this was found to produce the highest level of nematode leaf colonization in previous work (Zhen et al., 2012). After leaf injury, the leaf was wrapped in a 11 × 21 cm Kimwipe (Kimberly-Clark) which was lightly moistened with sterile water to make it adhere to the leaf surface. Leaves that received no nematodes had 100 µl of sterile water added to the surface of the leaf underneath the surrounding Kimwipe. The leaf was than enclosed in a sterile, plastic sample bag to maintain moisture close to the surface of the leaf. Leaves receiving nematodes had 100 µl of sterile water containing either 400 or 800 nematodes added to the leaf surface as described above. After nematode application, the leaf and Kimwipe were enclosed in a sterile sample bag to maintain leaf moisture. This was essential since nematodes require a water film for movement to allow tissue colonization. Trees were then placed in a warm greenhouse with supplemental lighting. Bags were kept on the treated leaves for 3 days to allow for nematode colonization and then removed. Trees were maintained in the greenhouse with a temperature between 12–25°C with a 12-hr day-night cycle. Trees were monitored for 5 months until leaf senescence and fall associated with the onset of plant dormancy.

Bud inoculations occurred in October 2018 using 4 trees with well-developed buds. On each tree, there were 4 bud treatments: uninjured bud, injured bud, uninjured bud that received a water suspension containing 170 nematodes and 80 eggs (Table 2), and an injured bud that received a water suspension containing 170 nematodes and 80 eggs. Buds were wrapped with a piece of Kimwipe as described above and wetted with sterile water to insure adherence to the bud surface. Then, 100 µl of sterile water was added under the Kimwipe to the bud surface. The buds that did not receive nematodes received a sterile water control treatment, but for buds receiving nematodes the sterile water suspension contained the nematodes as described above. After nematode or sterile water application, buds were carefully wrapped with parafilm to retain moisture against the bud surface (Figure 7). Buds were injured with a sterile dissecting needle such that 6 small holes were poked into each injured bud to facilitate nematode entry. Trees were then placed in a cold greenhouse where they underwent winter dormancy. Trees were kept under cold (4–10°C with ambient daylight), dormant conditions for 4 months prior to moving plants into a heated greenhouse (12–25°C) to break bud dormancy and stimulate leaf emergence and growth. Leaves were then monitored for BLD as they developed.

Additional bud inoculations were also made in April 2019 using two trees that had been dormant through winter. Due to low recovery of nematodes from dormant buds in April 2019, only one bud on each tree could be inoculated with nematodes. Therefore four total buds, two per tree, were treated as a part of this experiment. On each tree, one bud was injured with a dissecting needle as described above and then treated with sterile water as a no nematode control, and a second was injured but treated with a water suspension containing approximately 110 nematodes (see Table 2). Buds were covered with a Kimwipe and parafilm as a part of the inoculation as described above. The trees were placed in a heated greenhouse (12–25°C) to break bud dormancy under ambient light. Parafilm and Kimwipe were removed after 3 days, and buds broke dormancy and leaves emerged 2 weeks after bud inoculation. We monitored leaves for symptoms of BLD as they developed.

2.5 | DNA extraction and nematode detection post-inoculation

DNA was extracted from symptomatic leaf tissue collected from each tree and branch type. Leaf tissue was collected using a 1-cm sterile cork corer, and 1 leaf punch of BLD symptomatic tissue was extracted per tree. DNA was extracted using a bead-beating approach where tissue was transferred into a 1.5-ml bead beating tube which contained 300 mg of 400 µM sterile glass beads (VWR) and 200 mg of 1 mm sterile glass beads (Chemglass). About 750-µl of 2% cetyltrimethyl-ammonium bromide (CTAB) was added to each tube as the extraction buffer, and samples were bead beaten using a Precellys homogenizer (Bertin Technologies) for 80 s to lyse cells and release

TABLE 2 *Litylenchus crenatae mccannii* ssp. n. Leaf and bud inoculation results based on results obtained through 6 May 2019

Sample ID	Date Treat	Control	Injury + Control	Injury + 400 Nematodes	Injury + 800 Nematodes	Notes
Leaf Tree 1	10-3-18	Negative	Negative	Negative	Negative	See below
Leaf Tree 2	10-3-18	Negative	Negative	Negative	Negative	
Leaf Tree 3	10-3-18	Negative	Negative	Negative	Negative	
Leaf Tree 4	10-3-18	Negative	Negative	Negative	Negative	
Sample ID	**Date Treat**	**Control**	**Injury + Control**	**170 Nematodes + 80 eggs**	**Injury + 170 Nematodes + 80 eggs**	
Bud Tree 1	10-3-18	Negative	Negative	Negative	Not open (dead?)	
Bud Tree 2	10-3-18	Negative	Negative	Negative	Positive BLD	
Bud Tree 3	10-3-18	Negative	Negative	Positive BLD	Positive (slight)	
Bud Tree 4	10-3-18	Negative	Negative	Negative	Positive (slight)	
Bud Tree 5	4-23-19	NA	Negative	NA	Positive BLD	May 6 open
Bud Tree 6	4-23-19	NA	Negative	NA	Positive BLD	May 6 open

Note: For Bud tree inoculation 1–4, red and yellow label received about 140 juvenile nematodes, 30 adult nematodes and 80 eggs per bud if inoculated.

For bud trees 5–6 they received 110 nematodes and 40 eggs on average. Due to low nematode yield, we inoculated only injured buds.

NA equals not applicable; treatment was not included.

DNA. DNA was purified from the extract matrix using phenol-chloroform extraction (Burke, Smemo, López-Gutiérrez, & DeForest, 2012) followed by precipitation in 20% polyethylene glycol 8,000 with 2.5 M NaCl. Precipitated DNA was subsequently dried and suspended in 50 µl TE (Tris EDTA) buffer and stored in a 1.5-ml low retention microcentrifuge tube (Fisher Scientific) at −20°C until analysis. We used nematode specific ITS primers to detect nematodes within leaf and bud samples. Forward primer TW81 (GTTTCCGTAGGTGAACCTGC) and reverse primer 5.8MS (GGCGCAATGTGCATTCGA) (Tanha Maafi, Subbotin, & Moens, 2003; Vovlas, Subbotin, Troccoli, Liebanas, & Castillo, 2008) were used for nematode amplification. Amplification reactions were performed in an MJ Research PTC-200 thermal cycler (Bio-Rad Laboratories, Inc.). An initial denaturation for 2 min at 94°C was followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 45 s at 55°C and extension for 3 min at 72°C, with a final extension of 10 min at 72°C (Esmaeili, Heydari, & Ye, 2017). PCR product was visually checked using 1% agarose gels and ethidium bromide staining. PCR product positive to nematodes was purified using a Wizard SV Gel and PCR Clean Up System (Promega) and purified PCR used for direct sequencing of the nematode ITS region using Big Dye Terminator chemistry and a 3730 DNA Analyzer (Applied Biosystems Inc.). Sequences were generated through the Life Sciences Core Laboratories Center (Cornell University). Sequences were identified with the EMBL/GenBank/DDBJ database entries and the NCBI Blast tool through GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Morphological identification of nematodes: Leaves showing BLD symptoms and nearby bud tissue were also collected and sent to USDA-ARS Beltsville for microscopic examination and identification. Nematodes were dissected from plant tissue and stained with acid fuchsin (Byrd et al., 1983).

3 | RESULTS

Litylenchus crenatae Kanzaki et al. (2019) *mccannii* ssp. n. (Tables 3–5 and Figures 2–4).

3.1 | Description

Females have a nearly continuous, slightly offset lip (Figures 2a–d, 3a,b and 4a,b) region with 5 annules (Figure 4a,b), long and slender body shape (Figures 2, 3a,b and 6c), the stylet in females is 5% of the pharynx length and 7%–10% of the pharynx length in males, and there is a small, narrow, weak median bulb without an obvious valve. The vulval region is kinked and irregular (Figure 4c,d). The anterior gonad is relatively long [254 ± 79 (166–365) µm where n = 5], nearly five times the length of the post-uterine sac. The post-uterine sac is about three times the vulval body width and one quarter of the vulval anal distance (VAD). The VAD is 2.8 ± 0.3 (2.3–3.3) times the tail length. The rectum is approximately one quarter of the tail length, the anus pore-like and obscure in most specimens. There is a gradually tapering, slender, conical tail with an asymmetrically pointed, often mucronate extension. The distal tail in immature (Figures 2i and 4g,h) and mature females (Figure 4i,j) vary in shape.

3.1.1 | Localities and hosts

Perry, Ohio on leaves of American beech trees *Fagus grandifolia* Ehrh. and European beech trees, *Fagus sylvatica* L. specimens collected in September 2017 and received between 9/12/17, and 05/18/18; *Fagus grandifolia* specimens from Holden Arboretum, Kirtland, OH

TABLE 3 Morphometrics of live adult female *Litylenchus crenatae mccannii* ssp. n

Character	*L. c. mccannii* Perry OH 9-2017	*L. c. mccannii* Kirtland, OH 10-2018	*L. c. mccannii* Crawford, PA 9-2018	*L. c. mccannii* Potter, PA 10-2018	*L. c. mccannii* Ontario, Canada 11-2018
n	27	12	11	14	13
Body L µm	889 ± 119 (625–1084)	907.6 ± 28.6 (853.1–952.7)	739.8 ± 178.9 (562.0–1015.0)	788.8 ± 95.2 (564.4–891.2)	836 ± 115 (789–1109)
Body W µm	14.2 ± 1.0 (12.1–16.1)	13.2 ± 0.6 (12.1–14.0)	16.2 ± 2.4 (15.0–22.0)	12.8 ± 2.9 (10.6–22.2)	14.8 ± 2.2 (13.6–15.9)
Stylet µm	9.2 ± 0.5 (8.4–10.3)	9.5 ± 0.7 (8.8–11.4)	9.3 ± 1.2 (7.5–11.0)	9.6 ± 0.8 (8.8–11.5)	9.5 ± 0.7 (8.2 10.4)
Pharynx L µm	193.5 ± 35.7 (126.3–244.2)	209.8 ± 6.4 (200.1–221.0)	141.7 ± 36.7 (100–195)	193.7 ± 26.9 (126.0–224.1)	176.6 ± 12.7 (151.2–198.9)
PUS µm	34.3 ± 6.1 (22.7–45.0)	22.9 ± 5.8 (13.7–33.7)	—	27.9 ± 11.7 (17.7–64.0)	36.9 ± 9.4 (29.7–54.1)
Tail L µm	54.3 ± 6.1 (39.8–64.4)	55.6 ± 3.1 (51.6–61.9)	43.7 ± 11.3 (33.0–62.0)	50.6 ± 6.5 (31.5–57.0)	50.6 ± 5.5 (42.3–56.6)
a	63.0 ± 10.0 (43.8–76.8)	68.8 ± 4.0 (64.3–78.8)	46.3 ± 13.6 (31.2–67.7)	63.5 ± 11.8 (28.6–74.7)	61.4 ± 9.8 (40.9–73.4)
b	4.7 ± 0.7 (3.8–6.6)	4.3 ± 0.2 (4.1–4.6)	5.4 ± 0.6 (4.8–6.8)	4.1 ± 0.5 (3.5–5.0)	4.8 ± 0.3 (4.0–5.3)
c	16.4 ± 1.5 (13.3–20.1)	16.3 ± 0.7 (15.0–17.6)	16.8 ± 1.4 (15.0–18.8)	15.7 ± 1.7 (13.4–20.2)	16.6 ± 1.7 (12.6–19.6)
c′	5.7 ± 0.8 (4.3–7.9)	6.0 ± 0.5 (5.5–7.2)	—	5.3 ± 1.2 (2.2–6.8)	5.9 ± 0.9 (4.5–6.5)
V%	76.6 ± 1.4 (73–79)	77.1 ± 0.7 (76–78)	77.7 ± 0.07 (76.5–78.4)	76.6 ± 1.0 (75.3–78.3)	80.2 ± 6.6 (75.2–86.9)
PUS/VAD%	27 ± 8 (22–50)	15	—	20.9	25.5 ± 3.4 (20.4–30.6)
PUS/BW	2.8 ± 0.5 (1.9–3.8)	1.8 ± 0.5 (1.1–2.7)	—	2.3 ± 0.7 (1.6–3.9)	2.5 ± 0.5 (1.8–2.9)

TABLE 4 Morphometrics of fixed and live, adult female *Litylenchus crenatae mccannii* ssp. n

Character	*L. c. mccannii* Perry, OH, Live, young	*L. c. mccannii* Perry OH Fixed, young	*L. crenatae* Japan Fixed, young	*L. c. mccannii* N. America Live, mature	*L. crenatae* Japan, Fixed, mature
n	27	10	10	50	10
Body L µm	889 ± 119 (625–1084)	823 ± 61 (750–947)	863 ± 33 (837–915)	740–908 (625–1109)	816 ± 32 (758–870)
Body W µm	14.2 ± 1.0 (12.1–16.1)	11.4 ± 1.1 (9.9–13.5)	12.3 ± 0.9 (11.0–13.5)	12.8–16.2 (10.6–16.1)	22.9 ± 2.6 (18.4–27.7)
Stylet µm	9.2 ± 0.5 (8.4–10.3)	9.7 ± 0.9 (8.5–11.2)	8 ± 0.4 (7.4–8.5)	9.3–9.6 (7.5–11.4)	10.6 ± 0.5 (9.9–11.3)
Styl conus µm	4.6 ± 0.4 (3.6–5.2)	—	3.1 ± 0.2 (2.8–3.5)	—	3.3 ± 0.2 (3.9–4.6)
Pharynx L µm	193.5 ± 35.7 (126.3–244.2)	152.6 ± 16.2 (133–186)	203 ± 5.9 (192–213)	142–210 (100–244)	123 ± 6.7 (110–131)
PUS µm	34.3 ± 6.1 (22.7–45.0)	—	32 ± 3.4 (29–39)	23–37 (14–64)	68 ± 7.4 (57–81)
Tail L µm	54.3 ± 6.1 (39.8–64.4)	48.3 ± 6.2 (34.5–56.4)	55 ± 3.8 (50–63)	44–56 (31.5–64.4)	33 ± 2.3 (30–36)
a	63.0 ± 10.0 (43.8–76.8)	72.9 ± 9.3 (61–86)	67.5 ± 5.8 (60.7–74.4)	46–69 (31–79)	35.9 ± 3.4 (30.2–41.1)
b	4.7 ± 0.7 (3.8–6.6)	5.4 ± 0.7 (4.5–6.6)	5.3 ± 0.6(4.5–6.3)	4.1–5.4 (3.3–6.8)	6.6 ± 0.4 (6.1–7.6)
c	16.4 ± 1.5 (13.3–20.1)	17.4 ± 3.3 (13–25)	15.7 ± 0.7 (14.4–16.7)	15.7–16.8 (12.6–20.2)	24.5 ± 1.9 (18.5–25.1)
c′	5.7 ± 0.8 (4.3–7.9)	6.0 ± 1.0 (4.3–7.9)	6.3 ± 0.5 (5.5–7.4)	5.3–6.0 (2.2–7.9)	2.9 ± 0.3 (2.5–3.3)
V%	76.6 ± 1.4 (73–79)	76.9 ± 1.2 (75–79)	77.4 ± 0.5 (76.6–78.3)	77–80 (73–87)	81.5 ± 1.0 (79.4–83.2)
PUS/VAD%	27 ± 8 (22–50)		22.9 ± 2.1 (20.2–25.9)	15–25 (20–50)	57.9 ± 7 (47–73)
PUS/BW	2.8 ± 0.5 (1.9–3.8)		2.6 ± 0.4 (2.2–3.5)	1.8–2.8 (1.1–3.9)	3.5 ± 0.4 (2.8–4)

TABLE 5 Morphometrics of adult male *Litylenchus crenatae mccannii* ssp. n

Character	*L. c. mccannii* Kirtland, OH Live 2-2018	*L. c. mccannii* Crawford, PA Live 9-2018	*L. c. mccannii* Ontario, Canada Live 5-2018	*L. c. mccannii* Perry, OH Fixed 11-2017	*L. crenatae* Japan Fixed 6-2017
n	8	3	5	4	8
Body L µm	657 ± 64 (554–772)	586.3 ± 73.3 (502–635)	611.8 ± 109.1 (511.8–778.2)	548.0 ± 16.7 (534.5–566.7)	707 ± 41 (642–773)
Body W µm	16.7 ± 2.3 (13.5–20.3)	15 ± 0 (15)	15.4 ± 1.5 (13.1–17.6)	15.1 ± 2.5 (12.1–16.7)	12.4 ± 0.8 (11.3–13.5)
Stylet µm	11.2 ± 0.4 (10.6–12.0)	10 ± 0 (10)	9.8 ± 0.3 (9.6–10.1)	11.1 ± 0.5 (10.5–11.4)	10.2 ± 0.4 (9.9–11.0)
Styl conus µm	4.8 ± 0.3 (4.4–5.3)		3.4 ± 0.1 (3.4–3.6)		3.6 ± 0.3 (3.5–4.3)
Pharynx L µm	143.2 ± 11.8 (124.9–159.7)		121.2 ± 13.4 (117.8–134.9)	113.9 ± 5.0 (108.5–118.1)	135 ± 14 (116–157)
Tail L µm	34.9 ± 3.3 (30.1–41.5)		33.3 ± 3.9 (29.0–36.7)	35.3 ± 1.6 (33.7–37.9)	34 ± 2.6 (30–38)
a	40.0 ± 7.8 (31.1–57.3)	41.9 ± 0.6 (41.5–42.3)	45.6 ± 2.6 (41.1–47.8)	36.1 ± 5.4 (33.3–44.1)	57.2 ± 4.7 (48.9–61.9)
b	4.6 ± 0.4 (4.1–5.3)		4.5 ± 4.1 (3.9–5.0)	4.8 ± 0.2 (4.6–4.9)	4.3 ± 0.3 (3.9–4.8)
c	18.9 ± 2.0 (16.2–22.7)		19.1 ± 1.9 (17.0–22.7)	15.5 ± 0.2 (15.3–15.9)	21.1 ± 2.0 (18.5–25.1)
c′	3.2 ± 0.2 (2.9–3.5)		3.4 ± 0.3 (2.8–3.9)		3.6 ± 0.4 (3.0–4.1)
Spicule L µm	17.1 ± 2.4 (13.7–19.7)	15 ± 0 (15)	16.5 ± 2.1 (14.3–17.6)	16.3 ± 1.4 (14.9–17.6)	15.6 ± 1.2 (14.2–17.7)
Gubernaculm L µm	6.9 ± 0.7 (6.4–8.0)	6 ± 1 (5–7)	5.9 ± 0.2 (5.7–6.0)	5.3 ± 0.8 (4.3–6.1)	6.5 ± 0.4 (6.0–7.1)

8/27/18; (41°62′52.2x″N, 77°93′19.5x″W) Susquehannock State Forest, Potter County, PA 9/6/18; (41°64′41.95″N, 80°48′96.67″W) Pymatuning Reservoir, Jamestown, Crawford County, PA 9/21/18; Elgin County (42°39′52.49″N, 81°10′12.48″W) and Norfolk County, Ontario Canada 11/2018).

3.1.2 | Specimen designation and deposition

Thirty-seven slides (T709t (holotype), T6960- 6973p (paratypes), 7108-7110p, 7113-7126p, 7182-7186p) with 260 females and 10 males and 89 juveniles from Perry, OH were deposited in the United States Department of Agriculture Nematode Collection (USDANC) along with 5 vials (T-576-T-580p). In addition, 5 females and 5 males from Ontario, Canada were deposited in the USDANC, and 20 slides (T567 a-t) of the Ontario population were deposited in the Canadian National Collection of Nematodes Ottawa.

3.2 | Diagnosis

The *Litylenchus crenatae mccannii* ssp. n. young female population fixed and mounted in early autumn from North America had a longer stylet [9.7 ± 0.9 µm *n* = 10 (8.6–11.2) vs. 8.0 ± 0.4 (7.4–8.5) *n* = 10, $p < .001$] µm and stylet conus [4.6 (3.6–5.2) vs. 3.1 ± 0.2 (2.8–3.5) $p < .001$] µm than the type population from Japan. The pharynx was also significantly shorter (152.6 ± 16.2 vs. 203 ± 5.9 µm, $p < .001$) in immature females, although the "b" ratio was not different. However, the pharynx was longer in mature females of 3 populations ($p < .001$) but not for those in Crawford County (142 ± 36.7 vs. 123 ± 6.7) The post-uterine sac in mature females was shorter (36.9 ± 9.4 vs. 68 ± 7.4, $p < .001$) than the Japanese population. The tail was shorter in the fixed immature female populations (48.3 ± 6.2 vs. 55 ± 3.8 $p < .01$) but it was longer in the mature populations (43.7 ± 11.3 vs. 33 ± 2.3 $p < .01$, and $p < .001$ for 3 other populations) which was also reflected in different c (16.8 ± 1.4 vs. 24.5 ± 1.9, $p < .001$) and c′ (5.3 ± 1.2 vs. 2.9 ± 0.3, $p < .001$) ratios. The body width was narrower in all populations of mature females (16.2 ± 2.4 vs. 22.9 ± 2.6, $p < .001$). The male population from Perry, Ohio also had a longer stylet [11.2 (10.6–12) vs. 10.2 (9.9–11)] µm and stylet conus [4.8 (4.4–5.3) vs. 3.6 (3.5–4.3)] µm, and a wider body [16.7 (13.5–20.3)] µm than the fixed type population from Japan.

3.3 | Remarks

The morphological and host range differences in North American populations are nomenclaturally distinguished as *L. crenatae mccannii* ssp. n., after the plant pathologist who first observed the nematodes in BLD affected leaves. Live, mature males and females (Tables 1–3) had a great deal of variation with overlapping ranges, but certain differences were noted when population averages were compared. The degree of dimorphism between immature and mature

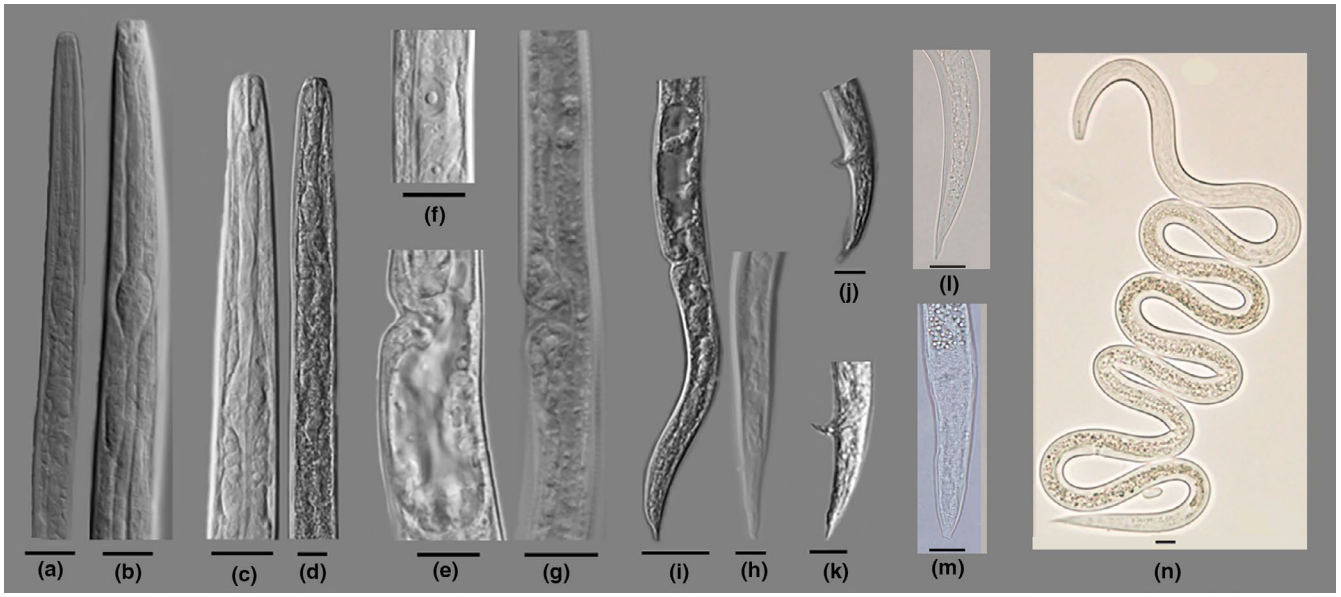
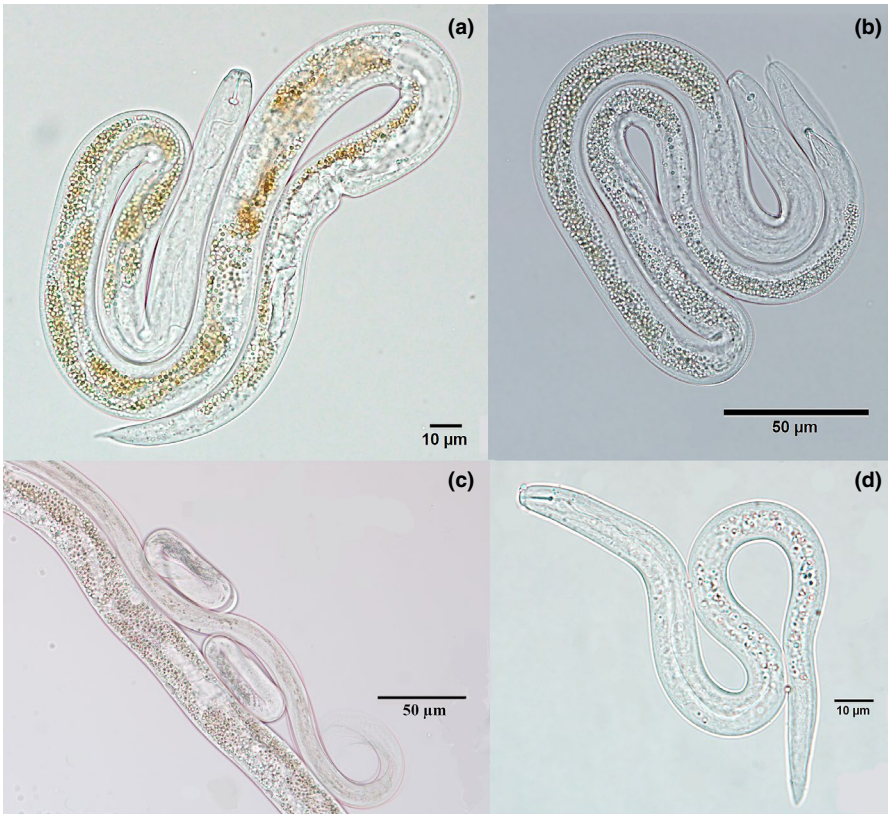


FIGURE 2 Light microscopy (LM) of *Litylenchus crenatae mccannii* ssp. n. fixed female specimens. (a–k), live, polarized light microscopy (PLM) females (l–n); (a-b) immature anterior pharynx; (c, d) mature anterior pharynx, (e) immature mid-body with vulva; (f) pharyngeal gland; (g) mature vulva, post-uterine sac; (h) immature tail. (i) mature vulva, tail; (j, k) male tails with spicules; (l) slender female tail with mucro, Perry, Ohio, USA; (m) obese female tail with asymmetric tip, Kirtland Ohio, USA; (n) mature female body, egg, Potter County, Pennsylvania, USA

FIGURE 3 Polarized light microscopy (PLM) of live *Litylenchus crenatae mccannii* ssp. n. (a) female; (b) male; (c) eggs; (d) juvenile



females seen in the population from Japan was less than that seen in the multiple North American populations, primarily reflected in the narrower body of mature females.

The young overwintering adults were primarily found in October - November, and reproductive, mature females predominated in spring through early autumn. Multiple nematode life stages, but not males, were found within buds, and males were found within leaves from spring through autumn. In Ontario, the young overwintering adults were only found in leaves on the ground in the wintering months, while mature adults could still be found in buds.

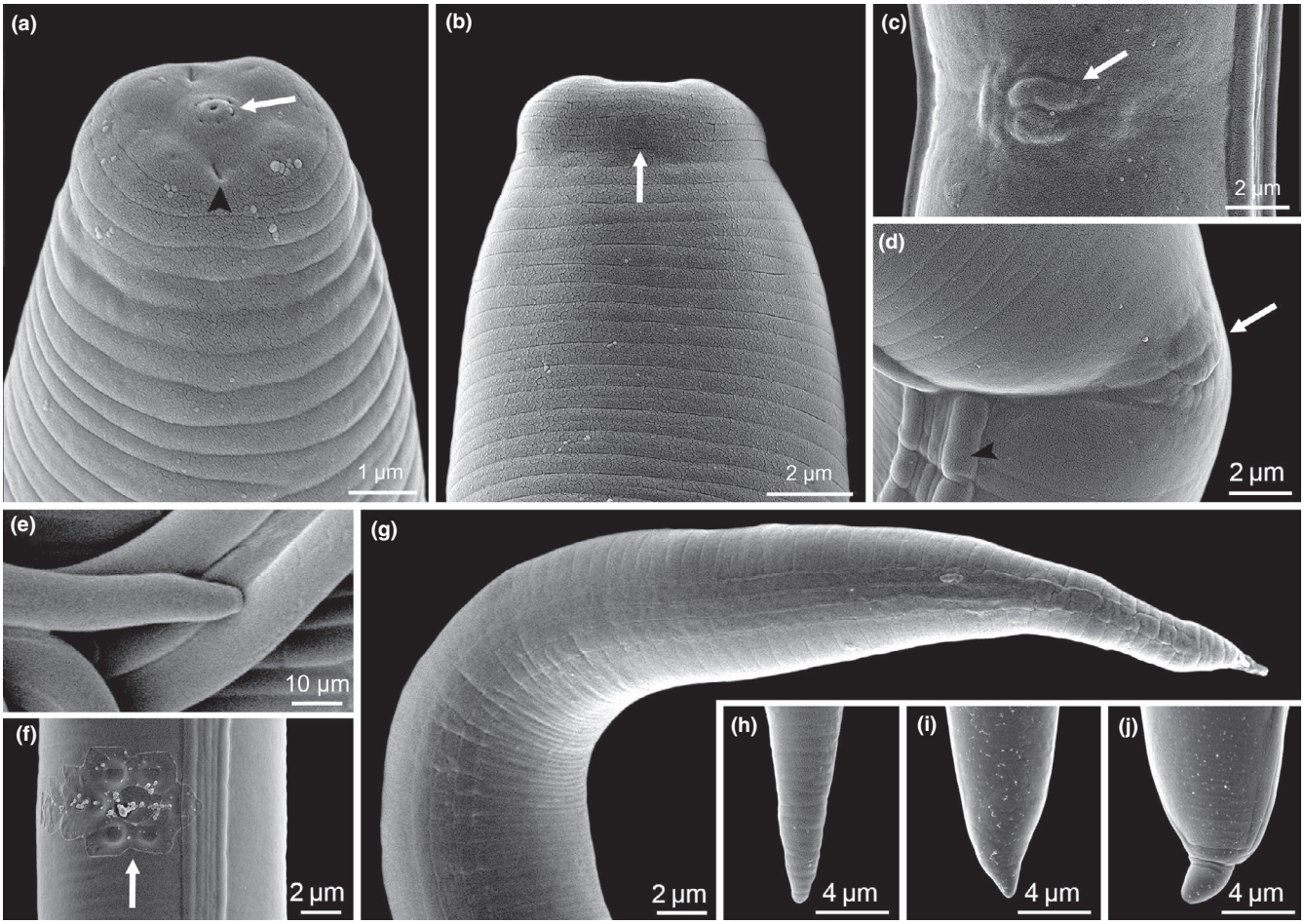


FIGURE 4 Low-temperature scanning electron microscopy of female morphology (a) face with stylet opening (vertical arrow), amphid opening (lateral arrow), oblique view; (b) lip region of head, lateral view; (c) vulva (top arrow) and lateral field (low arrow); (d) ventral view of vulva with puckered lips and lateral body depression in line with vulval opening (arrow); (e) nematode lips pressed on mid-body of a second nematode; (f) mid-body cuticle with six-sectored lip indentations from another nematode face, lateral view; conical tail tip variations. (g, h) young females; (i, j) mature females

Nematodes did not survive on potato dextrose agar plates with *Rhizoctonia solani*. Rinsed, surface-sterilized leaves embedded in either water agar or potato dextrose agar did not provide any better yield of nematodes than strips of leaves in sterile water from which thousands of nematodes per infested leaf could be harvested over a few weeks. Twigs below infested buds were cut and placed in Baermann funnels or dishes but no nematodes emerged.

3.4 | Bud associated nematodes

Litylenchus crenatae mccannii ssp. n. adults were inoculated to freshly dissected beech bud tips embedded in moist water agar plates that resulted in adult females swarming onto the bud tip (Figure 6a). Dissection demonstrated that nematodes entered the excised bud but did not develop.

Neither bud nor leaf explants could be maintained on water agar. Nematode females and eggs were exposed from within buds in March 2019 and imaged with a Hirox (Figure 5b–d) microscope and an LT-SEM (Figure 6a–c).

3.5 | Tree leaf and bud inoculation

We found that beech leaf inoculations failed to initiate any symptoms of BLD in trees after 5 months of growth (Table 4). Although some leaves developed browning or leaf margins over the course of 5 months, none developed interveinal darkening that is characteristic of BLD. We also observed no leaf mortality during the 5-month incubation until trees began to senesce and enter dormancy. Bud inoculation however was successful in initiating symptoms of BLD in buds where nematodes were applied (Table 4 and Figure 8). For buds inoculated in autumn prior to dormancy, a total of 3 of 4 buds which were injured prior to nematode inoculation showed evidence of BLD, while the fourth bud failed to open and died. Control buds which were injured or uninjured and which did not receive nematode inoculation all opened without signs of BLD. However, injured

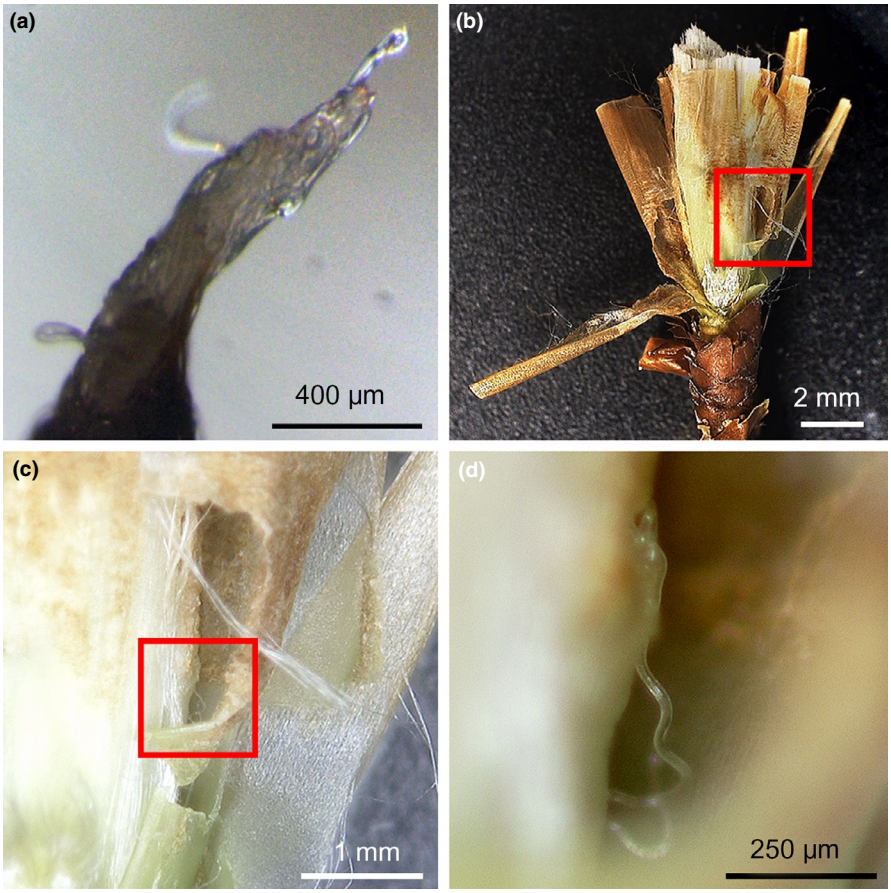


FIGURE 5 *Litylenchus crenatae mccannii* ssp. n. on and in buds. (a) LM image of adult females swarming on bud tip; (b–d) Hirox microscopy of nematode-infested beech bud from the Holden Arboretum, Kirtland, Ohio, USA

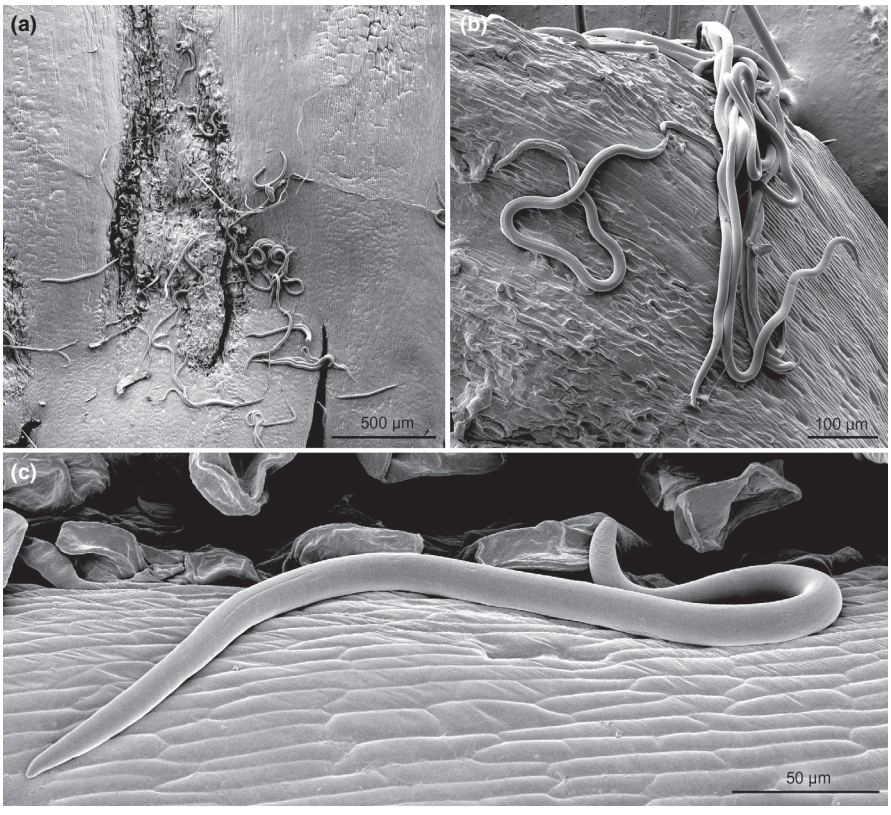


FIGURE 6 Low-temperature scanning electron microscopy images of *Litylenchus crenatae mccannii* ssp. n. on leaf sheath at bud base. (a) females, juveniles, eggs on bud sheath; (b) adult females on bud sheath; (c) adult female on bud sheath near leaf mesophyll

FIGURE 7 Inoculation of *Fagus grandifolia* seedlings with nematodes. Bud inoculation for nematodes involved wrapping the bud with a wet Kimwipe and adding nematode suspension under Kimwipe at bud surface. The bud was loosely wrapped in parafilm to retain moisture and permit nematodes to colonize it. Photographs show inoculation in April 2019 of dormant buds with nematodes collected from field grown trees. The "I" indicates the bud was injured prior to inoculation

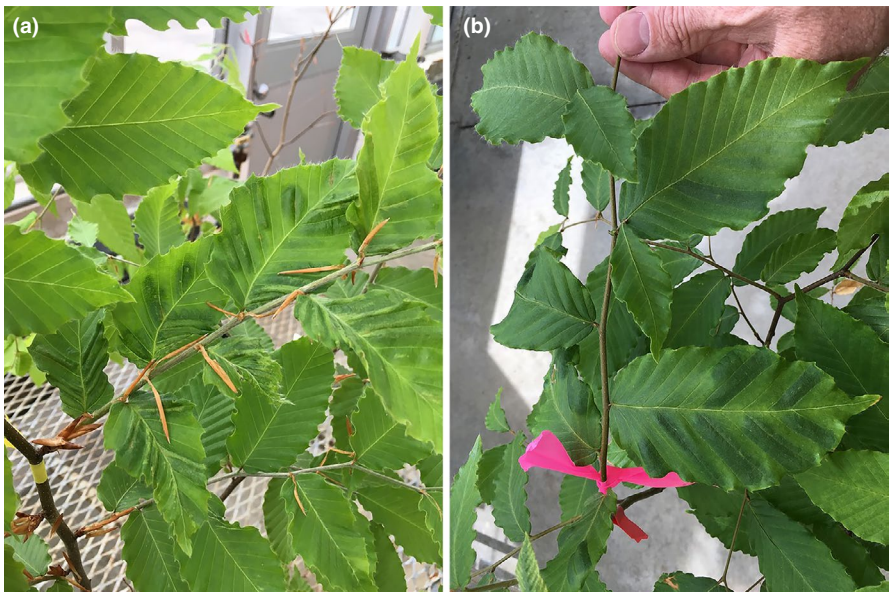


FIGURE 8 Symptoms from inoculated seedlings. (a) Bud tree two, injured with nematode added. 22 April 2019. (b) Bud tree three, no injury with nematode added. 29 May 2019

buds, even those without nematode inoculation, tended to open more slowly than uninjured leaves. Of the 4 buds which received nematodes but were not injured, only 1 developed symptoms of BLD while the other 3 buds opened normally and leaves appeared unaffected. For spring inoculated buds, both buds which were injured and received nematodes developed BLD symptoms even after only 2 weeks of incubation. Both buds which did not receive nematodes opened normally and were free of BLD symptoms.

3.6 | Nematode identification and sequence identity

Leaf tissue symptomatic for BLD was extracted from trees that underwent bud inoculation. Direct sequencing of PCR product and Blast matching returned matches to *Litylenchus crenatae* with 99% identity confirming the presence of the nematode in leaves developed from inoculated buds. GenBank sequence accession numbers: MN625146–MN625161. Leaves from Tree 4 that were bud inoculated with nematodes plus needle injury were harvested 4-22-19, after which symptomatic leaves were dissected and stained with acid fuchsin (Byrd et al., 1983) and stained nematodes recovered after 21 days (Figure 9a-c).

4 | DISCUSSION

Multiple symptomatic leaves received by the USDA-ARS MNGDBL and Agriculture Canada national nematology laboratories yielded many nematodes of one species only. In heavily infested areas where nematode counts were highest in symptomatic leaves, a very small number of nematodes were occasionally found on asymptomatic leaves, but across all samples nematodes were not found in the vast majority of asymptomatic leaves (S. Reed unpublished data).

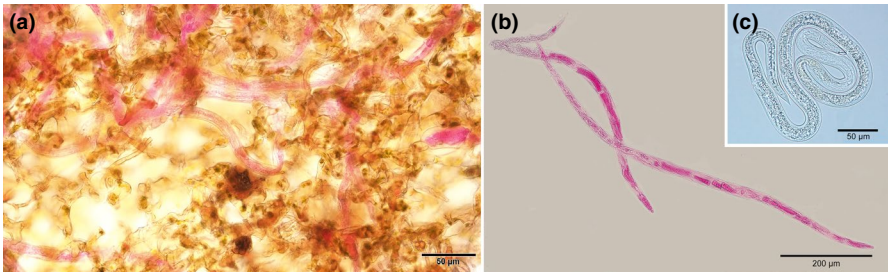


FIGURE 9 Acid fuchsin stains (a) female and male nematodes from peeled beech leaf mesophyll; (b) male nematodes stained from leaf of experimentally inoculated seedling, May 2019. (c) female nematode from inoculated seedling bud May 2019

Litylenchus nematodes with slender and obese morphs exhibit phenotypic plasticity as they develop, as with other nematodes in the family Anguinidae. Sclerotized, cuticular landmark structures like anal and vulval openings were much more difficult to identify in young adults and juveniles. However, the strongly sclerotized feeding stylet was always distinct, and consistently larger in mature than immature specimens.

As with *L. crenatae* in Japan (Kanzaki et al., 2019) and *L. coprosma* in NZ (Zhao, Davies, Alexander, & Riley, 2011), slender and obese morphs coexisted during much of the year, but obese morphs predominated in the late spring through autumn. As in Japan, males were found in leaves in late spring through autumn, but males were not found within buds in North America during September, November, March, or June. Eggs were found in buds rather than leaves during autumn months despite careful dissection and staining of leaves. However, it was possible to find eggs in leaves during late spring which hatched within leaves to produce many nematodes by late summer.

The fine resolution COI marker generated for this population employed the same primers designed for another phylogenetically distant foliar nematode (Kanzaki & Futai, 2002) associated with the laboratory of the author of the description of *L. crenatae*, but slightly different conditions. These included an especially thermostable polymerase enzyme and higher annealing temperature after the fifth PCR cycle. This different protocol may account for the sequence reported here that was not reported for the population from Japan.

Simple leaf inoculations with nematodes from leaves failed to produce symptoms in Ohio greenhouse tests reported here. However, a different experiment in Sault Ste. Marie, Ontario, Canada with two leaves per sapling injured with a needle, treated with a larger volume nematode inoculum but with a similar 540 total nematodes as in Ohio, was saturated to runoff in two 2 ml doses over a 72-hr period. In this September 2018 inoculation experiment, four out of the five surviving treated seedlings had BLD leaf symptoms at the beginning of June 2019, while all control seedlings were free of symptoms. Half of the initially treated seedlings were weakened by mites in the growth chamber and succumbed to freezing temperatures in a poly house. Leaves and buds of the surviving plants may have been injured by freezing temperature and mites. It is possible that the nematodes could have entered buds in the larger inoculum volume runoff during these ostensible leaf inoculations to produce symptoms. Entry into buds was the most reliable route for successful inoculation of nematodes, whether this occurred in the fall or the spring. Nematodes could enter buds on their own, but symptoms appeared more routinely with injury. In nature this might be facilitated by some type of vector or freezing and thawing. However, it appears that freezing during winter is not necessary to allow symptoms to develop since an early spring inoculation in the greenhouse produced symptoms.

While foliar nematodes are fairly common, nematodes that exist high in the tree canopy are not well-known, though endophytic *Aphelenchoides* were recently discovered in poplar leaves (*Populus* sp.) (Carta, Li, Skantar, & Newcombe, 2016). Nematodes like *Litylenchus crenatae mccannii* ssp. n. within the nematode family Anguinidae that are associated with hardwood leaves having similar swollen, chlorotic-becoming-necrotic mesophyll tissue include *Litylenchus coprosma* (New Zealand, Zhao et al., 2011), *L. crenatae* (Japan, Kanzaki et al., 2019), *Ditylenchus leptosoma* (Korea, Geraert & Choi, 1990) and *Subanguina chilensis* (Chile, Vovlas, Troccoli, & Moreno, 2000). Except for leaf symptoms in *L. coprosma*, the others were described as galls, though they are not discrete like the seed galls of *Anguina* or eriophyid mite galls. These other anguinid nematode leaf gallers were implicitly assumed to be the cause of their symptoms. Those plants were not very economically important nor did those symptoms accompany serious mortality, so there was no indication for the need to follow up with proof of nematode pathogenicity. None of these nematodes associated with galls have been reported to have a fundamental association with another pathogen since their description (Xu, Li, Ho, Alexander, & Zhao, 2017; citation updates of all species).

The sponginess of galled portions of the BLD leaves may result from pectinases, similar to host leaves of the related anguinid nematode *Ditylenchus dipsaci* (Myers, 1965). Physiological investigation of superficially similar leaf galls of the related *Ditylenchus gallaeformans* (Anguinidae) and leaf-galling mites (Eryiophidae) both demonstrated increased phenolics and carotenoids that counteract oxidative and light damage. However, the *Ditylenchus* nematode gall originated in the primordium, had a vascular connection, exhibited hypertrophied and hyperplastic mesophyll and promoted indeterminate growth. In contrast the simpler mite gall exhibited determinate growth in the epidermis (Ferreira et al., 2018). Therefore, the nematode gall affected the host plant more profoundly and systemically than the gall mite. Because there is likely to be a similar vascular connection for this related anguinid leaf galler, a *Litylenchus* metabolite or even an endophytic microorganism could have a profound effect on the plant. The nematode itself might be discovered to produce a toxin like that recently discovered in entomoparasitic nematode *Steinernema carpocapsae* (Lu et al., 2017).

Nematodes within the Anguinidae related to *Litylenchus* may harbour toxic *Rathayibacter* spp. bacteria specific to their plant hosts (*Anguina agrostis*, *A. funesta*, *A. tritici* on monocots: Dorofeeva et al., 2018 and *Mesoanguina picridis* on a dicot: Starodumova et al., 2017), but this appears to be a population-specific event among a few known species (Murray et al., 2017). In any event, preliminary evidence from subtractive leaf biome profiling by one of the authors found no suggestion of any *Rathayibacter* associated with the disease. However, work is underway to better understand the nematode and beech microbiomes. The microbiomes of the pinewood nematode, and beetle vectors are expected to illuminate the pathogenicity of pinewood disease (Alves et al., 2018).

Lack of symptomatic, naturally infested *Fagus crenata* leaves near infested *Fagus grandifolia* in North America suggests *F. crenata* may be resistant to the population from North America but not from Japan. As a result of the diversity of anguinids with similar leaf symptoms from the Pacific rim, that is a likely region of endemicity. The initial North American localities in Ohio, Pennsylvania, and New York, USA, and Ontario, Canada neighbour Lake Erie, a trade hub from which invasive species such as emerald ash borer (EAB) originated (Muirhead et al., 2006). Like EAB, human transport of wood may have distributed this probably invasive nematode. The nematode may have arrived on this continent through an invertebrate vector, as is also suspected for *Bursaphelenchus antoniae* that recently detected in the U. S. (Carta & Wick, 2018).

Transmission. Anguinid nematodes require water films to move. Certainly, windborne rain is a likely local means of disease transmission. Invertebrates are as well. For instance, a predatory mite was found entangled with nematodes, and we have collected various mites and insects from leaf surfaces. Spider mites were numerous in the summer in Ohio beech stands, and they can be windborne for many miles. There are many potential invasive beetle vectors associated with beech (Morrison, Sweeney, Hughes, & Johns, 2017; Rabaglia, Vandenberg, & Accivatti, 2009) (that may be present in the geographic regions where BLD occurs. Finding enough of any of these invertebrates with nematodes takes time and directed effort however.

Birds are another possible vector, as with transport of Lyme disease through ticks (Loss, Noden, Hamer, & Hamer, 2016). Beech nuts are a critical component of the food chain for birds in Northeastern and Appalachian forests. They are high in the canopy and difficult to harvest before the birds consume them. Since these nematodes inhabit leaf buds they may also inhabit flower buds. If so, birds might ingest nematodes and distribute them directly. They might carry mites, ticks or insects that carry nematodes as well.

Whether the nematode itself is the sole cause of the disease, or a vector of an elusive, hidden pathogen, it has had a consistent natural and experimental association with disease symptoms to date.

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