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Nematodes associated with terrestrial gastropod molluscs in Belgium and additional characterisation of *Pellioditis californica* and *P. hermaphrodita*

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Research Paper

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Abstract

A survey for slug- and snail-associated nematodes was conducted in forests, parks, botanical gardens, and nature reserves at 13 localities in Belgium to uncover more diversity of gastropod mollusc-associated nematodes and to characterise *Pellioditis* populations found in the country. A total of 319 slugs and snails belonging to nine species were examined. *Arion vulgaris* was the most commonly found mollusc species in this study (eight locations), and 19.4% of the examined mollusc specimens were found infected by nematodes. The highest prevalence of nematodes was observed in *Cornu aspersum* (60%) followed by *A. vulgaris* (34.8%), *Limax maximus* (28.6%), and *Cepaea* sp. (20%). Eleven nematode species belonging to eight families were isolated and identified from the mollusc hosts including *Alloionema appendiculatum*, *Angiostoma dentiferum*, *A. gandavense*, *Angiostrongylus vasorum*, *Cosmocerca longicauda*, *Panagrolaimus* cf. *subelongatus*, *Pellioditis californica*, *P. hermaphrodita*, *Rhabditis* sp., *Tetrameres* cf. *fissispina*, and *Troglostrongylus* cf. *brevior*. *Pellioditis* was the most commonly found nematode genus (at nine localities) and *C. longicauda* and *P. californica* were reported in Belgium for the first time. Co-infections of more than one nematode species were observed in eight (2.5%) molluscs specimens. Most co-infections consisted of two nematode species. In one *A. vulgaris* specimen, a co-infection of three nematode species (*A. vasorum*, *P. hermaphrodita*, and *Tetrameres* cf. *fissispina*) was observed. Four *ex vivo* cultures of *P. californica* and six *ex vivo* cultures of *P. hermaphrodita* were established from single hermaphrodites, and both species were described based on light microscopy, scanning electron microscopy, and morphometric, morphological, and molecular data.

Introduction

Terrestrial gastropod molluscs (slugs and snails; Mollusca: Gastropoda) play an important role in the environment. Most species are beneficial decomposers, feeding on decaying organic matter and fertilising soil (Barker 2001; Meyer *et al.* 2011). However, some species are adapted to home gardens and cultivated fields, causing substantial damage to quality and yield of many horticultural plants and field crops (Halwart 1994; Joshi 2007; Podgornaya *et al.* 2020; Abd El-Halim *et al.* 2021). Moreover, some species passively transmit spores of plant pathogens such as *Sclerotinia trifoliorum*, a fungus that infects forage legumes and causes stem rot and mold crown (Shakeel & Mowat 1992). As intermediate hosts of a range of helminth parasites and combined with their dispersal capacity, some gastropod molluscs have become a veterinary and medical concern. They may be able to spread zoonoses not only to pets, livestock, and wildlife, but also to humans (Spratt 2015; Giannelli *et al.* 2016; Ramos-de-Souza *et al.* 2021). It is estimated that more than 300 million people suffer from diseases caused by gastropod-borne helminths (Giannelli *et al.* 2016). For instance, the gastropod nematode *Angiostrongylus cantonensis* Chen, 1935, is the primary cause of human eosinophilic meningoencephalitis in many parts of the Indo-Pacific region (Barratt *et al.* 2016; Pandian *et al.* 2023).

Until recently, a common practise to control gastropod molluscs has been the use of chemical molluscicides, including both inorganic salts and organic molluscicides (Kumar 2020; Zheng *et al.* 2021). However, a significant breakthrough was made with the discovery of the slug-associated nematode *Pellioditis* (=*Phasmarhabditis*) *hermaphrodita* (Schneider 1859) Andrássy 1983 as a biological control agent, an alternative to molluscicides (Wilson *et al.* 1993; Rae *et al.* 2007; Tandingan De Ley *et al.* 2014; Pieterse *et al.* 2017). The infective dauer juveniles of *P. hermaphrodita* penetrate and infect slugs in the area beneath mantle surrounding the shell,

often causing disease with the characteristic symptom of swelling of the mantle (Wilson *et al.* 1993). The infection usually leads to death of the slug within a few days. The juveniles develop into adults and a new generation of dauer juveniles released from the slug cadaver spread into soil and infect new hosts. The remarkable efficacy of *P. hermaphrodita* as a biological control agent has led to a search for other species of *Pellioditis* suitable for use as biological control agents. Currently, two products are marketed – Nemaslug® launched in 1994, which is *P. hermaphrodita* formulated with the bacterial associates, *Moraxella osloensis* (Moraxellaceae) or *Psychobacter* spp. (Rae *et al.* 2007; Sheehy *et al.* 2022), and Nemaslug 2.0® launched in 2022, which is *P. californica* Tandingan De Ley, Holovachov, Mc Donnell, Bert, Paine, & De Ley 2016 also formulated with *M. osloensis* (Stenberg *et al.* 2021). The former is commercially available throughout Europe, whereas the latter is available in England, Scotland, and Wales (Rae *et al.* 2007; Mc Donnell *et al.* 2023).

Besides *Pellioditis*, slugs and snails also host members of many other nematode genera as either definitive (parasitic), intermediate, or paratenic (phoretic) hosts (Sudhaus 2018). These nematodes are known to belong to as many as eight families including Agfidae, Alaninematidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae, and Rhabditidae (Pieterse *et al.* 2017). In recent years, surveys focused in Canada, the USA, Europe, Africa, Australia, and New Zealand substantially increased the knowledge of diversity of gastropod mollusc-associated nematodes (Singh *et al.* 2019; Antzée-Hyllseth *et al.* 2020; Brophy *et al.* 2020). In Belgium, an exploratory survey in two provinces (East and West Flanders) in the northern part of the country revealed the presence of one new and six known slug-parasitic nematode species (Singh *et al.* 2019). To obtain additional information on diversity of gastropod mollusc-associated nematodes in Belgium and to specifically characterise *Pellioditis* populations found in the country, a follow-up survey was conducted in the current study.

Materials and methods

Slug and snail collection and identification, nematode extraction

Slugs and snails were collected from forests, parks, botanical gardens, and nature reserves at 13 localities in Belgium (Figure 1 shows locations) from September 2020 to May 2021. Purposive, non-probability sampling (Bhardwaj 2019) was done based on the animal's selectivity towards favourable micro-habitats (such as moist, cool spaces underneath leaf litter and in between rock rubbles, pieces of wood, etc.). Identification of the slugs and snails was based on morphology (Kerney *et al.* 1979; Mc Donnell *et al.* 2009; Thomas *et al.* 2010). The collected slugs and snails were placed in plastic containers lined with tissue paper and stored at 4°C. The tissue paper was regularly sprayed with water to retain moisture and the slugs and snails were fed with spinach (*Spinacia oleracea* L.). Before dissection, the molluscs were thoroughly washed with tap water to remove debris and nematodes adhering on body surfaces. Individual molluscs were dissected out on glass Petri plates. Nematodes detected in individual molluscs were removed using a needle and transferred into a glass embryo dish containing diluted M9 buffer solution (6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 1 L H_2O, 1 mL of 1 M $MgS0_4$; dilution: nine parts of the M9 buffer solution to one part H_2O). Some specimens were then used for DNA extraction, and the remaining were used for morphological analysis as described in the later sections.

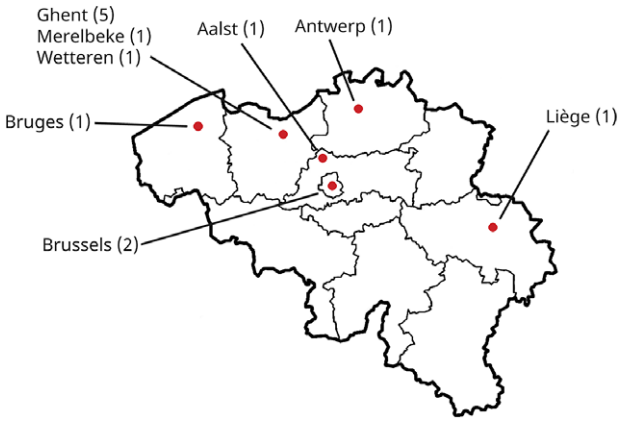

Figure 1. Map of the locations where snails and slugs were sampled in Belgium (n = 13). The numbers in parentheses indicate the number of sub-locations within a larger locality.

Nematode morphological analysis

Nematode morphological and morphometric characterisations were done using both live and fixed specimens mounted on glass slides. Temporary mounts of live nematodes were prepared as described in Singh *et al.* (2021), and some *Pellioditis* specimens were recovered from slides for culturing on plates as described further. For making permanent mounts of nematodes, they were killed and fixed in a microwave oven for 3 – 5 sec in a few drops of 4% formaldehyde buffered in phosphate-buffered saline + 1% glycerol and kept at 4°C for 24 – 48 h before they were processed to anhydrous glycerine for light microscopy (Seinhorst 1959 as modified by De Grisse 1965). Nematodes were examined, measured, and photographed using an Olympus BX50 DIC microscope equipped with a ToupTek UCMOS05100KPA USB microscope camera with an eyepiece adaptor and ToupView software.

For scanning electron microscopy, fixed specimens were first washed in 0.1M phosphate buffer (pH = 7.5) and dehydrated in a graded series of ethanol solutions, critical-point-dried with liquid CO_2, mounted on stubs with carbon tabs (double conductive tapes), coated with gold of 25 nm, and photographed with a JSM-840 EM (JEOL) at 12 kV (Nguyen *et al.* 2019).

Ex vivo culturing of *Pellioditis californica* and *P. hermaphrodita*

Ex vivo cultures of *P. californica* and *P. hermaphrodita* were established and maintained in Petri dishes (10-cm diameter) filled with humus agar (4 g agar, 130 mL humus, 60 µL cholesterol, 270 mL bi-distilled H_2O) (Grootaert & Maertens, 1976). Each Petri dish was inoculated with one hermaphrodite together with lung tissue of the slug or snail (as food source) from which the nematode was extracted, and the Petri dishes were incubated at 15°C.

Nematode molecular analysis

Before DNA extraction, nematode morphological vouchers were prepared as described in Singh *et al.* (2021). Vouchered nematodes were picked from the temporary mounts, cut into pieces in distilled water, and transferred to 200-µL Eppendorf tubes containing 20 µL of worm lysis buffer (50 mM KCl, 10 mM Tris at pH 8.3, 2.5 Mm $MgCl_2$, 0.45% NP 40 [Tergitol Sigma], 0.45% Tween 20) followed by incubation at –20°C (10 min), adding of 1 µL proteinase K (2.5 mg/mL), incubation at 65°C (1 h) and 95°C (10 min), and ending by

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Table 1. Slug and snail species, and associated nematode species collected from 13 localities in Belgium from September 2020 to May 2021

Location	GPS coordinates	Slug or snail species collected	Nematode species detected
Bruges	51°11'57.2"N, 3°13'30.18"E	*Arion vulgaris*	*Pellioditis hermaphrodita* *Pellioditis californica*
Ghent	51°2'7.84"N, 3°43'18.65"E	*Deroceras reticulatum* *Arion vulgaris*	*Angiostoma gandavense* *Pellioditis hermaphrodita*
Ghent	51°3'44.01"N, 3°39'45.28"E	*Arion hortensis* *Arion vulgaris* *Limax maximus*	*Alloionema appendiculatum* *Angiostoma dentiferum* *Pellioditis californica* *Pellioditis hermaphrodita* *Tetrameres* cf. *fissispina*
Ghent	51°2'9.29"N, 3°43'14.09"E	*Cornu aspersum* *Ambigolimax valentianus* *Limax maximus*	*Panagrolaimus* cf. *subelongatus* *Pellioditis californica* *Pellioditis hermaphrodita*
Ghent	51°3'24.29"N, 3°45'30.32"E	*Arion vulgaris*	*Angiostrongylus vasorum* *Alloionema appendiculatum* *Cosmocerca longicauda* *Pellioditis hermaphrodita* *Pellioditis californica* *Tetrameres* cf. *fissispina*
Ghent	51°2'39.41"N, 3°41'29.95"E	*Arion vulgaris* *Cepaea* sp. *Limax maximus*	*Cosmocerca longicauda* *Pellioditis hermaphrodita* *Rhabditis* sp.
Merelbeke	–	*Arion vulgaris*	*Pellioditis hermaphrodita*
Wetteren	50°59'8.4"N, 3°54'26.5"E	*Deroceras reticulatum* *Arion hortensis* *Arion vulgaris*	*Alloionema appendiculatum* *Angiostoma gandavense* *Angiostoma dentiferum*
Aalst	–	*Cornu aspersum*	–
Antwerp	–	*Deroceras reticulatum* *Arion hortensis* *Ambigolimax valentianus*	–
Brussels	50°50'37.94"N, 4°21'49.57"E	*Deroceras reticulatum*	*Pellioditis hermaphrodita*
Brussels	50°52'28.87"N, 4°15'41.51"E	*Deroceras reticulatum* *Limax maximus*	–
Liège	50°37'46.79"N, 5°34'33.73"E	*Arion vulgaris* *Cornu aspersum*	*Angiostoma gandavense* *Pellioditis californica* *Troglostrongylus* cf. *brevior*

centrifugation at 14,000*g* for 1 min. Amplification of the partial sequences of 18S of ribosomal DNA (rDNA) was done using the primer pair SSU18A: 5′-AAA GAT TAA GCC ATG CAT G-3′/SSU26R: 5′-CAT TCT TGG CAA ATG CTT TCG-3′ (Mayer *et al.* 2007) following the thermal profile of 94°C for 5 min, 35x (94°C for 1 min, 52°C for 1.5 min, and 68°C for 2 min), 68°C for 10 min and a final hold at 4°C. The D2-D3 expansion segment of 28S rDNA was amplified using the primer pair 391F: 5′-AGC GGA GGA AAA GAA ACT AA-3′/501R: 5′-TCG GAA GGA ACC AGC TAC TA-3′ (Nadler *et al.* 2007) following the thermal profile of 94°C for 4 min, 42x (94°C for 30 sec, 53°C for 30 sec, and 72°C for 1 min), 72°C for 5 min, and a final hold at 12°C (Nadler *et al.* 2007). For the internal transcribed spacer region of rDNA (ITS1, ITS2, 5.8S), the primer pair Vrain2F: 5′-CTT TGT ACA CAC CGC CCG TCG CT-3′/Vrain2R: 5′-TTT CAC TCG CCG TTA CTA AGG GAA TC-3′ was used (Vrain *et al.* 1992) following the thermal profile as described in Singh *et al.* (2019). Finally, for amplification of the *COI* region of mitochondrial DNA, the primer pair JB3: 5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′/JB4.5: 5′-TAA AGA AAG AAC ATA ATG AAA ATG (Bowles *et al.* 1992) was used following the thermal profile of 94°C for 3 min, 34x (94°C for 30 sec, 45°C for 30 sec, and 72°C for 1 min), 72°C for 10 min, and a final hold at 4°C. An additional primer pair for *COI* was also used (i.e., COIF1: 5′-CCT ACT ATG ATT GGT GGT TTT GGT AAT TG-3′/COI-R2: 5′-GTA GCA GCA GTA AAA TAA GCA CG-3′) (Kanzaki & Futai 2002) with thermal profile from Etongwe *et al.* (2020). The polymerase chain reaction products were purified according to Singh *et al.* (2020) and sent for sequencing to Macrogen (https://dna.macrogen.com).

Phylogenetic analysis

All the received forward and backward sequences were assembled using Geneious 10.0.9 (https://www.geneious.com) to generate contigs, and BLAST search was performed using the newly obtained sequences to collect closely related species sequences from GenBank. The partial sequence of 18S rDNA and the D2-D3 expansion segment of 28S rDNA were used to analyse the phylogenetic relationships of the nematode species detected in this study. All the generated sequences and the sequences of closely related species from GenBank were separately aligned for each gene segment using MUSCLE with default parameters, followed by manual trimming of the poorly aligned ends. Phylogenetic trees were generated using the GTR + I + G nucleotide substitution model.

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Table 2. Number and point prevalence of nematode species associated with specific slug and snail species collected from 13 localities in Belgium from September 2020 to May 2021

Slug or snail species	Positive (total)	Point prevalence (%)	Nematode species
Agriolimacidae			
Deroceras reticulatum	2 (91)	2.2	*Angiostoma gandavense*
			Pellioditis hermaphrodita
Arionidae			
Arion hortensis	4 (43)	9.3	*Angiostoma gandavense*
			Cosmocerca longicauda
			Pellioditis hermaphrodita
			Tetrameres cf. *fissispina*
Arion vulgaris	39 (112)	34.8	*Alloionema appendiculatum*
			Angiostoma dentiferum
			Angiostoma gandavense
			Angiostrongylus vasorum
			Cosmocerca longicauda
			Pellioditis californica
			Pellioditis hermaphrodita
			Tetrameres cf. *fissispina*
Helicidae			
Cepaea nemoralis	0 (8)	0	–
Cepaea sp.	1 (5)	20.0	*Cosmocerca longicauda*
Cornu aspersum	6 (10)	60.0	*Panagrolaimus* cf. *subelongatus*
			Pellioditis californica
			Troglostrongylus cf. *brevior*
Limaceae			
Ambigolimax valentianus	4 (28)	14.3	*Pellioditis californica*
Limax maximus	6 (21)	28.6	*Angiostoma dentiferum*
			Pellioditis californica
			Pellioditis hermaphrodita
			Rhabditis sp.
Oxychilidae			
Oxychilus alliarus	0 (1)	0	–
Total	**62 (319)**	**19.4**	–

Markov chains were set with 1×10^6 generations, four runs, 20% burn-in, and subsampling frequency of 500 generations (Huelsenbeck & Ronquist 2001).

Results

In total, 319 gastropods were collected from 13 localities in Belgium, and nine slug and snail species were identified (Table 1). The number of slug and snail species found per locality varied from one (five localities) to three (five localities). *Arion vulgaris* was the most commonly found mollusc species in this study (at eight localities). About 20% of the total gastropods collected were found parasitized by 11 nematodes species (Table 2), and the majority of the gastropod species contained nematodes (Table 2); only in *Cepaea nemoralis* and *Oxychilus alliarus*, no nematodes were found. The highest prevalence of nematodes was observed in *C. aspersum* (60%) followed by *A. vulgaris* (34.8%), *Limax maximus* (28.6%), and *Cepaea* sp. (20%). The highest numbers of nematode taxa were isolated from the slug species, *A. vulgaris* (eight) followed by *L. maximus* and *A. hortensis* (four each). Co-infections of more than one nematode species were observed in eight of the 319 (2.5%) slugs and snails collected (i.e., in all the species except in *Ambigolimax valentianus*, *Cepaea* sp., and *L. maximus*).

The 11 nematode species that were isolated are *Alloionema appendiculatum* Schneider, 1859, *Angiostoma dentiferum* Mengert, 1953, *Angiostoma gandavense* Singh, Couvreur, Decraemer & Bert, 2019, *Angiostrongylus vasorum* Baillet, 1866, *Cosmocerca*

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longicauda Linstow, 1885, *Panagrolaimus* cf. *subelongatus*, *P. californica*, *P. hermaphrodita*, *Rhabditis* sp., *Tetrameres* cf. *fissispina*, and *Troglostrongylus* cf. *brevior*. The identification of the seven confirmed nematode species were based on morphology, morphometrics, and molecular data; however, comprehensive characterisations of only *P. californica* and *P. hermaphrodita* are presented in this paper. The four unconfirmed nematode species were based on limited morphological and molecular data. Three of the nematode species (i.e., *A. gandavense*, *P. californica*, and *P. hermaphrodita* were found respectively at three, five, and eight localities), whereas the remaining nematode species were found at either one or two localities (Table 1), and at three localities, no nematodes were found in the collected slugs and snails (47 specimens). *Pellioditis* was the most commonly found nematode genus (at nine localities). Most of the nematode co-infections consisted of two nematode species; however, in one *A. vulgaris* specimen, a co-infection of three nematode species (*A. vasorum*, *P. hermaphrodita*, and *Tetrameres* cf. *fissispina*) was observed.

In total, 40 partial 18S, 38 D2-D3 of 28S and 4 ITS of rDNA sequences, and 31 *COI* of mitochondrial DNA sequences were generated from ten nematode species of which the *COI* sequences for *A. appendiculatum* and the 18S and 28S sequences for *C. longicauda* were generated for the first time (Table 3).

Four *ex vivo* cultures of *P. californica* and six *ex vivo* cultures of *P. hermaphrodita* were established from single hermaphrodites. Ten to 15 days after initiation of the cultures, the first generation of juveniles was observed that developed into gravid hermaphrodites in 24 – 48 h. The cultures were maintained for one month to check for the emergence of males; however, no males were observed in any of the ten cultures.

Pellioditis californica Tandingan De Ley, Holovachov, Mc Donnell, Bert, Paine and De Ley, 2016

Morphological description (Figure 2; Table 3)

Hermaphrodite: (Based on 28 specimens cultured *ex vivo* on lung tissue of their host.) Body straight or slightly curved in the middle when heat-killed, robust, elongate, gradually tapering to blunt anterior end. Annules fine; lateral field with six incisures. Lip region 16.9 (15 – 19) µm wide, flattened anteriorly, continuous with body, six lips grouped in pairs. One labial papilla emerging from each lip with clear apical dendrite of inner labial papilla. One outer cephalic papilla prominent on each subventral lip and two less prominent on each dorsal lip. Stoma triangular in cross-section, 19.2 (16 – 22) µm long, cheilostom, gymnostom, and stegostom prominent. Stegostom ends in two subventral metarhabdions. Pharyngeal collar reaching up to level of gymnostom. Corpus cylindrical, 119 (101 – 130) µm long, 2.5 times longer than isthmus, gradually expanding into non-valvular metacorpus, narrowing into isthmus, and ending in a bulbous, valvular basal bulb. Nerve ring surrounding anterior of isthmus, at 68.9% of pharynx length. Deirids observed. Secretory-excretory pore opening posteriorly, near base of basal bulb or cardia level. Cardia conoid. Reproductive system didelphic–amphidelphic, anterior and posterior ovaries reflexed, occupying 23.6% and 22.9% of body length, respectively. Oviduct short, filled with sperm in young gravid hermaphrodites; gonads of mature hermaphrodites filled with elliptical eggs (59 x 38 µm), juveniles observed hatching inside hermaphrodites. Vulva transverse slit; vulval lips slightly protruding, located at 50% of body length. Intestine ends in balloon-shaped rectum, 29.8 (23.6 – 37.9) µm long, somewhat shorter than anal body diameter, with four associated cell bodies and sphincter. Anus transverse slit. Anal body diameter 37.7 (27 – 51) µm, about 30% of tail length. Phasmids prominent, located at 37% of tail length. Tail elongate conoid, wider part funnel-shaped.

Male: No males were found in the *ex vivo* cultures.

Pellioditis hermaphrodita (Schneider, 1859) Andrássy 1983

Morphological description (Figure 3; Table 3)

Hermaphrodite: (Based on 28 specimens cultured *ex vivo* on lung tissue of their host). Body straight or slightly curved when heat-killed, robust, elongate, gradually tapering to blunt anterior end. Annules fine. Lateral field with six incisures. Lip region 15.9 (14 – 19) µm wide, flattened anteriorly, continuous with body; six lips grouped in pairs. One labial papilla emerging from each lip with clear apical dendrite of inner labial papilla. One outer cephalic papilla prominent on each subventral lip and two less prominent on each dorsal lip. Stoma triangular in cross-section, 17.6 (16 – 20) µm long; cheilostom, gymnostom, and stegostom prominent. Stegostom ends in two subventral, isomorphic metarhabdions. Pharyngeal collar reaching up to level of gymnostom. Corpus cylindrical, 105 (93 – 116) µm long, 2.3 times longer than isthmus, gradually expanding into non-valvular metacorpus, narrowing into isthmus, and ending in pyriform, valvular basal bulb. Nerve ring surrounding anterior of isthmus at 68.4% of pharynx length. Deirids prominent. Secretory–excretory pore opening at level of the middle or near base of basal bulb. Cardia conoid. Reproductive system didelphic–amphidelphic, anterior and posterior ovaries reflexed, occupying 25.2% and 23.7% of body length, respectively. Oviduct short, filled with sperm in young gravid hermaphrodites; gonads of mature hermaphrodites filled with elliptical eggs (48 x 35 µm); juveniles observed hatching inside hermaphrodites. Vulva transverse slit; vulval lips slightly protruding, located at 50% of body length. Intestine ends in balloon-shaped rectum, 28.7 (24 – 37) µm long, almost as long as anal body diameter, with four associated cell bodies and sphincter. Anus transverse slit. Anal body diameter 30.5 (24 – 37) µm, about one-third of tail length. Phasmids prominent, located at 37% of tail length. Tail elongate conoid, tapering to slightly filiform end.

Male. No males were found in the *ex vivo* cultures.

Molecular characterisation and phylogenetic relationships

Four identical 18S (OL468618 – OL468621; up to 847 bp), 3 identical D2-D3 (OL468695 – OL468697; up to 888 bp), and three *COI* (OL468716, 681bp; OL468717 – OL468718, up to 423 bp; upon alignment of both regions gave a contig of 903 bp) sequences were generated for *P. californica*. The 18S sequences were found to be identical to sequences of *P. californica* from the USA (KM510210), Canada (MT135094), and New Zealand (MT179851) (Tandingan de Ley *et al.* 2014; Brophy *et al.* 2020; Howe *et al.* 2020). The D2-D3 sequences were also identical to sequence of a *P. californica* isolated from the USA (KM510199) (Tandingan de Ley *et al.* 2014). The *COI* sequence (OL468716) was 98% similar (11-bp difference) to the only available *COI* sequence (KM555043) of *C. californica* (Tandingan de Ley *et al.* 2014, 2016).

Nine identical 18S (OL468624 – OL468632; up to 865 bp), nine identical D2-D3 (OL468686 – OL468694; up to 873 bp), and two *COI* sequences (OL468731, 627 bp; and OL468732, 414 bp; upon alignment gave a contig of 900 bp) were generated for

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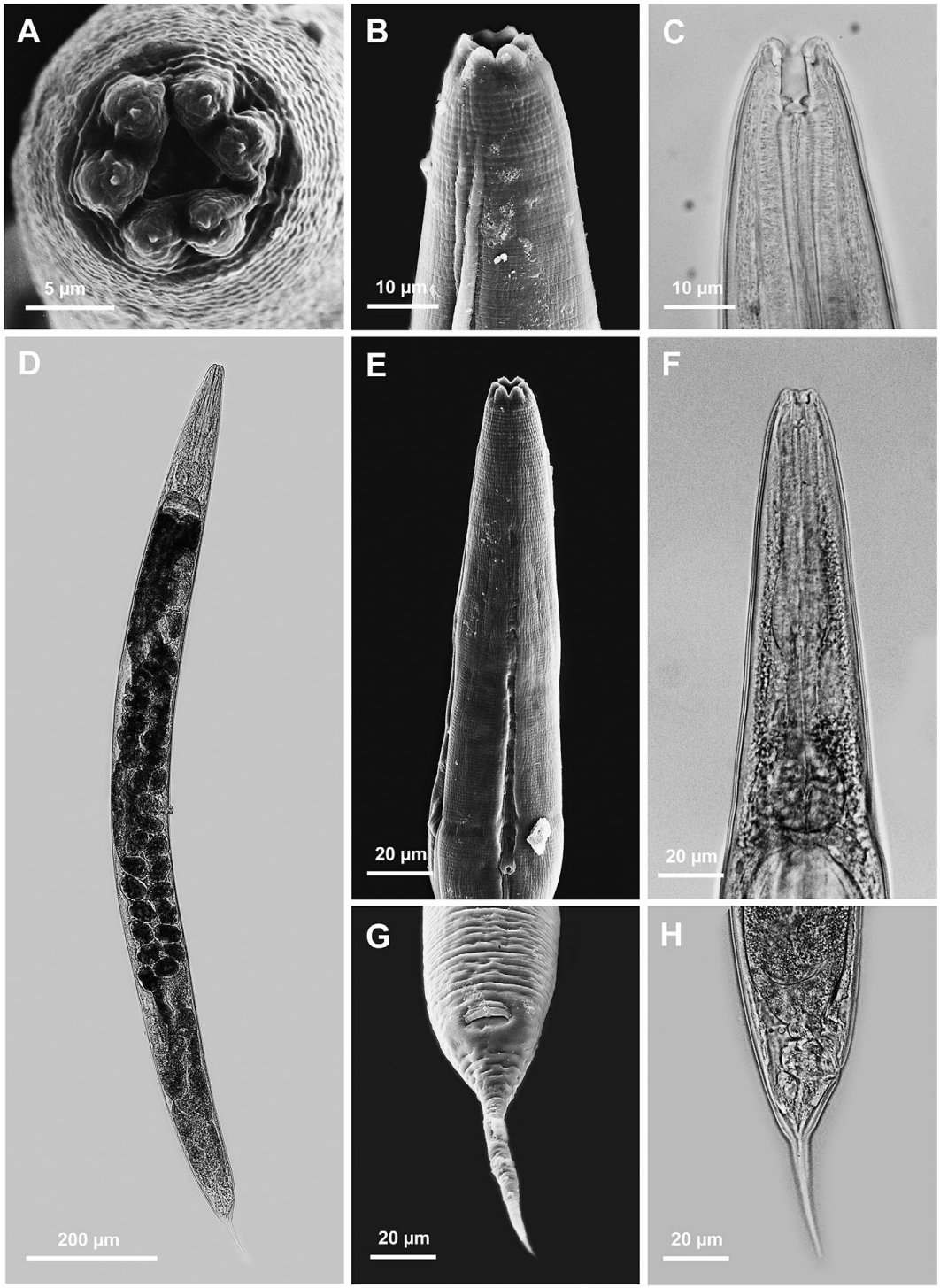

Figure 2. Light microscopy (LM) microphotographs and scanning electron microscopy (SEM) images of *Pellioditis californica* hermaphrodites. (A) Face view; (B – C) anterior end showing lip region; (D) total body; (E – F) anterior body showing up to pharynx end; (G – H) tail region.

P. hermaphrodita. The 18S sequences were found identical to sequences of previously isolated *P. hermaphrodita* from Belgium (MK214808), New Zealand (JQ965811), and Norway (KC883637), and 99.9% (1-bp difference) similar to isolate from the USA (KM510208) (Wilson *et al.* 2012; Tandingan de Ley *et al.* 2014; Ross *et al.* 2016; Singh *et al.* 2019). The D2-D3 sequences were also identical to sequences of previously isolated *P. hermaphrodita* from Belgium (MK214812) and the USA (KM510194) (Tandingan de Ley *et al.* 2014; Singh *et al.* 2019). Finally, the *COI* sequence (OL468731) was 96.5% similar (12-bp difference) to *P. hermaphrodita* isolate (KM555041) from the USA (Tandingan de Ley *et al.* 2014), and 97% similar (11-bp difference) to that (MK205359) collected from Belgium (Singh *et al.* 2019).

In the inferred phylogenetic tree based on the 18S alignment, our *P. californica* sequences formed an unresolved clade together with other sequences of *P. californica*, *P. papillosa*, and unidentified *Pellioditis* spp. (Posterior probabilities, PP=1) (Figure 4). On the

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Table 3. GenBank accession numbers of submitted sequences of the slug- and snail-associated nematode species found in this study. Accession numbers in bold are for sequences generated for the first time

Nematode species	Partial 18S rDNA	D2-D3 of 28S rDNA	ITS of rDNA	COI of mtDNA
Alloionema appendiculatum	OL468601 – OL468606	OL472312 – OL472320	–	**OL468709** – **OL468713**
Angiostoma dentiferum	–	OL468670 – OL468671	–	OL468701 – OL468702
Angiostoma gandavense	OL468588 – OL468595	OL468674 – OL468680	–	OL468703 – OL468706
Angiostrongylus vasorum	OL468608 – OL468611	OL472304 – OL472307	–	OL468723 – OL468726
Cosmocerca longicauda	**OL468612** – **OL468616**	**OL468682** – **OL468684**	OL472308 – OL472311	–
Pellioditis californica	OL468618 – OL468621	OL468695 – OL468697	–	OL468716 – OL468718
Pellioditis hermaphrodita	OL468624 – OL468632	OL468686 – OL468694	–	OL468731 – OL468732
Rhabditis sp.	OL468664 – OL468665	OL468698 – OL468699	–	OL468734
Tetrameres cf. *fissispina*	OR663993	–	–	–
Troglostrongylus cf. *brevior*	OR663994	–	–	–

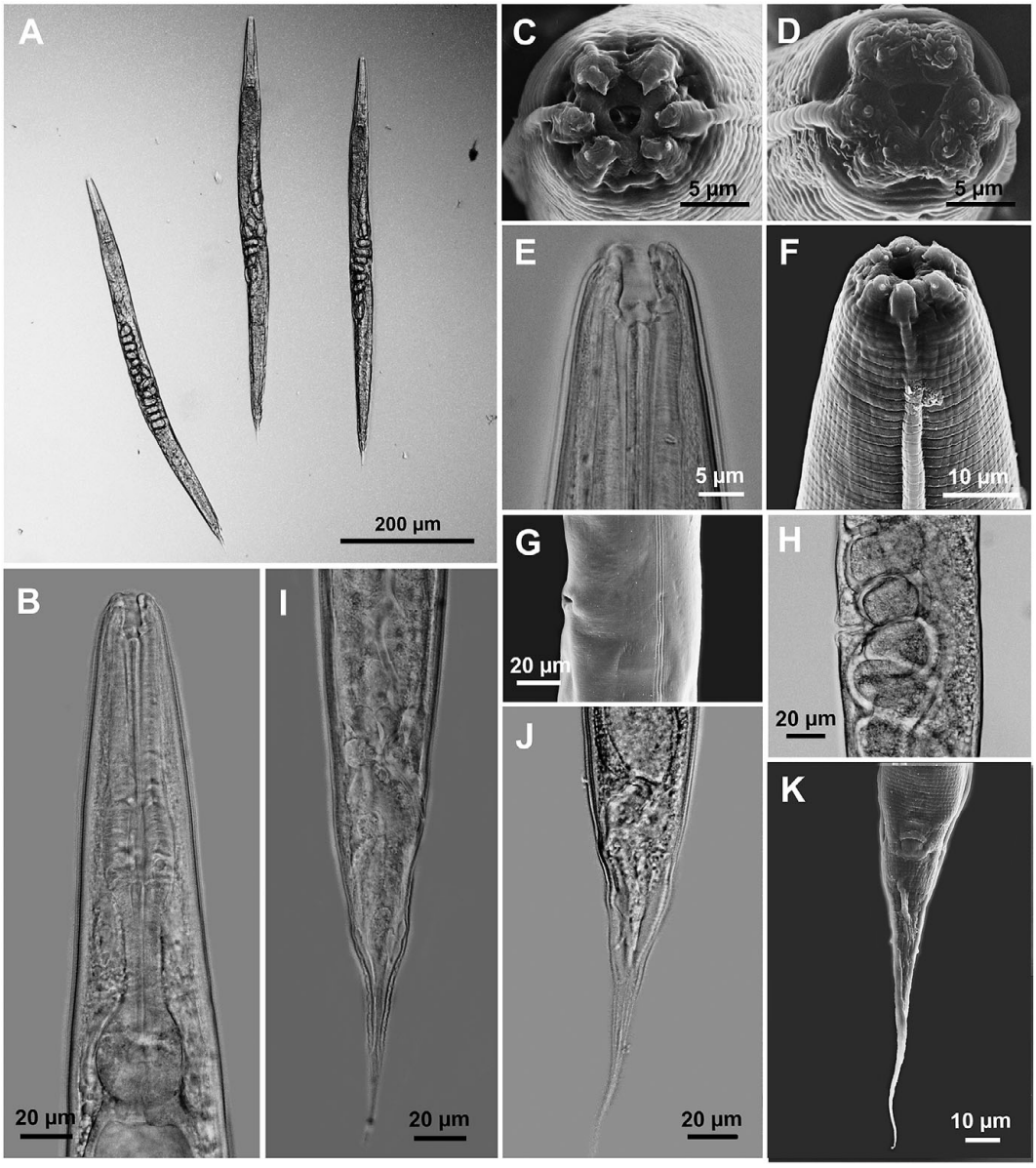

Figure 3. Light microscopy (LM) microphotographs and scanning electron microscopy (SEM) images of *Pellioditis hermaphrodita* hermaphrodites. (A) Total bodies; (B) anterior body showing up to pharynx end; (C – D) face view; (E – F) anterior end showing lip region; (G) lateral field; (H) eggs; (I – K) tail region.

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Figure 4. Phylogenetic tree based on partial 18S rDNA sequences of snail and slug parasitic nematodes as inferred by Bayesian Inference (BI) using the GTR + I + G nucleotide substitution model. The branch support is indicated by posterior probabilities at each node. Sequences highlighted in green were generated in this study.

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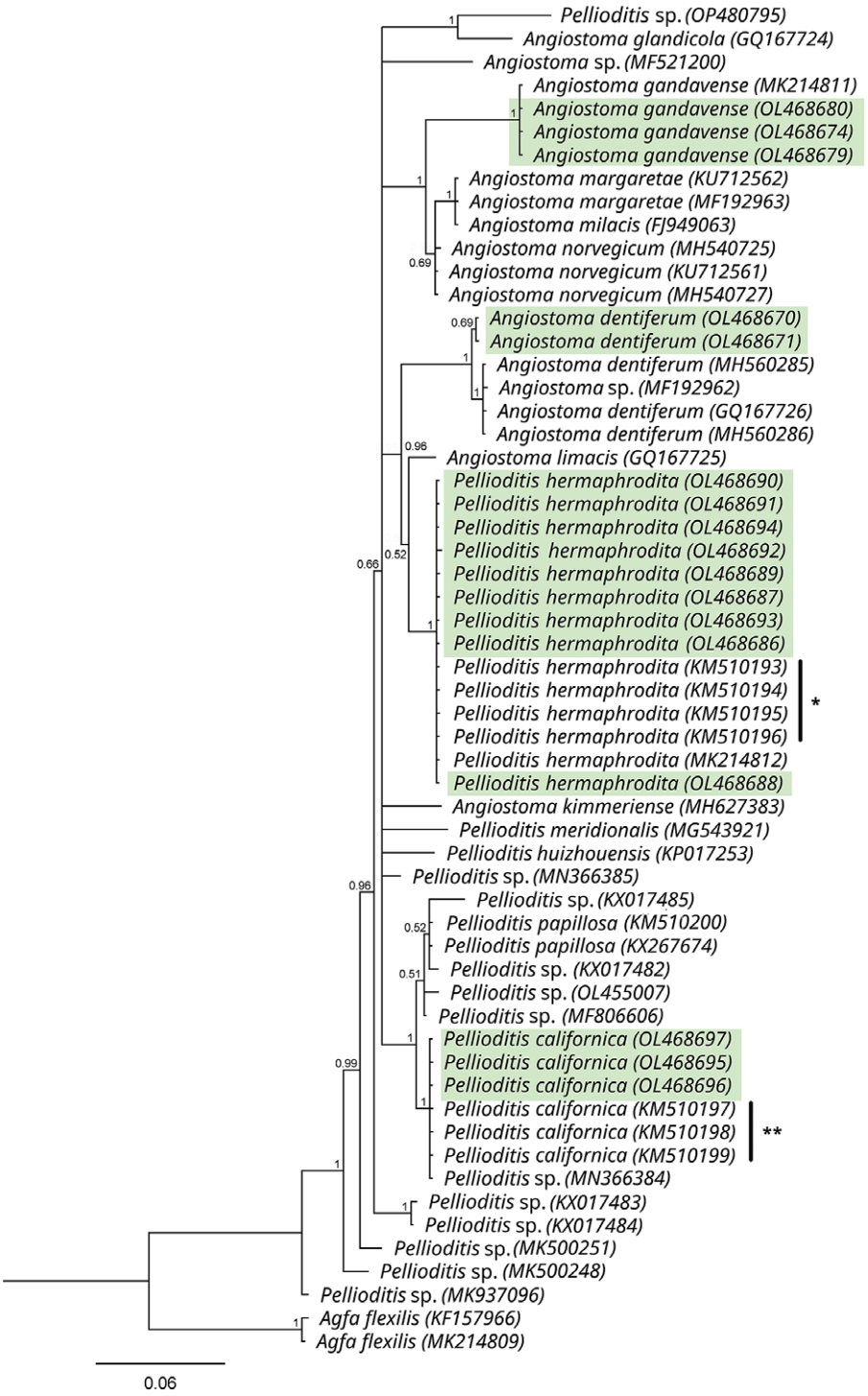

Figure 5. Phylogenetic tree based on the D2-D3 of 28S rDNA sequences of *Angiostoma* spp. and *Pellioditis* spp. as inferred by Bayesian Inference (BI) using the GTR + I + G nucleotide substitution model. The branch support is indicated by posterior probabilities at each node. Sequences highlighted in green were generated in this study. (*Sequences of *P. hermaphrodita*; **Sequences of *P. californica* from the USA published in Tandingan De Ley et al., 2014).

other hand, our *P. hermaphrodita* sequences formed a clade together with other sequences of the same species (PP=0.82) and appeared as sister to *P. tawfiki* (PP=1). The 18S sequences of the other mollusc parasitic nematodes generated in this study were also included in the tree (Figure 4), and sequences of the genera *Pellioditis* and *Angiostoma* appeared together in an unresolved clade (PP=1) that is sister to *Agfa* (PP=1). The observed close relationship between these two genera were similarly reported by Tandingan De Ley *et al.* (2023). A further check in the resolution of this clade was done by constructing D2-D3 tree using an alignment of only sequences of *Pellioditis* and *Angiostoma* species (Figure 5). In this tree, *P. californica* appeared as sister to *P. papillosa* (PP=1), and

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Table 4. Comparison of the morphometrics of hermaphrodites of the *Pellioditis hermaphrodita* and *P. californica* populations from Belgium with the morphometrics of *P. hermaphrodita* and *P. californica* populations from the UK and the USA. Measurements were made from fixed specimens and are given in μm and in the format mean ± standard deviation (range)

	P. hermaphrodita	*P. californica*	*P. hermaphrodita*		*P. hermaphrodita*	*P. californica*
	(Current study)	(Current study)	(Hooper et al., 1999)		(Tandingan de Ley et al., 2014)	(Tandingan De Ley et al., 2016)
	Belgium	Belgium	UK	UK	USA	USA
Character	Culture (host tissue)	Culture (host tissue)	Culture (host tissue)	Culture (bacteria)	Culture (bacteria)	Culture (bacteria)
n	28	28	20	20	10	20
L	1307 ± 150	1500 ± 170	1799 ± 279	1354 ± 115	1542 ± 161	1501 ± 117
	(992-1540)	(1135-1931)	(1509-2372)	(1186-1525)	(1284-1721)	(1298-1757)
a	16.0 ± 2.1	14.5 ± 1.6	19.5 ± 3.1	15.2 ± 1.6	17.2 ± 1.6	18.9 ± 2.9
	(12.7-21.1)	(11.6-17.2)	(13.6-28.9)	(12.4-17.9)	(15.1-19.5)	(15.1-23.1)
b	6.6 ± 0.7	6.9 ± 0.7	7.2 ± 1.1	5.9 ± 0.4	8.0 ± 0.6	7.0 ± 0.4
	(5.3-7.9)	(5.9-9.0)	(5.9-9.3)	(5.1-6.4)	(6.9-8.9)	(6.3-8.0)
c	13.1 ± 1.7	15.5 ± 2.5	15.8 ± 2.8	13.1 ± 0.7	15.6 ± 1.2	18.3 ± 2.2
	(10.9-18.9)	(12.2-21.8)	(13.2-24.0)	(11.6-14.3)	(13.4-17.4)	(14.1-22.5)
c’	3.3 ± 0.4	2.7 ± 0.5	3.0 ± 0.3	2.9 ± 0.2	2.5 ± 0.2	2.8 ± 0.5
	(2.1-3.9)	(1.8-3.6)	(2.4-3.6)	(2.4-3.2)	(2.2-3.0)	(2.4-4.0)
V%	51.2 ± 1.70	51.4 ± 1.9	51.0 ± 2.6	50.0 ± 1.6	50.4 ± 1.5	52.0 ± 0.9
	(45.5-55.6)	(46.3-57.7)	(48.0-60.0)	(48.0-55.0)	(48.2-52.9)	(50.8-53.7)
Max. body diameter	83.8 ± 17.1	103 ± 18.0	94.0 ± 15.8	90.0 ± 11.2	90.0 ± 8.3	81.0 ± 12.3
	(54.8-118)	(86.3-159)	(71.0-118)	(75.0-106)	(82.0-107)	(60.0-99.0)
Lip region diam.	15.9 ± 1.0	16.9 ± 1.1	18.0 ± 1.2	18.0 ± 0.7	17.7 ± 1.9	16.0 ± 1.8
	(13.5-18.5)	(14.6-19.0)	(16.0-20.0)	(17.0-19.0)	(14.1-20.4)	(12.4-18.0)
Stoma length	17.6 ± 1.22	19.2 ± 1.6	19.1 ± 1.5	18.0 ± 1.3	18.4 ± 1.0	18.8 ± 2.1
	(15.7-19.8)	(15.5-22.1)	(17.0-20.0)	(16.0-21.0)	(17.0-20.3)	(16.0-21.5)
Corpus length	105 ± 6.59	119 ± 6.9	115 ± 5.6	107 ± 5.2	97.0 ± 6.1	108 ± 9.0
	(93.2-116)	(101-130)	(109-126)	(96.0-114)	(86.0-107)	(83.0-119)
Metacorpus diam.	24.0 ±1.5	28.2 ± 3.3	–	–	23.2 ± 2.2	29.9 ± 3.3
	(22.1-27.4)	(23.0- 7.9)			(17.7-25.4)	(25.7-37.5)
Isthmus length	46.6 ± 6.1	47.3 ± 5.0	64 ± 2.8	59.0 ± 3.4	42.7 ± 3.5	42.7 ± 4.1
	(34.0-59.4)	(32.9-54.2)	(58.0-69.0)	(54.0-63.0)	(37.3-48.2)	(37.4-49.2)
Isthmus diam.	14.1 ± 1.2	16.0 ± 2.2	18.0 ± 1.2	18.0 ± 0.7	–	–
	(10.9-15.8)	(13.8-22.5)	(16.0-20.0)	(17.0-19.0)		
Basal bulb length	37.0 ± 3.3	41.4 ± 3.90	40.0 ± 1.4	35.0 ± 2.2	36.9 ± 4.4	45.3 ± 2.3
	(30.2-46.4)	(29.4-47.6)	(37.0-42.0)	(31.0-40.0)	(32.1-45.9)	(41.6-49.3)
Basal bulb diam.	29.6 ± 2.3	35.4 ± 3.4	–	–	29.6 ± 1.9	39.5 ± 2.5
	(25.3-34.5)	(28.3-43.9)			(27.2-32.4)	(35.2-43.5)
Cardia length	8.79 ± 1.13	8.0 ± 1.5	–	–	8.5 ± 1.9	12.2 ± 3.2
	(6.8-11.2)	(6.0-11.2)			(5.1-11.5)	(7.4-17.1)
Ant. end to pharynx base	197 ± 13.17	217 ± 13.4	–	–	193 ± 7.2	213 ± 12.5
	(161-215)	(175-239)			(126-149)	(180-230)
Ant. end to nerve ring	134 ± 8.9	149 ± 11.2	166 ± 7.4	141 ± 6.9	139 ± 7.2	149 ± 13.3
	(113-149)	(116-167)	(154-177)	(131-154)	(126-149)	(115-165)
Ant. end to nerve ring as % of ant. end to pharynx base	68.4 ± 4.44	68.9 ± 4.53	–	–	72.2 ± 2.9	69.8 ± 3.0
	(61.6-77.8)	(52.4-78.7)			(67.2-75.4)	(64.0-74.0)

(*Continued*)

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Table 4. (*Continued*)

	P. hermaphrodita	*P. californica*	*P. hermaphrodita*		*P. hermaphrodita*	*P. californica*
	(Current study)	(Current study)	(Hooper et al., 1999)		(Tandingan de Ley et al., 2014)	(Tandingan De Ley et al., 2016)
	Belgium	Belgium	UK	UK	USA	USA
Character	Culture (host tissue)	Culture (host tissue)	Culture (host tissue)	Culture (bacteria)	Culture (bacteria)	Culture (bacteria)
Ant. end to excretory pore	172 ± 14.2	193 ± 16.8	212 ± 11.0	172 ± 12.5	–	197 ± 6.6
	(137-193)	(150-228)	(192-231)	(157-189)	–	(187-204)
Vulva body diam.	78.7 ± 15.6	92.9 ± 5.9	–	–	89.0 ± 8.5	81.0 ± 11.8
	(51.2-109)	(82.4- 106)			(80.0-107)	(60.0-97.0)
Rectum length	28.7 ± 3.3	29.8 ± 3.9	–	–	39.1 ± 5.3	38.8 ± 7.3
	(23.7-36.6)	(23.6-37.9)			(33.6-52.3)	(27.3-48.7)
Anal body diam.	30.5 ± 4.0	37.7 ± 5.3	–	–	39.5 ± 4.3	29.7 ± 4.1
	(24.0-37.0)	(27.1-50.6)			(34.0-48.2)	(22.2-38.6)
Anus to phasmid	37.5 ± 4.1	33.9 ± 4.9	–	–	47.0 ± 9.7	26.8 ± 7.8
	(31.4-44.0)	(82.4- 106.0)			(38.0-70.1)	(17.6-43.8)
Tail length	84.0± 26.0	98.7 ± 13.9	114 ± 7.8	104 ± 8.6	99.0 ± 10.4	83.0 ± 8.9
	(37.3-131)	(63.9-120)	(99.0-129)	(82.0-113)	(85.0-117)	(66.0-94.0)
Anus to phasmid as % of tail length	37.1 ± 4.01	31.5 ± 3.0			47.1 ± 5.8	32.0 ± 6.8
	(31.5-44.6)	(24.7-33.7)			(41.6-62.9)	(26.5-47.6)
Anal body diam./tail length	30.5 ± 4.4	38. 9 ± 7.7	–	–	40 ± 3.2	35.8 ± 5.1
	(25.6-48.4)	(27.8-56.2)			(33.3-45.0)	(25.1-41.9)
Rectum length/anal body diam.	1.0 ± 0.1	0.8 ± 0.2	–	–	1.0 ± 0.1	1.4 ± 0.3
	(0.7-1.2)	(0.5-1.3)			(0.8-1.3)	(0.9-1.9)

P. hermaphrodita appeared as a clade with a sister relationship to *A. limacis* with a weak support (PP=0.52).

Remarks

The hermaphrodite morphology and morphometrics of *P. californica* largely agreed with the original description of the species (Tandingan de Ley *et al.* 2016). Similarly, our *P. hermaphrodita* population agreed with the original description by Mengert (1953) and the morphometrics data also fall within the range of the measurements from cultured nematodes in previous descriptions of the species (Hooper *et al.* 1999; Tandingan de Ley *et al.* 2014). However, the general morphometric data of both of our populations showed some variations such as in the body length and width, and tail length (see Table 4 for details). This was because of the inclusion of both young and matured hermaphrodites in our measurements. Nevertheless, the most important morphological differences observed between the two *Pellioditis* species was the tail shape (i.e., conical that appears almost like a funnel for *P. californica vs* more elongate conoid for *P. hermaphrodita*). However, in addition to tail shape, Tandingan de Ley *et al.* (2014) considered tail length as also one of the main distinguishing features between the two species; however as noted in the current study, measurements can be influenced by maturity level of the nematodes. In addition, they can also be affected by the diet on which nematodes are cultured, for instance, nematodes grown in slugs tend to be larger than those grown on bacterial agar plates (Hooper *et al.* 1999). Therefore, it appears that tail shape rather than length is more reliable in separating the two species. However, molecular confirmation of *Pellioditis* spp. is generally required because of the high morphological similarities and overlapping morphometrics amongst the species (Tandingan de Ley *et al.* 2014, 2016).

Discussion

The diversity of the gastropods and their associated nematodes in Belgium is remarkable (Singh *et al.* 2019; current study). In the current work, a total of nine slug and snail species belonging to five families and 11 mollusc-associated nematode species belonging to eight families were identified. Of the seven slug species collected in Belgium by Singh *et al.* (2019), only one species (i.e., *L. maximus*) was recollected in this study, whereas the remaining species (i.e., *A. ater*, *A. flagellus*, *A. fasciatus*, *A. lusitanicus*, *L. flavus*, and *L. valentiana*) were not found. On the other hand, *A. vulgaris*, the most commonly detected slug species in this study, was not found in the former study. Similarly, of the seven molluscs nematode species detected by Singh *et al.* (2019), three species (i.e., *A. flexilis*, *A. limacis*, and *A. norvegicum*) were not redetected in this study; however, six other nematode species not detected by the previous

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study were found here. In the survey of Singh *et al.* (2019), the slugs were collected from household gardens and a forest, whereas in this study the molluscs were collected mainly from forests, parks, botanical gardens, and nature reserves. This difference in habitats may explain the difference in the biodiversity of the collected gastropods and their associated nematode species. It underlines the importance of collecting samples from diverse habitats to obtain a better picture of the biodiversity of slugs and snails and associated nematode species in a region.

Two of the nematode species found in this study (i.e., *C. longicauda* and *P. californica*) were recorded for the first time in Belgium, whereas five other species (i.e., *A. vasorum*, *A. appendiculatum*, *A. dentiferum*, *A. gandavense*, and *P. hermaphrodita*) were previously reported in the country (Jolly *et al.* 2014; Singh *et al.* 2019;). *Angiostoma gandavense*, described from East Flanders of Belgium (Singh *et al.* 2019) was herein revealed to be more widespread in the country, as evidenced by its presence in Liège of Southeastern Belgium. In this study, on average, about one out of five slugs and snails examined (20%) was infected with at least one nematode species. This prevalence of nematodes is comparable to that of the survey conducted in Norway which found 18.7% of 611 slugs collected as infected by nematodes (Ross *et al.*,2016), and to that in the former survey in Belgium (i.e., infection of 22.8% of 239 slugs) (Singh *et al.* 2019). In contrast, a survey of native and introduced species of terrestrial slugs conducted in the Western Cape Province, South Africa, found only 6% of 521 slugs collected as infected by nematodes (Ross *et al.* 2012). The higher prevalence of nematodes associated with slugs and snails in Belgium suggests that gastropod fauna in Belgium is more susceptible to infection by nematodes. Also, some similarities and differences in the nematode infection rate of a few well-known slug species were found when compared with existing reports; for instance, in Canada, Brophy *et al.* (2020) reported a nematode infection rate of 3.2% of 2,158 examined *Deroceras reticulatum* specimens and 24.6% of 65 examined *A. valentianus*, whereas our study found the infection rate of 2.2% of the 91 examined *D. reticulatum* specimens and 14.3% of the 28 examined *A. valentianus* specimens, respectively.

Pellioditis was the most commonly found nematode genus in our study (i.e., it was found at nine of the 13 localities sampled and in six out of the nine slug and snail species collected). Based on morphometrics and morphology, and molecular characterisations, the presence of *P. californica* and *P. hermaphrodita* could be confirmed. Following the first description of *P. californica* from the USA (Tandingan De Ley *et al.* 2016), this species was subsequently reported in Europe (Carnaghi *et al.* 2017) and, more recently, in Germany (Keyte *et al.* 2022). It was also recovered in other countries including Canada (Brophy *et al.* 2020) and New Zealand (Wilson *et al.* 2016). The current study reports *P. californica* for the first time in Belgium. On the other hand, *P. hermaphrodita* was recovered for the second time in Belgium after it was found by Singh *et al.* (2019), but the identification of the species was based only on molecular data. This species has a cosmopolitan distribution and has been recovered from gastropod molluscs worldwide including Germany (Mengert 1953), France (Maupas 1900; Coupland 1995), the UK (Wilson *et al.* 1993), Iran (Karimi *et al.* 2003), Czech Republic (Nermuť *et al.* 2010), Egypt (Genena *et al.* 2011), New Zealand (Wilson *et al.* 2012), South Africa (Ross *et al.* 2012), the USA (Tandigan De Ley et al. 2014), China (Huang *et al.* 2015), Norway (Ross *et al.* 2016), and Japan (Waki 2017). It is most well-known for its biocontrol efficacy against molluscan pest (Wilson *et al.* 1993; Rae *et al.* 2007; Mc Donnell *et al.* 2020; Tandingan De Ley *et al.* 2020). A comprehensive morphological and molecular characterisations of *P. californica* and *P. hermaphrodita* are presented from Belgium for the first time in this paper.

As also reported in previous studies, some nematodes such as *Agfa* spp. and *Angiostoma* spp. can complete their life cycles inside mollusc hosts (Ivanova and Wilson, 2009, Ross *et al.* 2010, Singh *et al.* 2019) indicating their obligate parasitism, the current study also observed adults and juveniles of *Angiostoma* spp. within the same infected hosts. Another interesting observation, which was also similarly made by Singh *et al.* (2019), was the co-infections of *A. dentiferum* and *P. hermaphrodita* within the same host where the former occupies the intestine and the latter occupies below the mantle, oftentimes in the pallial cavity of the host. Although both the nematode significantly differ in their morphology (e.g., stoma shape and size) and mode of parasitism (obligate vs facultative), phylogenetic inference showed that *Angiostoma* and *Pellioditis* are closely related (see phylogenetic analysis above; Tandigan De Ley *et al.* 2023). The significance of such co-infection of nematodes with different modes of parasitism should be further investigated to enhance our understanding of mollusc parasitism by the nematodes, and also to determine whether *Angiostoma* has potential as biocontrol agents. Further interesting observation made in this study was the recovery of J4 (4th-stage juveniles) of *A. appendiculatum* from the digestive tubules of the digestive gland in one slug specimen and from below the mantle cavity in two slug specimens. This nematode species has an alternate parasitic-saprophytic life cycle – i.e. infective juveniles invade the foot muscle to develop into J4 and thereafter leave their host to continue their life cycle in the soil as free-living nematodes (Nermuť *et al.* 2019). To our knowledge, this is the first record of the recovery of J4 of *A. appendiculatum* from host digestive tubules other than the foot muscle. Juveniles inhabiting the pallial cavity (a double fold of the mantle which encloses a water space) vs the foot was one of the characters used to differentiate *Neoalloionema tricaudatum* from *A. appendiculatum* (Ivanova *et al.* 2015). However, our above observations suggest that the difference in infection site in the host may not be a reliable character to differentiate these two nematode species.

Remarkably, this study also revealed the presence of an important vertebrate nematode parasite in the mollusc specimens (i.e., juveniles [J1 – J3] of *A. vasorum* were found in the foot muscle of *A. vulgaris*). *Angiostrongylus vasorum*, also known as French heartworm, is a lungworm of foxes and, *inter alia*, of domestic dogs that utilizes terrestrial gastropods as intermediate hosts. Recently, its spread in North America and Europe has increased the veterinary relevance of this nematode species (Morgan *et al.* 2021). In Belgium, the first fatal case of canine angiostrongylosis caused by *A. vasorum* was reported in 2013 (Jolly *et al.* 2014). A subsequent sero-epidemiological study demonstrated a high seroprevalence of *A. vasorum* in southern Belgium (Lempereur *et al.* 2016). The recovery of *A. vasorum* in the current study revealed that this important canine parasite is also present in the northern part of the country.

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Competing interest. None.

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