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Morphological and molecular characterisation of *Punctodera mulveyi* n. sp. (Nematoda: Punctoderidae) from a golf course green in Oregon, USA, with a key to species of *Punctodera*

Mihail R. KANTOR [1], Zafar A. HANDOO [1,*], Andrea M. SKANTAR [1], Maria N. HULT [1], Russell E. INGHAM [2], Nadine M. WADE [2], Weimin YE [3], Gary R. BAUCHAN [4] and Joseph D. MOWERY [4]

[1] *Mycology and Nematology Genetic Diversity and Biology Laboratory, USDA, ARS, Northeast Area, Beltsville, MD 20705, USA*

[2] *Botany and Plant Pathology Nematology Research Program & Extension Nematode Testing Service, Oregon State University, Corvallis, OR 97331, USA*

[3] *North Carolina Department of Agriculture & Consumer Services, 4300 Reedy Creek Road, Raleigh, NC 27607, USA*

[4] *Electron & Confocal Microscopy, USDA, ARS, Northeast Area, Beltsville, MD 20705, USA*

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Summary – *Punctodera mulveyi* n. sp. is described and illustrated from turf grass (*Poa annua*) in golf course greens with other fescues in Bandon, Coos County, Oregon, USA. Females and cysts are characterised by a saccate, globose to ovoid or pear-shaped body with a protruding neck. The cuticle has a lace-like pattern of ridges and heavy punctations on the subsurface. Cysts have distinctive vulval and anal circumfenestral patterns with heavy bullae scattered around the fenestral area, these being absent in young cysts. Second-stage juveniles (J2) vermiform, tapering to a long and cylindrical tail with a bluntly rounded to occasionally clavate tail terminus. Morphologically the new species resembles all known species of *Punctodera* using both light microscopy and scanning electron microscopy observations, but differs from the other species either by the J2 body and stylet length, shape of head, tail and tail terminus, female and male stylet or spicule length, and in having distinctive vulval and anal circumfenestral patterns in the cysts. Molecular analysis with sequence alignments and phylogenetic trees of ITS rDNA, nuclear heat shock protein 90 and mitochondrial *COI* sequences separated *P. mulveyi* n. sp. from *P. matadorensis*, *P. punctata*, *P. stonei* and *P. chalcoensis*, but 18S and 28S were relatively conserved with a few bp differences and there were insufficient *Punctodera* species sequences to give strong support to a new species designation. A morphologically most closely related species, *P. stonei* from Canada, further supported the status of *P. mulveyi* n. sp. An identification key to all five nominal species of *Punctodera* is given.

Keywords – 18S, 28S, *COI*, description, Hsp90, ITS, molecular, morphology, morphometrics, new species, phylogeny, *Poa annua*, SEM, taxonomy, turf grass.

Punctodera spp. are important plant pathogens displaying marked sexual dimorphism. Males and second-stage juveniles (J2) are vermiform and active. Females and cysts are saccate with a globose to ovoid or pear-shaped, spherical or subspherical shape with eggs inside the body and with a protruding neck, and cysts lacking a posterior protuberance or cone. *Punctodera* Mulvey & Stone, 1976 currently includes four species, namely: *P. punctata* (Thorne, 1928) Mulvey & Stone, 1976, *P. chalcoensis* Stone, Sosa Moss & Mulvey, 1976, *P. matadorensis* Mulvey & Stone, 1976 and *P. stonei* Brzeski, 1998. So far, *P. punctata* has been reported from several states in the USA, such as California, Michigan, Minnesota, New Jersey, North Dakota, South Dakota, and Texas, and a detailed distribution from other countries is given by Subbotin *et al*. (2010). *Punctodera matadorensis* is reported from North Dakota (Handoo *et al*., 2010) and Saskatchewan, Canada (Mulvey & Stone, 1976). *Punctodera chalcoensis* is a serious pest of corn in Mexico and is listed as a harmful organism in Brazil, Ecuador and South Korea

* Corresponding author, e-mail: zafar.handoo@ars.usda.gov

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(CAPS, 2017) and there may be trade implications with these countries if this nematode becomes established in the USA. *Punctodera stonei* is reported from Europe in several provinces of Poland and Slovakia, in Germany, Greece, The Netherlands, Spain, UK, and in Canada (Subbotin *et al*., 2010). They are mostly parasitic on grasses, with *Poa annua* L. being indicated as a common host for this genus (Subbotin *et al*., 2010). Morphologically, the species of *Punctodera* can be differentiated by the presence or absence of bullae in cysts, the stylet length, shape and length of tail, and tail terminus in the J2, female and male stylet lengths, and male spicule length. Two species of *Punctodera* have bullae present in all the cysts (*P. chalcoensis* and *P. matadorensis*), one species (*P. stonei*) has bullae only in the mature cysts but not in the younger cysts, and one species (*P. punctata*) does not have bullae (Subbotin *et al*., 2010).

In 2019, a cyst nematode from turf grass (*P. annua*) in golf course greens in Bandon, Coos County, Oregon, USA, was initially characterised and identified as *P. stonei* (Kantor *et al*., 2019). However, upon further examination *via* morphology, SEM and molecular analysis using additional markers, this population appeared to be quite different from *P. stonei* and is herein described as *P. mulveyi* n. sp. To help identify the species in this genus, a key to all five nominal species is presented.

Materials and methods

Morphological observation

In the spring and autumn of 2019 and in March 2020, 3 inch (*ca* 7.5 cm) core soil and root samples collected from turf grass (*P. annua*) in golf course greens with other fescues from Bandon, Coos County, Oregon, were mailed overnight by Oregon State University, Corvallis, OR, USA to the USDA, ARS (MNGDBL), Beltsville, MD, USA, for nematode analyses and species identification purposes. A high number of juveniles, cysts and eggs were separated from soil by sieving and Baermann funnel extraction. A few white females and males were recovered from the samples received in March 2020. Juveniles were fixed in 3% formaldehyde and processed to glycerin by the formalin glycerin method (Hooper, 1970; Golden, 1990). Females and some cysts were removed from roots after fixation for 12 h in 3% formaldehyde solution. Photomicrographs of both females and cysts, males, and J2 were taken with an automatic 35 mm camera attached to a compound microscope having an interference contrast system. Whole cysts were photographed under a Nikon SMZ 18 dissecting microscope using a Nikon DS-Ri2 16-megapixel camera. The light microscopic images of fixed nematodes were taken on a Nikon Eclipse Ni compound microscope using the same type of camera. Measurements were made with an ocular micrometer on a Leica WILD MPS48 Leitz DMRB compound microscope. In evaluation of the species for development of the key, our own data for the new species and the original descriptions of four other known species, as well as any subsequent re-descriptions, were utilised for the data included in Tables 1-3.

Low-temperature scanning electron microscopy (LT-SEM) was used to observe juveniles and cyst posterior ends of females. The specimens were observed using the techniques described in Carta *et al*. (2020). Briefly, nematodes were collected and placed into 1.5 ml Eppendorf tubes filled with a fixative composed of 2% paraformaldehyde, 2.5% glutaraldehyde, 0.05 M Na cacodylate, 0.005 M $CaCl_2$ for at least 12 h, rinsed in distilled water and individual nematodes were placed onto ultra-smooth, round (12 mm diam.) carbon adhesive tabs (Electron Microscopy Sciences) that were secured to 15 × 30 mm copper plates. The specimens were frozen conductively, in a styrofoam box, by placing the plates on the surface of a pre-cooled (−196°C) brass bar whose lower half was submerged in liquid nitrogen. After 20-30 s, the brass plate containing the frozen sample was transferred to the Quorum PP2000 cryo transfer system (Quorum Technologies) attached to an S-4700 field emission scanning electron microscope (Hitachi High Technologies America). The specimens were freeze-etched inside the cryotransfer system to remove any surface contamination (condensed water vapour) by raising the temperature of the stage to −90°C for 10-15 min. Following etching, the temperature inside the chamber was lowered below −130°C, and the specimens were coated with a 10 nm layer of platinum using a magnetron sputter head equipped with a platinum target. The specimens were transferred to a pre-cooled (−130°C) cryostage in the SEM for observation. An accelerating voltage of 5 kV was used to view the specimens. Images were captured using a 4pi Analysis System.

One of us (WY), from the NC Department of Agriculture, provided the DNA and one preserved cyst of a *P. stonei* population from turfgrass in Ottawa, ON, Canada, in ethanol. The cyst was cut off in a few drops of distilled water and four juveniles were recovered from the cyst. After morphometric data (see Tables 1, 2) and photomicro-

Table 1. Morphometrics of second-stage juveniles (J2) and males of *Punctodera mulveyi* n. sp. and other *Punctodera* species.

Character	*P. mulveyi* n. sp.		*P. punctata*		*P. matadorensis*		*P. chalcoensis*		*P. stonei*		
	This paper		After Mulvey & Golden (1983)	After Chizhov & Idekh (1980)	After Mulvey & Stone (1976)		After Stone *et al.* (1976)		This paper, ex. Ontario, Canada	After Subbotin *et al.* (2010) (several populations)	After Brzeski (1998)
	J2	Male	J2	Male	J2	Male	J2	Male	J2	J2	Male
	Paratypes	Paratype									
n	15	1	128	10	20	0	50	12	4	?	13
L	493 (465-510)	960	620 (520-680)	910-1050	526 (500-570)		533 ± 29	985 ± 69	505 ± 22.7 (485-535)	520 (470-590)	880 (840-960)
a	25.0 (22.0-28.0)	28.0	–	29.7-40.0	–		–	–	20.4 ± 1.0 (19.4-21.4)	26 (23.0-29.0)	31 (28.0-33.0)
b	4.3 (3.6-4.9)	6.0	–	4.8-6.2	–		–	–	–	–	7.3 (6.5-8.2)
b′	2.2 (1.9-2.6)	–	–	–	–		–	–	3.4 ± 0.3 (3.0-3.8)	2.6 (2.1-3.4)	–
c	7.0 (6.5-7.8)	320	–	162-226	–		–	–	6.8 ± 0.3 (6.2-7.1)	6.7 (5.9-7.9)	–
c′	5.3 (4.0-6.3)	–	–	–	–		–	–	–	5.7 (4.5-6.7)	–
Stylet	26.5 (24.0-28.0)	28.0	25 (24.2-25.8)	26-29	24.6 (24-25)		24.7 ± 0.6	26.8 ± 0.8	25.7 ± 1.2 (25.0-27.5)	25.4 (24.0-26.5)	27.4 (26.5-28.0)
Anal body diam.	13.0 (11.0-13.5)	–	–	–	–		–	–	–	–	–
Max. body diam.	20.0 (18.0-23.0)	28.0	–	–	–		–	–	24.7 ± 0.5 (24.0-25.0)	–	–
Head to pharyngo-intestinal junction	115 (100-126)	–	–	–	–		–	–	–	118 (95-132)	–
Head to gland tip	231 (195-260)	–	–	–	–		–	–	147 ±18.3 (127-175)	201 (148-257)	–
Anterior end to excretory pore	94 (88-100)	–	–	–	–		108 ± 4.9	131.3 ± 7.7	–	99 (94-111)	123 (117-132)
Tail length	70 (62-75)	3.0	70.1 (62.7-77.5)	–	61.7 (58-65)		63.2 ± 3.3	2.8 ± 1.4	74 ± 2.6 (70-77)	78 (60-95)	5-8
Hyaline tail terminus	47 (35-55)	–	40 (38.5-41.4)	–	39 (35-42)		38.2 ± 3.1	–	48 ± 3.4 (45-53)	50 (41-66)	–
Spicule	–	36.0	–	28.0-34.0	–		–	32 ± 1.8	–	–	33 (29-36)
Gubernaculum	–	–	–	8.0-10.0	–		–	7.3 ± 0.8	–	–	–

Table 2. Morphometrics of females and cysts of *Punctodera mulveyi* n. sp. and other *Punctodera* species.

Character	*P. mulveyi* n. sp.			*P. punctata*		*P. matadorensis*		*P. chalcoensis*		*P. stonei*	
	This paper			After Horne (1965)	After Chizhov & Idekh (1980)	After Mulvey & Stone (1976)	After Mulvey & Golden (1983)	After Stone *et al.* (1976)		After Brzeski (1998)	
	Female		Cyst	Female	Cyst	Female	Cyst	Female	Cyst	Female	Cyst
	Holotype	Paratypes	Paratypes								
n	–	8	10	20	?	12	?	20	20	0	25
L	425	419 (300-695)	469 (421-550)	370 (330-420)	565 (392-823)	483 (430-560)	500-600	473 ± 105	441 ± 69	–	0.6 (0.5-0.7)
Diam.	321	333 (237-520)	230 (183-272)	236 (170-320)	387 (298-710)	403 (320-530)	480-500	429 ± 120	416 ± 61	–	0.44 (0.4-0.6)
Stylet length	18	18 (18-18)	–	27 (20-25)	–	22 (21-23)	–	25.8 ± 0.9	–	–	–
Length/Diam. ratio	1.3	1.3 (1.1-1.7)	–	1.6 (1.2-2.5)	1.5 (1.2-1.9)	1.2 (1.0-1.4)	–	1.2 ± 0.9	1.1 ± 0.1	–	1.3 (1.0-1.5)
Neck length	60	69 (50-85)	75 (60-85)	123 (110-150)	–	115-140	–	142 ± 24	95 ± 26	–	80-130
Neck diam.	40	45 (38-60)	37 (29-50)	–	–	–	–	–	–	–	–
Vulval slit length	15	15 (15-15)	8.8 (7.5-10.0)	–	–	4	–	4.0 ± 0.5	4.2 ± 0.4	–	–
Vulval fenestra length	27.5	28.7 (16-35)	26.5 (23.0-27.5)	16.5	–	–	24	30.7 ± 7.0	18.1 ± 2.7	–	26.6 (22.0-36.0)
Vulval fenestra diam.	27.0	27.2 (25-30)	23.0 (20.0-27.5)	20.3	–	20	–	32.1 ± 7.0	19.8 ± 2.9	–	26.6 (22.0-36.0)
Anal fenestra length	–	–	25.5 (18.0-35.0)	–	–	20	25	29.5 ± 5.7	21.1 ± 3.4	–	23.0 (18.0-32.0)
Anal fenestra diam.	–	–	21.5 (16.0-25.0)	–	–	20	–	1.1 ± 5.3	22.4 ± 3.1	–	23.0 (18.0-32.0)
Distance between fenestra	–	–	53 (45-65)	–	27 (17-42)	–	–	67.7 ± 14.5	42.3 ± 8.8	–	59 (32-140)
Vulval fenestra diam.	–	–	–	–	33 (25-42)	–	–	–	–	–	27 (22-36)

Table 3. Genetic distances within mitochondrial *COI* sequences (expressed as bp differences) among *Punctodera mulveyi* n. sp. and selected *Punctodera* spp. and *Globodera pallida*.

Species and GenBank accession no.	*P. mulveyi* n. sp. MN267176	*P. stonei* MN267175	*P. chalcoensis* HM640928	*P. punctata* KC172917	*G. pallida* MH399819
P. mulveyi n. sp.	–	28	21	25	80
P. stonei	28	x	29	6	83
P. chalcoensis	21	29	x	26	76
P. punctata	25	6	26	x	81
G. pallida	80	83	76	76	

graphs were taken, the juveniles were then submitted for molecular analysis.

DNA EXTRACTION AND PROCESSING

Single juveniles were mechanically disrupted in 20 μl nematode extraction buffer. The internal transcribed spacer region 1, 5.8S and region 2 (ITS), large ribosomal subunit 28S D2-D3 (28S), small ribosomal subunit 18S (18S), mitochondrial cytochrome oxidase subunit I (*COI*), and heat shock protein 90 (Hsp90) molecular markers were examined. The ITS was amplified with primers TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) as described in Skantar *et al*. (2012). The PCR products were cleaned with the Monarch DNA Gel Extraction Kit (NEB) and then cloned using the Strataclone PCR Cloning Kit (Agilent). Seven ITS clones representing two J2 were prepared with the Monarch Plasmid Miniprep Kit (NEB) and sequenced by Genewiz, Inc. The 28S was amplified using primers D2A (5′-ACAAGTACCGTGAGGGAAAGTT-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) as previously described (De Ley *et al*., 2005; Ye *et al*., 2007). The 18S was amplified with primers 18S-G18S4 (5′-GC TTGTCTCAAAGATTAAGCC-3′) and 18S-18P (5′-TG ATCCWKCYGCAGGTTCAC-3′) according to Carta *et al*. (2016) and sequenced using the above primers and internal primers 550F (5′-GGCAAGTCTGGTGCCA GCAGCC-3′) and 1108R (5′-CCACTCCTGGTGGTGCC CTTCC-3′). Hsp90 sequences were amplified with primers U288 (5′-GAYACVGGVATYGGNATGACYAA-3′) and L1110 (5′-TCRCARTTVTCCATGATRAAVAC-3′) according to Skantar & Carta (2004). Hsp90 PCR and cloning were performed according to Skantar *et al*. (2020); clones were sequenced with M13F and M13R vector primers and the internal primer Punc3R (5′-GTTC AGCTCTTCGTCCTCAATG-3′). Mitochondrial *COI* was amplified with primers Het-coxiF (5′-TAGTTGATC GTAATTTTAATGG-3′) and Het-coxiR (5′-CCTAAAA CATAATGAAAATGWGC-3′) and amplified as described previously (Subbotin, 2015). GenBank accession numbers were assigned to new sequences as follows: 18S (MN123231); ITS rDNA (MN121000-MN121006); 28S rDNA (MN123246-MN123248, MT845117-MT845118); COI (MN267175-MN267178); and Hsp90 (MN182655, MN182656, MT661444-MT661447).

Multiple sequence alignments of newly obtained DNA sequences and those available from GenBank were created for each marker using Geneious Prime 2019.0.3 (www.geneious.com) with built-in parameters or MAFFT. Outgroup taxa for each gene were selected in accordance with prior published studies of Punctoderidae. Best fitting models of nucleotide substitution were estimated using jModelTest based on the Akaike Information Criterion. Phylogenetic relationships were estimated with Bayesian inference (BI) on the CIPRES Science Gateway (http://www.phylo.org/; Miller *et al*., 2010). The parameters for BI analyses were implemented in CIPRES as described in Skantar *et al*. (2012), with a random starting tree, two independent runs with four chains (1.0×10^6 generations). Markov chains were sampled at intervals of 500 generations and burn-in of 10 000. The 50% majority rule consensus trees were generated with posterior probabilities (PP) calculated for each clade.

Results

Genus *Punctodera* Mulvey & Stone, 1976

AMENDED DIAGNOSIS (AFTER MULVEY & STONE, 1976; SIDDIQI, 2000)

Punctoderidae: Mature female and cyst: without posterior protuberance, colour pale to dark brown, darkening with age, globose, spherical to subspherical, ovoid, or

pear-shaped, with short projecting neck and heavy subcrystalline layer. Cuticle with lace-like pattern of ridges and subcuticle with punctations. D-layer present. Vulval slit very short, underbridge and perineal tubercles absent. Bullae present or absent (in two species including *P. stonei* and *P. mulveyi* n. sp., older cysts with heavily cuticularised bullae scattered around fenestra but absent in younger cysts). A circumfenestrate fenestra surrounding both vulva and anus and of similar size. Eggs retained in body, no egg sac. Anus offset towards ventral margin of anal fenestra. J2: vermiform, body less than 1 mm long, stylet length less than 32 μm long. Pharyngeal glands long, overlapping mostly ventrally but also laterally, filling body cavity. Tail conical, 60-93 μm long, hyaline tail region 38-64 μm long. Lateral field with four incisures. Phasmid openings punctiform, without a lens-like structure in muscle layer. Male: vermiform, less than 1.5 mm long. Labial disc present but with low profile. Cloacal opening thickened, prominent. Spicules 28-36 μm long with smooth tip. Tail less than half body diam. long. Parasites of monocotyledonous plants. Only one generation occurs each year for *P. punctata*, but situation unknown for other species.

TYPE SPECIES

Punctodera punctata (Thorne, 1928) Mulvey & Stone, 1976
= *Heterodera punctata* Thorne, 1928
= *Heterodera* (*Globodera*) *punctata* Thorne, 1928 (Skarbilovich, 1959)
= *Globodera punctata* (Thorne, 1928) Skarbilovich, 1959

OTHER SPECIES

P. chalcoensis Stone, Sosa Moss & Mulvey, 1976
P. matadorensis Mulvey & Stone, 1976
P. mulveyi n. sp.
P. stonei Brzeski, 1998

Punctodera mulveyi* **n. sp.**
(Figs 1-4)

MEASUREMENTS

See Tables 1 (J2 and male) and 2 (white female and cyst).

* The species name is given in honour of Dr R.H. Mulvey for his outstanding contributions to our knowledge of cyst nematodes.

DESCRIPTION

J2

Morphometric details from 15 juveniles of *P. mulveyi* n. sp. from Oregon and four juveniles of *P. stonei* from Canada are given in Table 1.

Slightly arcuate upon relaxation with distinct cuticular annulation. Lateral field with four lines, outer two lines areolated, starting from anterior end above pharyngeal region and extending to hyaline portion of tail. Labial region offset, bearing four or five annules with distinct basal annule when seen in light microscopy. SEM observations showing four annules, extra annule seen in LM being part of lips. Oral disc distinct, elongated dorso-ventrally to more than twice its width and surrounded by distinct lateral lips bearing amphidial apertures, submedian lips fused. Stylet strong, knobs anchor-shaped, rounded anteriorly, 6-8 μm in diam. Pharyngeal gland lobe with long ventral and lateral overlap, *ca* 113 (90-125) μm long from pharyngo-intestinal junction to gland tip. Phasmids not prominent, located 11-13 μm posterior to anal opening. Genital primordium clearly visible, body annules prominent, tail tapering evenly in anterior portion, becoming thin, long and cylindrical in posterior part, variation in tail terminus varying from bluntly rounded to slightly pointed terminus with few specimens having slightly clavate-shaped terminus, otherwise mostly rounded.

Cyst

Globose, ovoid to spherical or subspherical with posterior end rounded, light to medium or dark brown in colour. Young cysts with subcrystalline layer. Fenestral area with distinct punctation and cuticle with transverse rows, ridge-like near fenestral area, occasionally zigzag in some parts of body. Dark large bullae present between vulval fenestra or scattered around fenestral area in mature cysts, absent in young or small cysts.

Female

White, spherical or subspherical with projecting neck and without vulval cone. Head with two annules. Stylet slender, 18 μm long with faint rounded knobs. Excretory pore located near base of neck. Cuticle with lace-like to ridge-like pattern with heavy punctations. Vulval fenestra and anal fenestra usually of about equal size, separated by a distance of *ca* 1-3 vulval fenestral widths. Bullae usually absent, vulval slit small, anal fenestra 18-35 μm long.

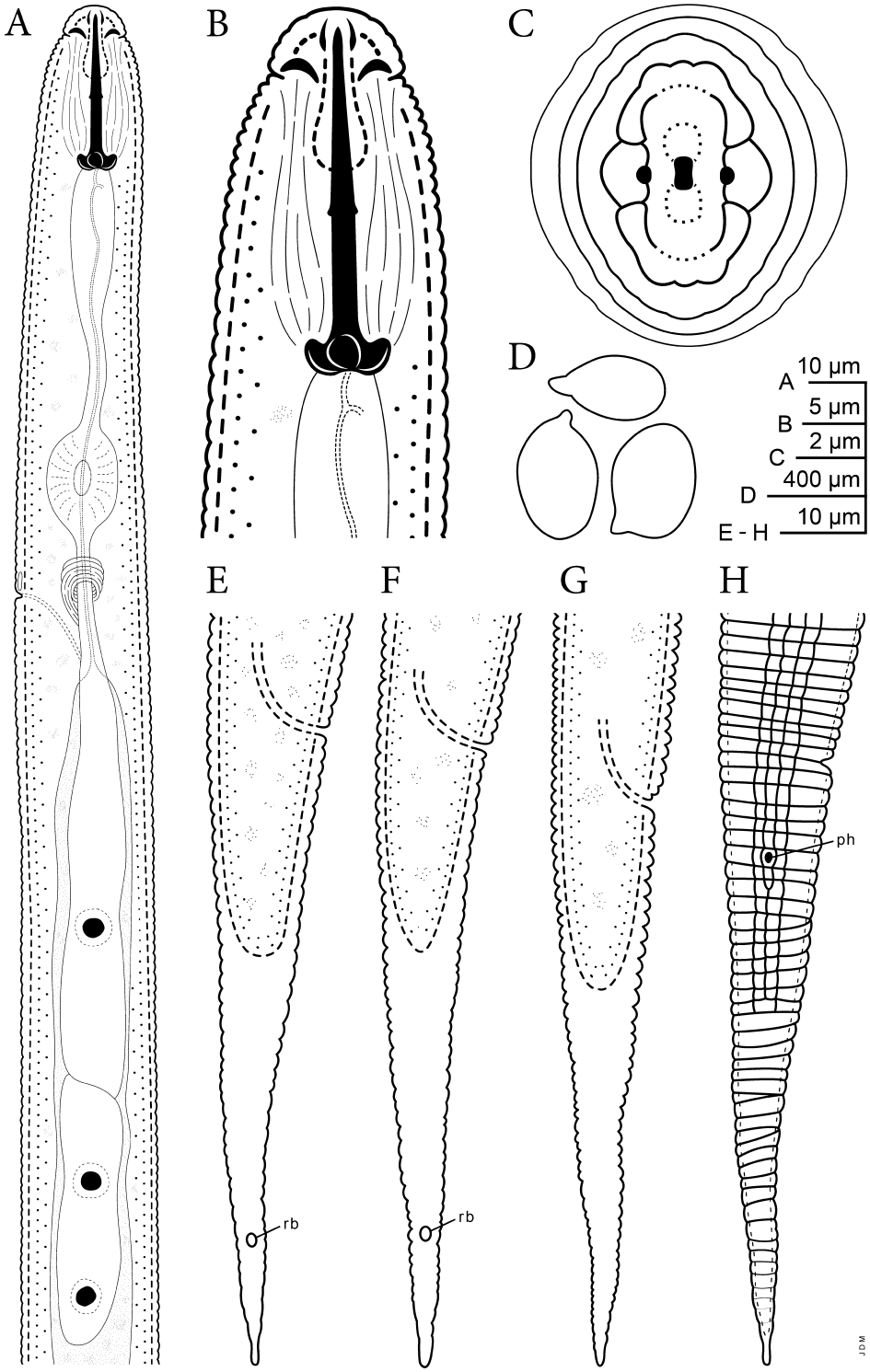

Fig. 1. Line drawings of *Punctodera mulveyi* n. sp. Second-stage juveniles and female cysts. A: Pharyngeal region; B. Lip region; C: Details of lip region showing oral disc (*en face* view); D: Female cysts; E-H: Juvenile tails, with E and F showing refractile bodies (rb) in the hyaline region, and H showing the phasmid (ph) between the areolated lateral field.

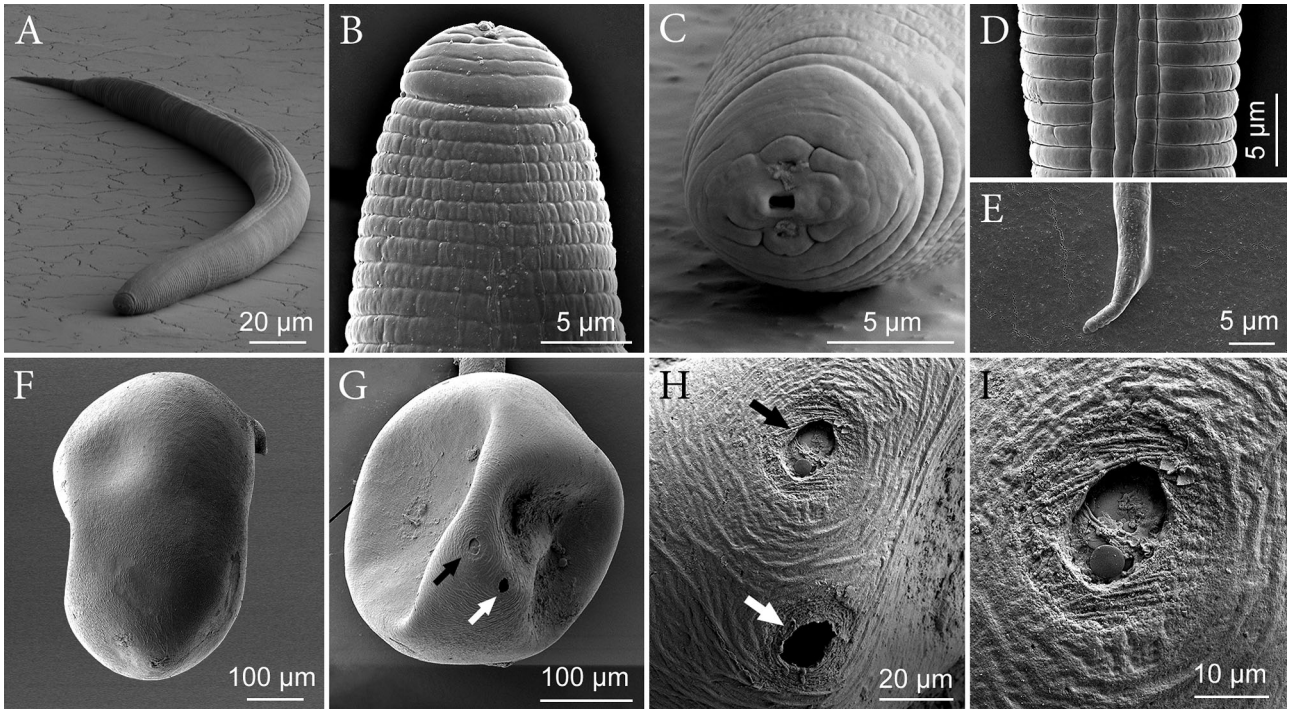

Fig. 2. SEM of *Punctodera mulveyi* n. sp. A-E: Second-stage juvenile. A: Entire body; B: Lateral view of head region; C: *En face* lip pattern; D: Lateral incisures at mid-body; E: Lateral view of tail. F-I: SEM of cyst. F: Entire cyst; G, H: Terminal regions showing vulval (black arrow) and anal (white arrow) fenestral regions with image in H magnified; I: Showing more magnified view of vulval fenestral area.

Male

Body vermiform with ventral curvature upon heat relaxation or when killed and fixed in 3% formaldehyde. Head offset with 5-7 fine annules and a labial disc. Cuticular annulation distinct. Lateral field with four incisures. Stylet well developed, 28 μm long with flat to concave knobs anteriorly. Tail bluntly rounded, less than 0.25 body diam. long. Spicules tapering distally and curved ventrally with smooth tip, single non-ornamented gubernaculum. Phasmids indistinct.

Type host and locality

Recovered from roots and around soil of turf grass (*Poa annua*) with other fescues in golf course greens from Bandon, Coos County, OR, USA, global positioning coordinates 43.188221°N 124.390174°W.

Type material

Holotype (female): slide T-727t deposited in the United States Department of Agriculture Nematode Collection, Beltsville, MD, USA. Paratypes (females, cysts, J2 and male): same data and repository as holotype, slides T-7384p-T7399p (seven slides with cysts, three slides with females, five slides with J2 and one slide with a male). Additional cysts and J2 on slide numbers T-7400p-T-7401p at University of California, Riverside, CA, USA, and T-7402p to T-7403p at FERA, Plant Pest Disease Cultures and Collections, York, UK.

Diagnosis and relationships

Punctodera mulveyi n. sp. is characterised by having females and cysts saccate, globose to ovoid, or pear-shaped with rounded posterior ends, vulval fenestra and anal fenestra of almost equal size, cuticle and fenestral area with distinct punctations, ridge like pattern with dark bullae present in older cysts located under surface in between the fenestra, while young cysts lack bullae, J2 vermiform, tapering to a long cylindrical tail, long hyaline tail terminus that is bluntly rounded to occasionally clavate, and lateral field with four lines.

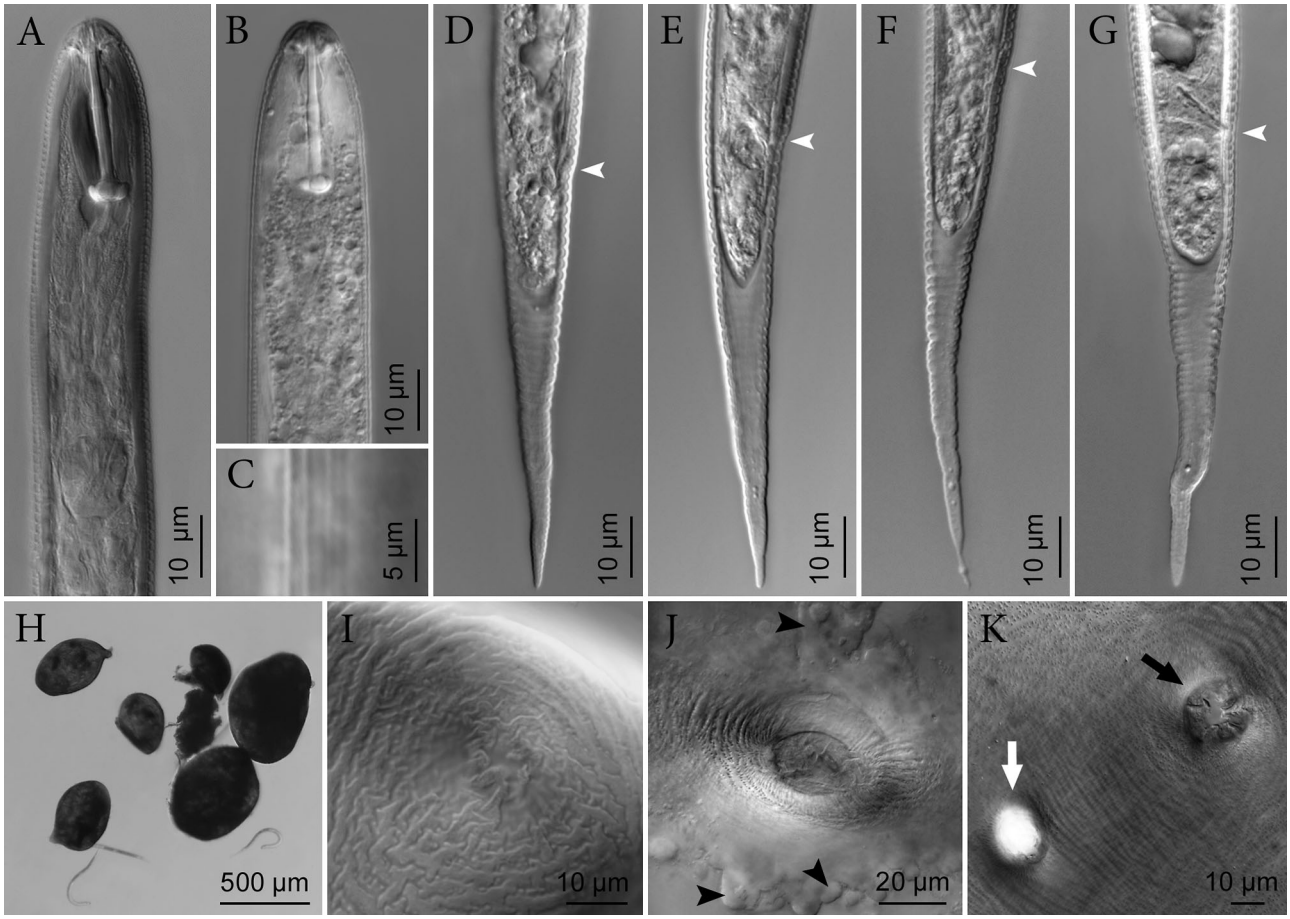

Fig. 3. Photomicrographs of *Punctodera mulveyi* n. sp. A-G: Second-stage juvenile. A, B: Anterior region; C: Lateral field showing four incisures near mid-body; D-G: Tail region showing variations in tail shape (arrowheads pointing to anal opening). H-K: Cyst. H: Entire cysts; I-K: Terminal region (I: Ridge like pattern; J: Vulval fenestral area with arrowheads showing bullae; K: Anal fenestra (white arrow) and vulval fenestra (black arrow)).

Morphologically, the new species resembles all four known species of *Punctodera*. It differs from *P. stonei* (additional data from a Canadian population are given in Table 1) by having a slightly shorter J2 body length of 493 (465-510) *vs* 520 (470-590) μm, the distance from the anterior end to the excretory pore is slightly shorter at 94 (88-100) *vs* 99 (94-111) μm, and by having a shorter tail and hyaline tail terminus of 70 (62-75) *vs* 78 (60-95) μm and 47 (35-55) *vs* 50.0 (41.0-66.0) μm, respectively. In addition, the cysts are smaller (470 (420-550) *vs* 600 (470-740) μm) and have a shorter distance between the fenestra (53.0 (45.0-65.0) *vs* 59.0 (32.0-140.0) μm).

The J2 can be separated from that of *P. chalcoensis* by a slightly shorter body length of 493 (465-510) *vs* 542 $\pm$ 26 μm, longer stylet, 26.5 (24.0-28.0) *vs* 24.7 $\pm$ 0.6 μm, and longer tail (70 (62-75) *vs* 63.2 $\pm$ 3.3 μm) and longer hyaline tail region (47 (35-55) *vs* 38.2 $\pm$ 3.1 μm). The cysts have a shorter distance between the fenestrae (53.0 (45.0-65.0) *vs* 142.3 $\pm$ 8.8 μm) and large dark bullae are present between the fenestra or scattered around the fenestral area in mature cysts, but absent in young or small cysts *vs* bullae lacking from many cysts but may occasionally be present in some specimens when they are small and scattered in the perineal region. The females have a shorter stylet length of 18.0 (18-18) *vs* 25.8 $\pm$ 0.9 μm and the male stylet and spicule length are slightly longer at 28.0 *vs* 26.8 $\pm$ 0.8 μm and 36.0 *vs* 32. 0 $\pm$ 1.8 μm, respectively.

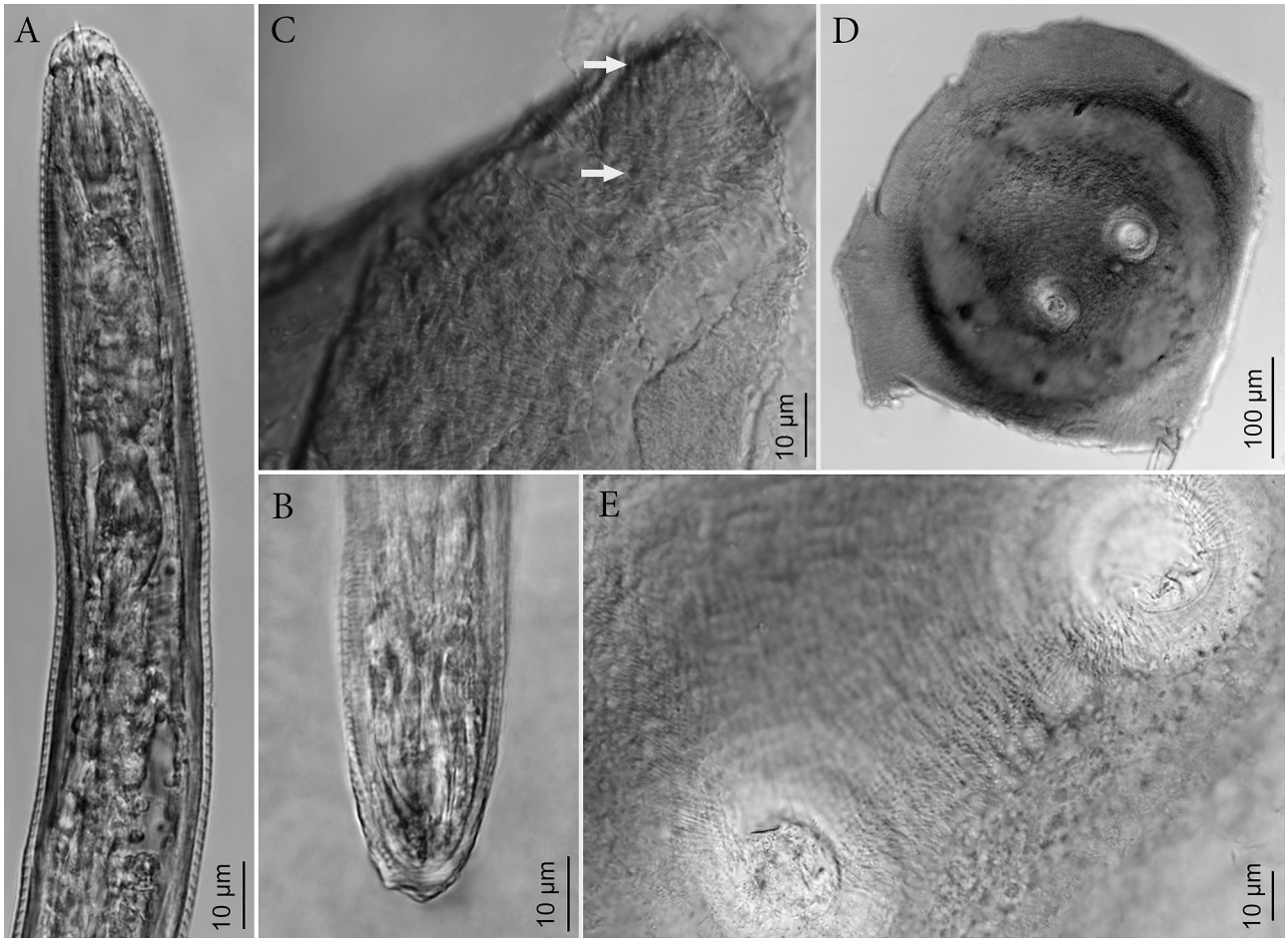

Fig. 4. Photomicrographs of *Punctodera mulveyi* n. sp. male and young females. A, B: Anterior and posterior end of male; C: Anterior region of young female with arrows showing stylet; D, E: Cone mounts of young females showing the fenestra.

The J2 of the new species can be distinguished from *P. punctata* by having a slightly shorter body length of 493 (465-510) *vs* 620 (520-680) μm, shorter tail (70 (62-75) *vs* 80 (68-93) μm) and shorter hyaline tail terminus (47 (35-55) *vs* 53.0 (37.0-64.0) μm). The cysts have bullae present *vs* absent, are smaller in size, 470 (420-550) *vs* 560 (400-820) μm, and the females have a shorter stylet, 18.0 (18-18) *vs* 23.2 (20.0-25.0) μm.

From *P. matadorensis*, the new species J2 differs in having a slightly longer stylet (26.5 (24-28) *vs* 24.6 (24-25) μm), with anchor-shaped knobs that are rounded anteriorly *vs* knobs strongly concave anteriorly, longer tail of 70 (62-75) *vs* 62 (58-65) μm and hyaline tail terminus 47 (35-55) *vs* 39.1 (35-42) μm. The female has a shorter stylet, 18 (18-18) *vs* 22 (21-23) μm, and males are present *vs* absent.

Molecular analysis

Amplification of DNA markers from *P. mulveyi* n. sp. yielded sequences of the following lengths: 1643 bp from 18S rDNA; 750 bp from 28S rDNA; 985 bp from ITS rDNA; 1982 bp for nuclear Hsp90; and 497 bp for mitochondrial *COI*. These sequences were compared by BlastN to existing sequences in GenBank.

18S showed 99.8% similarity with 3 bp difference to a 1703 bp sequence of *P. stonei* from The Netherlands (EU682391; Holterman *et al*., 2008), 5 bp differences to the 18S portion of the 2662 bp-long sequence *Punctodera* sp. QY-2011 from Canada (JF895515), and with 1-2 bp difference from several shorter *ca* 750 bp 18S sequences of *P. stonei* from the type locality in Poland (KC852178-KC152181) (Dobosz *et al*., 2013). As re-

ported in that paper, the Canadian population QY-2011 was confirmed to be molecularly identical to *P. stonei*. Unfortunately, no other 18S is available for the other three *Punctodera* species. Two of the seven ITS rDNA clones from *P. mulveyi* n. sp. came from one J2 and five from another. The intraclonal variation within ITS ranged from 98.9-99.8% identity; two clones amplified from J2-1 varied at 2 bp and the five clones from J2-2 varied from 3-10 bp, so variation was not limited to one J2 or the other. ITS rDNA sequences from *P. mulveyi* n. sp. varied at 8-11 bp with *P. chalcoensis* (98.6-99% identity); 10-14 bp with *P. matadorensis* (98.5-98.9%); and 20-29 bp with *P. punctata* (96.8-97.8%). ITS from *P. stonei* from Canada (JF895515) was only 97.0-97.4% similar, varying at 24-27 bp; no ITS rDNA sequences were available for the type specimens of *P. stonei* from Poland, thus no direct ITS comparison to that population was possible.

For 28S, similarity was highest to *P. matadorensis* at 99.6% (MK660273; 2-3 bp difference), and 99.1% to *P. punctata* (MK660274; 6-7 bp) and *P. stonei* QY-2011 from Canada (JF895516; 9-10 bp), and 97.8% to *P. stonei* from Poland (KC852182-KC852185; 10-11 bp). It should be noted that the 28S of *P. mulveyi* n. sp. overlapped with the latter sequences only partially over 569 bp due to their amplification of a slightly different 28S fragment (Dobosz *et al*., 2013).

Phylogenetic trees were constructed from Bayesian Inference (BI) using a 917-bp ITS rDNA sequence alignment as shown in Figure 5. Sequences from *P. mulveyi* n. sp. grouped in a strongly supported clade that was distinct from those of *P. matadorensis*, *P. punctata*, and notably distinct from Canadian *P. stonei*. Trees inferred from BI analysis of a 911-bp alignment of 28S rDNA (Fig. 6) showed that *P. mulveyi* n. sp. was separated from the main clade that included *P. stonei* from Poland and *P. punctata* from Belgium. However, these differences are minor as shown by short branch length difference.

While ITS showed clear separation of *P. mulveyi* n. sp. from *P. stonei* (Fig. 5), the initial analysis of 18S and 28S left the new species status somewhat in doubt due to the relative lack of sequences to provide context for the observed variation. Subsequently, we obtained specimens of *P. stonei* from Canada, allowing a direct comparison using additional markers from the two populations. Excluding the degenerate primer ends, Hsp90 from the two *P. mulveyi* n. sp. sequences varied from one another at 5 bp (0.3%). The four clones from *P. stonei* varied from each other at 4-19 bp (0.2-1%), most of which was within introns. The conserved protein domains within Hsp90 anchor the size of exons across species but the introns may vary in length and composition, providing another source of phylogenetic signal with which to separate species. The amplified region of Hsp90 in this dataset encompasses six exons and five introns. Due to the conservation of protein domains reflected in the coding regions, the length of exons typically varies only slightly among species, if at all. However, between *P. mulveyi* n. sp. and *P. stonei*, two of the five Hsp90 introns differ substantially in length; intron 1: 569 *vs* 580 bp and intron 2: 75 *vs* 113 bp. The phylogenetic tree constructed from Hsp90 genomic DNA sequences (Fig. 7) shows strong separation of *P. mulveyi* n. sp. from *P. matadorensis* (differing at 349-353 bp;17% difference); the clade comprising these two species was clearly distinct from a clade containing several Hsp90 clones representing *P. stonei* from Canada (differing at 400-406 bp; 20% difference) and the clade for *P. punctata* from MA (differing at 599-607 bp; 28% difference). The variation observed amongst clones from the same population was significantly smaller than the bp differences between the species.

As shown in Table 3, mitochondrial *COI* sequences from *P. mulveyi* n. sp. differed from *P. stonei* from Canada by 28 bp (8.4%), by 21 bp (6.3%), from *P. chalcoensis*, and by 25 bp (7.5%) from *P. punctata*, adding strong support to its designation as a new species. The *COI* tree likewise reflected this separation of *P. mulveyi* n. sp. from other *Punctodera* species (Fig. 8), but closer to *P. stonei* and *P. punctata*. Alignment of translated *COI* sequences shows that the *P. mulveyi* n. sp. amino acid sequence is identical to that of *P. stonei* and differs from *P. chalcoensis* at one position and from *P. punctata* at four positions. The majority of base differences occur at the 3rd codon positions since most of the amino acids do not vary.

Identification of *Punctodera* species

In some *Punctodera* species, the known range of variation is limited to observation of specimens in single populations from the type locality. Further morphological studies, including SEM and more specimens from a broader spectrum of habitats, are needed to examine the relationships and identities of some species. It is a challenge to identify these species solely by morphology and we agree with Subbotin *et al*. (2010) that more detailed molecular and morphological studies are required to evaluate the reliability of some of the characters for

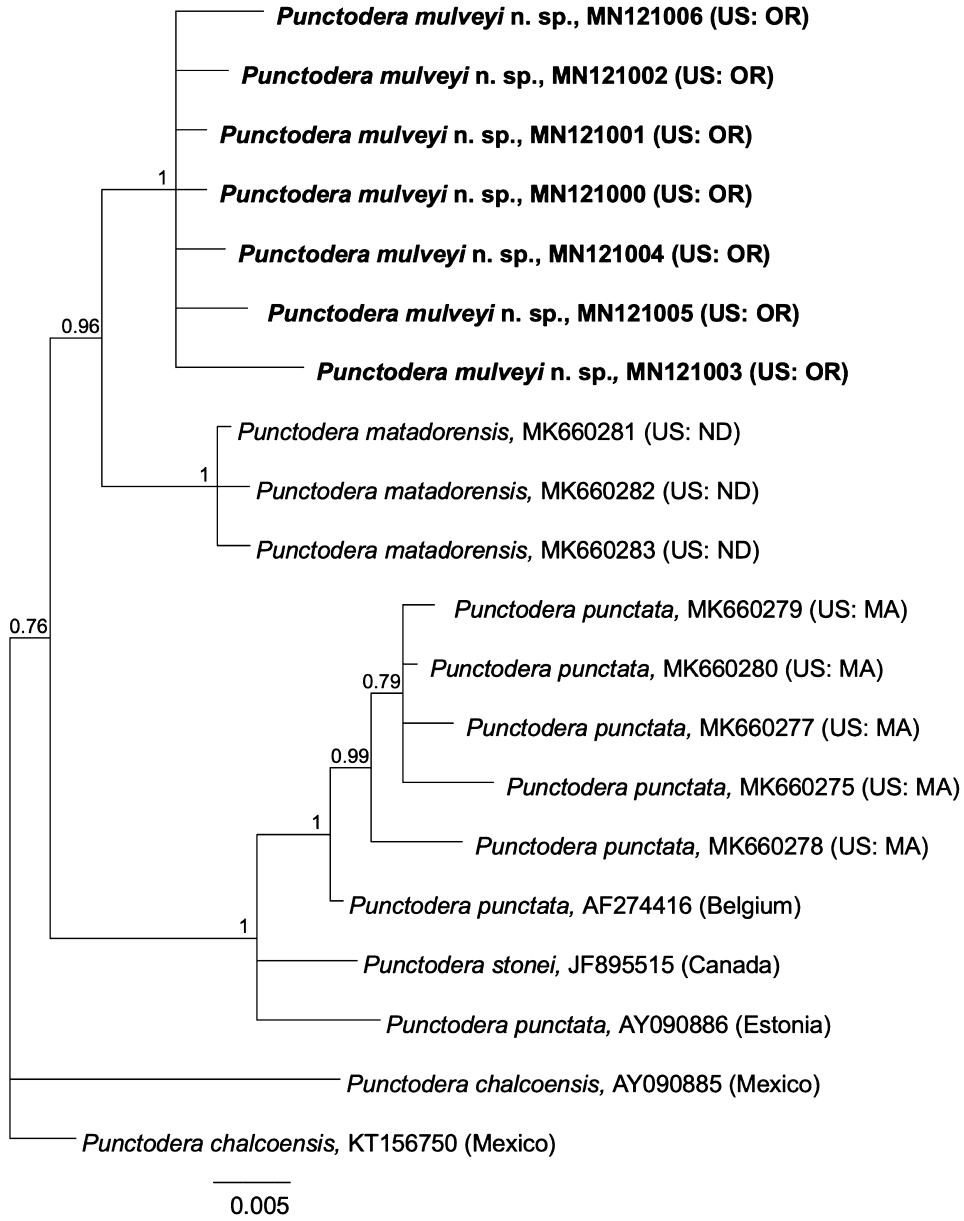

Fig. 5. Phylogenetic relationships of *Punctodera mulveyi* n. sp. and other selected cyst nematodes, as inferred from a 917 bp alignment of ITS rDNA, according to the GTR + I + G model of nucleotide substitution. The parameters for BI analyses were implemented in the Geneious CIPRES plug-in for MrBayes, with a random starting tree, two independent runs with four chains for 1.0×10^6 generations. Markov chains were sampled at intervals of 500 generations and burn-in was 10 000. A 50% majority rule consensus tree was generated with posterior probabilities (PP) shown on appropriate branches and *P. chalcoensis* as the outgroup. New sequences are indicated in bold.

diagnostic purposes and to further test the validity of *Punctodera* species.

The key to species is based on overall morphology of cysts, females, males and J2 and works well with all five species, including this new species, as well as the specimens of this genus deposited in the USDA Nematode Collection (Handoo *et al*., 1998, 2018). A compendium of

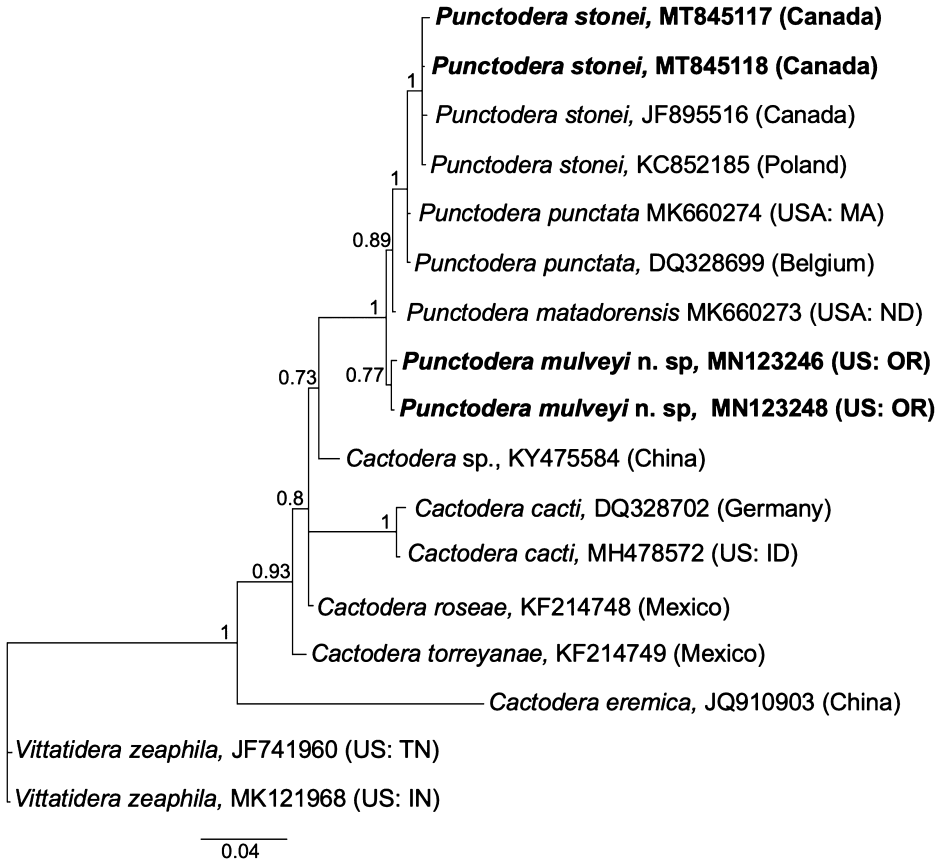

Fig. 6. Phylogenetic relationships of *Punctodera mulveyi* n. sp. and other selected cyst nematodes, as inferred from a 911 bp alignment of 28S rDNA, according to the GTR + I + G model of nucleotide substitution and incorporated into MB as described in Figure 5. A 50% majority rule consensus tree was generated with posterior probabilities (PP) shown on appropriate branches with *Vittatidera zeaphila* as the outgroup. New sequences are indicated in bold.

Punctodera spp. morphometric data is provided (Tables 1, 2).

Key to species of *Punctodera*

1. Cysts pear- or oval-shaped, bullae absent, males present with dorsal contour of tail convex, parasitising wheat and grasses . . . . . *P. punctata*
   Cysts spherical to subspherical or oval-shaped, bullae present, males present or absent, if present, dorsal contour near tail terminus concave or broadly rounded 2
2. Bullae small and scattered; female stylet >25 μm long; males present with conical tail and bluntly rounded terminus, parasitising maize in Mexico . . . . . *P. chalcoensis*
   Bullae massive, located between vulval and anal fenestra, female stylet 18-23 μm long; males present or absent, parasitising grasses 3
3. J2 stylet 24.6 (24-25) μm long, with knobs strongly concave anteriorly, tail 62 (58-65) μm and hyaline region 39.1 (35-42) μm long; female stylet 22 (21-23) μm long; males absent . . . . . *P. matadorensis*
   J2 stylet 26.7 (26.7-27.0) μm long, knobs anchor-shaped, tail 78 (60-95) μm long, hyaline region 50 (41-66) μm long; males present, stylet and spicule 27.4 $\pm$ 0.8 (26.5-28.0) μm and 33 (29-36) μm long, respectively . . . . . *P. stonei*
   J2 stylet 26.5 (24-28) μm long, with knobs anchor-shaped and or rounded anteriorly; tail 70 (62-75) μm long, hyaline region 47 (35-55) μm long; female stylet

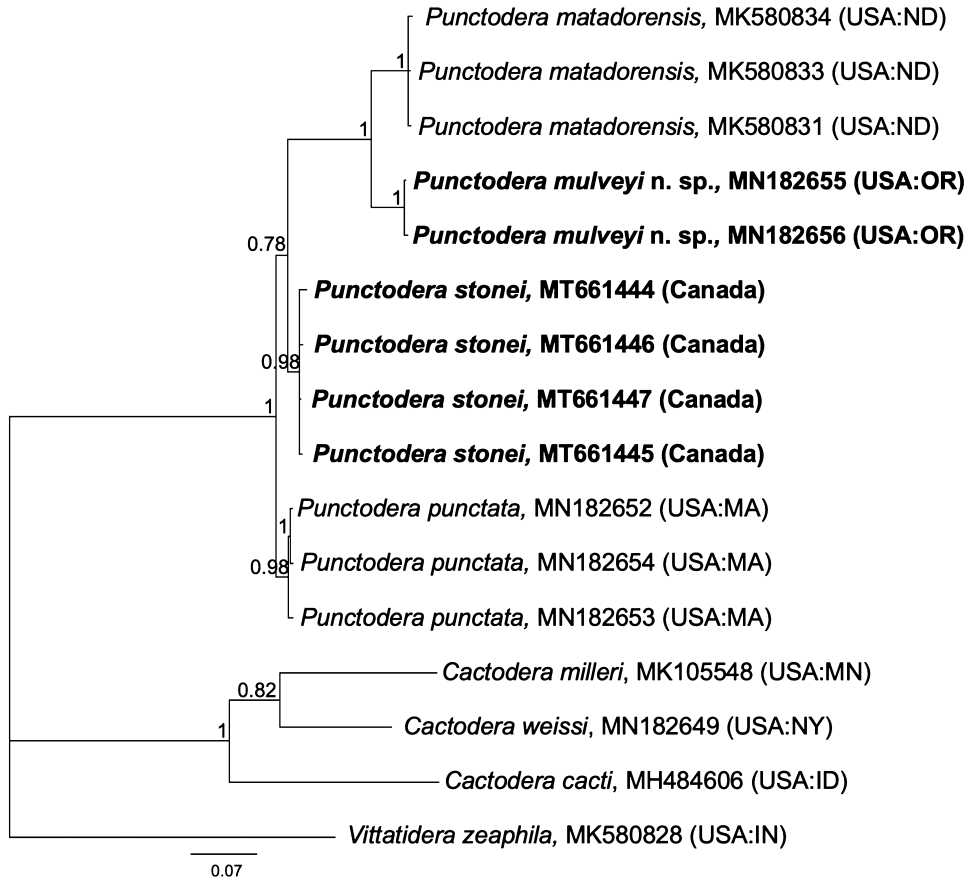

Fig. 7. Phylogenetic relationships of *Punctodera mulveyi* n. sp. and other selected cyst nematodes, as inferred from a 2326 bp alignment of Hsp90 genomic DNA, according to the GTR + I + G model of nucleotide substitution. The parameters for BI analyses were implemented in the Geneious CIPRES plug-in for MrBayes and incorporated into MB as described in Figure 5. A 50% majority rule consensus tree was generated with posterior probabilities (PP) shown on appropriate branches with *Vittatidera zeaphila* as the outgroup. New sequences are indicated in bold.

shorter 18 μm long; males present, stylet and spicules 28 and 36 μm long, respectively . . . . . *P. mulveyi* n. sp.

Discussion

Cryptic species have been described for several groups of plant-parasitic nematodes (Palomares-Rius *et al*., 2014). The *Xiphinema* species complex presents an especially good example of cryptic species. With more than 55 *Xiphinema* species described, all are quite similar in morphology and with overlapping morphometrics. Nevertheless, molecular data supported the establishment of several valid new species (Gutiérrez-Gutiérrez, 2010, 2012; Zasada *et al*., 2014; Zhao *et al*., 2017). Among the cyst nematodes, both *Globodera rostochiensis* and *G. pallida* have been proposed to contain cryptic species, supported by molecular approaches including RAPD, satellite DNA, ITS-rRNA RFLP, and sequence analysis (Grenier *et al*., 2010; Madani *et al*., 2010; Subbotin *et al*., 2010; 2011). Within *Punctodera*, Wouts & Baldwin (1998) recognised five different species: *P. punctata* from wheat, with short juveniles; *P. matadorensis* and *P. stonei* from native grasses and with distinct bullae; *P. chalcoensis* from maize; and an undescribed species from grasses. We do not know the source of this undescribed species from grasses, but our study refers to the subject of this paper, previously undescribed until now from Oregon. We agree with the remarks given in Subbotin *et al*. (2010) that several authors (Oostenbrink, 1960; Mulvey, 1972; Solovjeva & Vasiljeva, 1973; Wouts *et al*., 1986) consider that *P.*

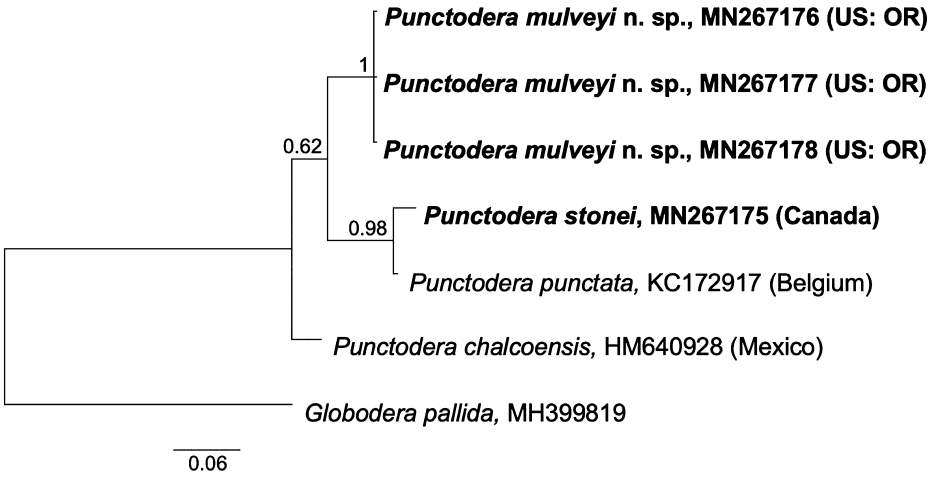

Fig. 8. Phylogenetic relationships of *Punctodera mulveyi* n. sp. and other select cyst nematodes, as inferred from a 332 bp alignment of mitochondrial COI, according to the GTR + I + G model of nucleotide substitution and incorporated into MB as described in Figure 5. A 50% majority rule consensus tree was generated with posterior probabilities (PP) shown on appropriate branches with *Globodera pallida* as the outgroup. New sequences are indicated in bold.

punctata might represent a complex of several closely related species.

Morphological characteristics of the cysts and juveniles of *P. mulveyi* n. sp. were very close to *P. stonei* so it was critical to analyse multiple molecular markers to strengthen the diagnosis. In particular, direct comparison with *P. stonei* from Canada played a central role in allowing us to propose this new *Punctodera* species. Despite the relatively low number of sequences representing different *Punctodera* populations, molecular comparisons, particularly Hsp90, supported separation of *P. mulveyi* n. sp. from other known species. For most markers the closest species was *P. matadorensis*, a nematode of grasses previously found in Saskatchewan and North Dakota; however, the present population does not fit within this species based on morphometrics. Based on the collective morphological and molecular data, this Oregon isolate is herein described as *P. mulveyi* n. sp. We agree with Subbotin *et al*. (2010) that more detailed molecular and morphological studies of geographically diverse populations are required to evaluate the reliability of characters for diagnostic purposes and to further test the validity of *Punctodera* species. Integrated taxonomies that also consider life histories, geographical distribution, and behaviour are needed to clarify relationships further within the genus.

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