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Integrated characterisation of *Daubaylia burnupiae* n. sp. (Nematoda: Daubayliidae) from a freshwater gastropod in South Africa, with comments on the biology of *Daubaylia* spp.

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ABSTRACT

Gastropod-nematode associations are underreported worldwide. In the present study, juvenile and adult nematodes were found in the freshwater gastropod *Burnupia stenochorias* (Melvill & Ponsonby, 1903), from the Vaal River, South Africa. The nematodes were confirmed to belong to the genus *Daubaylia* chitwood & chitwood, 1934 (Daubayliidae). This is the first report of *Daubaylia* from a snail belonging to the family Burnupiidae, and the first report of this nematode taxon in southern Africa. Like *D. pearsoni* and *D. malayanum* from Australia and Malaysia respectively, adult females of the current species possess multiple well-developed eggs in the uteri, with larvae developing *in utero*. Morphological and molecular characteristics showed that the nematodes are distinct from all the described species of *Daubaylia*. Thus, they are considered a new species, *Daubaylia burnupiae* n. sp. The species differs from its congeners based on spicule shape, the short tail of the male, an anal cuticular knob-like protrusion on the female, and oesophagi with short isthmi and short glandular basal bulbs in both sexes. Three club-shaped pharyngeal lobes, extending slightly above the surface of the cephalic lips in both sexes and a precloacal median papilla on the male were described using scanning electron microscopy, the first of such observations for the daubayliids. Genetic analyses showed that partial sequences of *D. burnupiae* n. sp. differed from species for which genetic data are available, by at least 26 and 9 base pair differences for 28S and 18S rDNA, respectively. Our results show that low prevalence and abundance of nematodes in the snails, corresponded with increased pollution in the river. We suspect that exposure to pollutants reduces the viability of the infective gravid female nematode during transmission. Therefore, the nematode is a potential bioindicator for aquatic pollution.

1. Introduction

Molluscs have diverse associations with parasitic helminths. However, compared with mollusc-trematode associations, nematodes of molluscs are understudied (Morley, 2010). According to Grewal et al. (2003), Rhabditida is the main nematode group that parasitise molluscs, and at least 61 species are known to use molluscs as intermediate hosts, while 47 species complete their life cycles in molluscs. In general, comprehensive investigations on nematodes that use molluscs as hosts have centred around species that infect livestock and humans. The widely studied species are *Angiostrongylus vasorum* (Baillet, 1866), *Angiostrongylus cantonensis* (Chen, 1935), *Strongyloides stercoralis* (Bavay, 1876) and *Muellerius capillaris* (Mueller, 1889), whose larvae occur in terrestrial gastropods, with the adults inhabiting vertebrates (Morley, 2010; Igbinosa et al., 2016). Consequently, compared with their terrestrial counterparts, nematodes that parasitise aquatic molluscs are understudied.

Studies from various parts of the world show that at least eleven rhabditid species complete their life cycles in freshwater gastropods (Grewal et al., 2003; Zimmermann et al., 2011a, 2011b). For some, nematode infections result in minimal pathology, while in others, infections lead to host mortality (Anderson and Bartlett, 1993; Grewal et al., 2003). The latter case is predominant in species of the family Daubayliidae Chitwood and Chitwood, 1934. Daubayliids reproduce rapidly in their hosts, causing fatal infections, and then the infective adult females leave their dying hosts, to invade new ones (Poinar and Richards, 1979; Anderson and Bartlett, 1993; Zimmermann et al., 2011a; Zimmermann et al., 2013). *Daubaylia* Chitwood and Chitwood,

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1934 is the only genus in the family; the species are slender, slow-moving parasites of aquatic snails and leeches (Poinar, 2016). Currently, *D. seistanensis* Baylis and Daubney, 1922; *D. potomaca* Chitwood and Chitwood, 1934; *D. dewiti* Schuurmans-Stekhoven, 1956; *D. elegans* Honer and Jansen, 1961; *D. malayanum* Sullivan and Palmieri, 1978; *D. helicophilus* Poinar and Richards, 1979; *D. olsoni* Poinar, 1984; *D. pearsoni* Anderson and Bartlett, 1993 and *D. bonaerensis* Camino and Gonzalez, 2011, are the known species of the genus.

Detailed morphological and genetic characterisation is still lacking for most species. Only *D. bonaerensis* was studied using scanning electron microscopy (Camino and Gonzalez, 2011) and the remainder have been described based primarily on features observed through light microscopy. Regarding DNA characterisation, there are only two 28S and one 18S rDNA sequences of *Daubaylia* on GenBank (Holovachov et al., 2015; Schultz and Adema, 2017). The current study is a novel report of a species of *Daubaylia* from a freshwater snail, *Burnupia stenochorias* (Melvill & Ponsonby, 1903) (Burnupiidae), in the Vaal River, South Africa. The taxonomic study applies an integration of genetic characterisation and morphometric description using light and scanning electron microscopy. The diagnostic features that distinguish the current nematode from its congeners are described.

Aquatic organisms are influenced by the chemical and physical conditions of their habitats (Chovanec et al., 2003). Consequently, the sensitivity of aquatic biota to changes in environmental conditions makes them important tools for biomonitoring. For instance, free-living aquatic invertebrates and vertebrates, are often used as accumulation and effect indicators (Chovanec et al., 2003; Outa et al., 2020a). However, biomonitoring using parasites tends to receive less attention (Gilbert and Avenant-Oldewage, 2017). It is unfortunate as the prevalence of diseases of aquatic fauna might decrease or increase in habitats associated with chronic or high levels of pollution (Morley et al., 2003). Generally, reports on the bioindication ability of aquatic parasites tend to focus more on fish parasites (Gilbert and Avenant-Oldewage, 2017; Sures et al., 2017). According to Gilbert and Avenant-Oldewage (2017), there is a high likelihood of a decline in ectoparasites (monogeneans and crustaceans), and an increase in endoparasites (nematodes, cestodes and acanthocephalans) of fish, with increased pollution. However, since fish are mobile and intermixing of individuals from polluted and unpolluted habitats in an ecosystem is likely to occur, interpretation of pollution effect on parasitism in fish from field studies should be done cautiously (Morley and Lewis, 2004). Gastropods on the other hand are less mobile, and likely to offer better results in point source pollution in field surveys (Outa et al., 2020a). Unfortunately, the response of parasites of molluscs to environmental changes is often overlooked, with only a few studies focused on digenean trematodes (Morley et al., 2003; Sures et al., 2017; Outa et al., 2020b). Consequently, the effect of pollution on nematodes of gastropods remains understudied.

In the Vaal River system, anthropogenic pressures have led to a rise in the levels of various pollutants, that have caused alterations in the distribution of biota (Gilbert and Avenant-Oldewage, 2016; Pretorius and Avenant-Oldewage, 2022). Therefore, the present study offers an opportunity, to compare the prevalence and abundance of nematodes in snails obtained from sites, that are affected by different levels of pollution, in the Vaal River. Considering that pollutants can induce physiological stress and depress host immunity, thereby favouring parasitism (Morley et al., 2006), we postulate a high prevalence and abundance of nematodes in snails from the polluted site.

2. Material and methods

2.1. Study area

As indicated in Fig. 1, snails were sampled from two sites in the Vaal River. The first site (S1) is located below the Vaal Dam wall (26.872364 °S, 28.117173 °E) and the second site (S2), is situated downstream, below the Vaal River Barrage Reservoir (26.734854 °S, 27.634372 °E). The site where S2 is located, has a long history of pollution, being the recipient of point discharge of industrial and municipal wastewater through the Rietspruit and Taaibosspruit tributaries (Wepener et al., 2011; Gilbert and Avenant-Oldewage, 2016). Pretorius and Avenant-Oldewage (2022) reported that electrical conductivity, salinity and the levels of total dissolved solids, chlorides, ammonia and nitrates, in water were at least three times higher at S2 than at S1. In addition, the sediment at S2 is polluted with chromium, copper, zinc, arsenic and lead (Pretorius and Avenant-Oldewage, 2022).

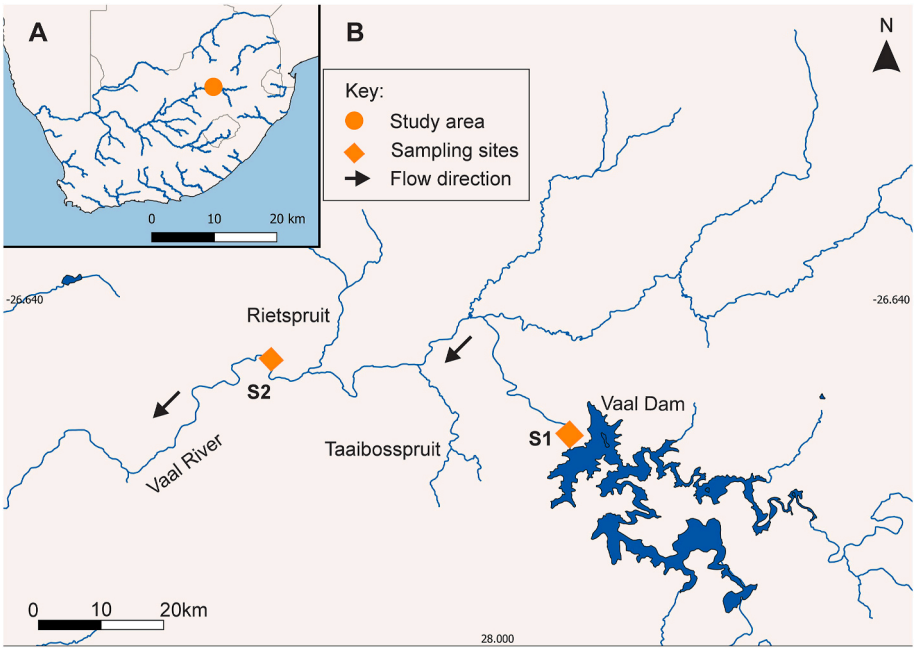

Fig. 1. Map showing Southern Africa (A) and the study area (B). S1: below Vaal Dam (26.872364 °S, 28.117173 °E) and S2: below Vaal Barrage (26.734854 °S, 27.634372 °E).

2.2. Sampling and sample preparation

Burnupia stenochorias specimens were collected from the study sites at the end of summer, in March 2022. March was chosen for the survey since the recruitment of *B. stenochorias* begins in spring (September–November) and the population peaks towards the end of summer (February–March) (Davies-Coleman, 2001). The snails were found attached onto submerged rocks or reed stems. The specimens were placed into plastic containers, half-filled with water from their respective habitats, protected from direct sunlight and transported alive to an onsite field laboratory. Identification of the snails was based on their morphology using keys (Brown, 1994) and all specimens were examined within 24 h of sampling. In total, 746 specimens of *B. stenochorias* (442 from S1 and 304 from S2), were examined. Based on the procedures outlined by Sullivan and Palmieri (1978), each specimen was necropsied in 0.3% saline, followed by an inspection of the body wall, body cavities and visceral organs using a dissecting microscope.

Fixation and preparation of nematodes for morphological examination followed the standard procedures used for daubayliids (Chernin et al., 1960; Sullivan and Palmieri, 1978; Anderson and Bartlett, 1993; Poinar, 2016). Accordingly, specimens isolated from the snails, were fixed in hot 70% ethanol and preserved in either 5% glycerine-alcohol for light microscopy, or 70% ethanol for scanning electron microscopy. In addition, some were preserved in 96% ethanol for molecular analyses. For morphometrics, 53 adult nematodes obtained from 30 snails from the two sampling sites, were used. Twenty-eight female and eleven male nematodes were slowly cleared in glycerol-alcohol for two months, in a Sanpla dry keeper desiccator cabinet (Kita-Ku, Osaka, Japan). The specimens were mounted in pure glycerol on glass slides, and the cover slips were sealed with clear nail polish. A Zeiss Axioplan 2 epifluorescence microscope and AxioVision 4.3 imaging software (Göttingen, Germany), were used to obtain micrographs and measurements of the mounted specimens. For scanning electron microscopy, ten females and four males were prepared based on the methods described by Nation (1983) and Rindoria et al. (2020). Accordingly, specimens were dehydrated through successions of 70%, 80%, 90%, 96% and 100% ethanol; followed by 40%, 70% and 100% hexamethyldisilazane (Merck, Darmstadt, Germany). Subsequently, the specimens were mounted on a strip of double sided adhesive conductive carbon tape, fixed to a cone-shaped copper stub and dried in a Sanpla dry keeper desiccator cabinet (Kita-Ku, Osaka, Japan) for at least 24 h. The nematodes were then coated with gold, using an Emscope SC500 sputter coater (Quorum Technologies, Newhaven, UK) and studied using a scanning electron microscope (Vega 3 LMH, Tescan, Brno, Czech Republic) at 6 kV.

For genetic identification, twelve nematodes isolated from twelve snails were used. DNA was extracted from the mid sections (fixed in 96% ethanol) of three females and two males, whose anterior and posterior body parts had been fixed separately (glycerine-alcohol) for morphological examination. Additionally, DNA was extracted from seven whole specimens that had been fixed in 96% ethanol. E.Z.N.A.® Tissue DNA Kit (Omega, Bio-tek, Inc, Georgia, USA), was used for DNA extraction, based on the manufacturer's instructions. Two genetic markers (18S and 28S rDNA) were used for the molecular identification of the specimens. Nuclear 18S rDNA was amplified using nematode specific primers Nem 18S F (5' –CGCGAATRGCTCATTACAACAGC – 3′) and Nem 18S R (5' –GGGCGGTATCTGATCGCC – 3′) (Floyd et al., 2005) and 136R (5' –TGATCCTTCTGCAGGTTCACCTAC – 3′) (Nadler et al., 2007). Fragments of 28S rDNA were amplified using primer sets LSU5 (5' –TAGGTCGACCCGCTGAAYTTAAGCA – 3′) and 1500R (5' – GCT ATC CTG AGG GAA ACT TCG – 3′) (Olson et al., 2003; Schultz and Adema, 2017). PCR conditions set by Olson et al. (2003), were modified by adjusting the initial denaturation (5 min), annealing temperature (52 °C) and final extension (10 min). PCR products were verified for successful amplification visually in agarose gel (1%), impregnated with SafeView™ FireRed (abm), using a SmartDoc™ 2.0 ultra-violet transilluminator (Benchmark Scientific, NJ, USA). DNA sequencing was done using PCR forward and reverse primers, according to the procedures outlined by Avenant-Oldewage et al. (2014). Following Kearse et al. (2012), Geneious Prime 2020.2.2 was used to inspect, edit where necessary and align the generated sequences. The sequences were blasted to identify the most similar sequences from GenBank, on the NCBI website. Subsequently, the sequences were compared to the three nuclear rDNA sequences published for the Daubayliidae. Finally, the distances and number of base pair differences between the sequences, were determined using MEGA7 (Tamura et al., 2013).

2.3. Data analyses

Descriptive numerical ratios and qualitative expressions of the specimens' morphology were determined, following the guidelines by Fortuner (1990). Accordingly, ratio a (body length/body width); ratio b (body length/oesophagus length); ratio c (body length/tail length) and index V (distance of anterior end to the vulva, as a % of the body length), were calculated.

Prevalence, 95% confidence interval (CI) of the prevalence, mean intensity (MI) and mean abundance (MA), of the nematodes in the snails, were determined following Bush et al. (1997). IBM SPSS 21 was used to perform additional statistical analyses on nematode infection parameters. Data were tested for normality of distribution using the Kolmogorov-Smirnov test, followed by the appropriate parametric or non-parametric test analyses. The prevalence of nematodes from the sampling sites were compared using Chi-square test, while MI and MA were compared using Mann-Whitney's *U* test.

3. Results

3.1. Daubaylia burnupiae n. sp. (Figs. 2-5)

3.1.1. Taxonomic summary

Type host and locality: The nematode was collected from *Burnupia stenochorias* (Melvill & Ponsonby, 1903) (Gastropoda, Burnupiidae), from the Vaal River, South Africa.

Etymology: The name refers to the genus of the type host.

Type material and deposition: one holotype (male), one allotype (female) and two paratypes (one male and one female) deposited at the Iziko South African Museum, Cape Town, South Africa: number MB-A094927 - MB-A094930. Four paratypes; two females and two males deposited in the Natural History Museum, Vienna, Austria: number NHMW-ZOO-EV-M-5876 - NHMW-ZOO-EV-M-5879.

Genetic material: The representative 28S and 18S rDNA sequences; accession number OQ269618 and OQ269617, respectively.

Zoobank Registration: In compliance with the International Commission on Zoological Nomenclature (ICZN) guidelines, details of the new species have been submitted to Zoobank. Accordingly, the Life Science Identifier (LSID) of the article is **urn:lsid:zoobank.org:pub:C648B28F-0159-44A9-8589-B4E063D3643B**. The LSID for the species is: **urn:lsid:zoobank.org:act:89EC2CB8-C3C0-4F97-874F-383827EF99D7**.

3.1.2. Morphological description

The nematodes collected in this study belong to the genus *Daubaylia* Chitwood and Chitwood, 1934 based on the following morphological features: presence of an elongate oesophagus, with distinguishable corpus, isthmus and glandular basal bulb that lacks a valve; nerve ring that surrounds the isthmus just below the junction of the corpus with the isthmus; six cephalic lips; dorsolateral amphids; reduced stoma; single ovary; single, reflexed testis; paired, similar and separate spicules; a gubernaculum and genital papillae. In all morphometric descriptions, unless stated, the measurements are in micrometres, as mean values, followed by the range in parentheses.

Females (n = 28): Longer than male (Fig. 2), length 1.61 (1.20–1.91) mm, width at vulva 32 (27–37). Stoma reduced, largely occupied by

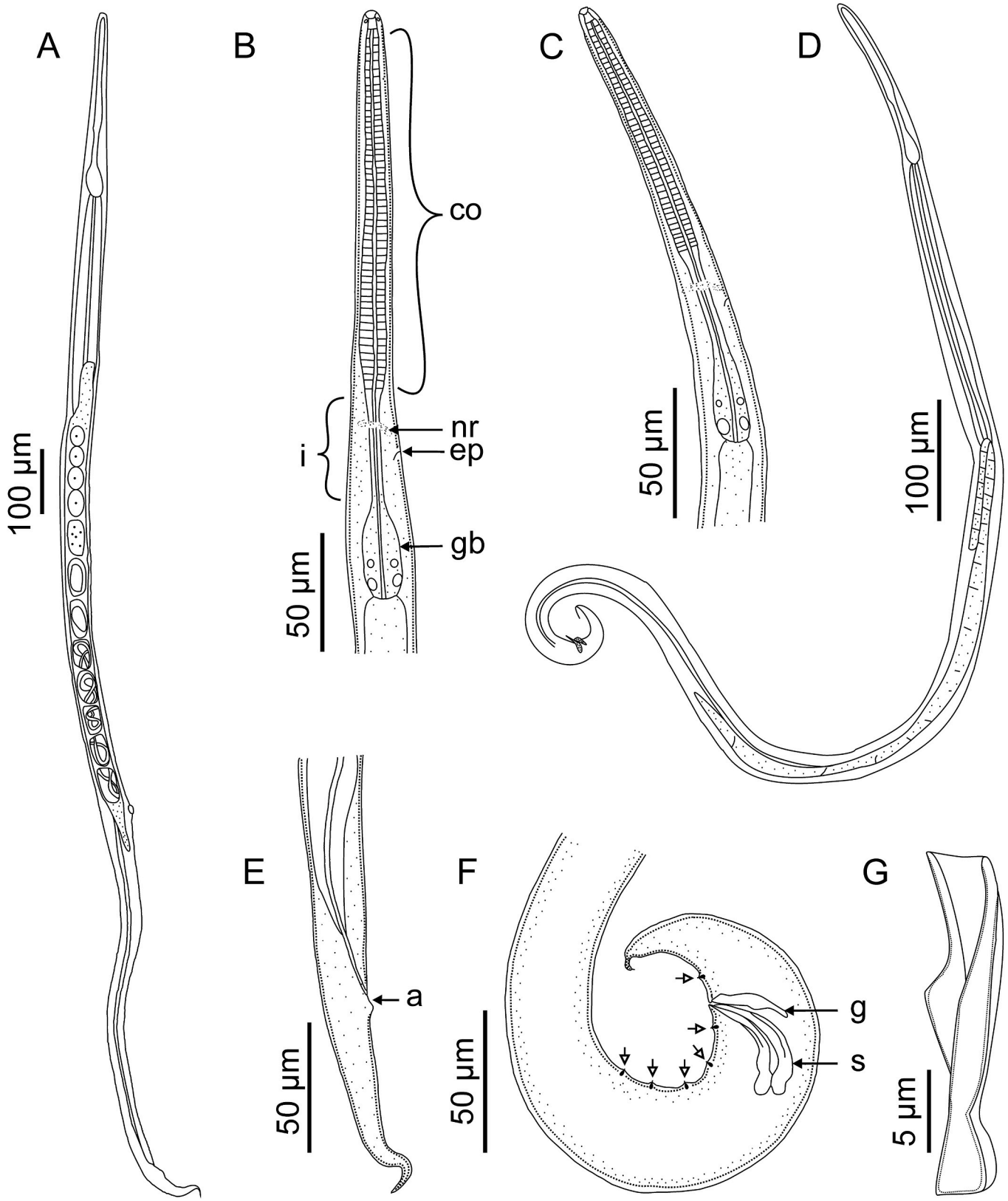

Fig. 2. Line drawings of *Daubaylia burnupiae* n. sp. A, female; B, anterior end female; C, anterior end male; D, male; E, posterior end female; F, posterior end male (arrows with no fill show lateroventral genital papillae) and G, gubernaculum. A-F, lateral view; G, subventral view. Abbreviations: a, anus; co, corpus; ep, excretory pore; g, gubernaculum; gb, glandular basal bulb; i, isthmus; nr, nerve ring and s, spicule.

three club-shaped pharyngeal lobes, that extend anteriorly, slightly above level of cephalic lips (Fig. 3D). Ten papillae: one pair on lateral lips, two pairs on latero-dorsal lips and two pairs on latero-ventral lips. Papillae on latero-dorsal and latero-ventral lips unequal in size: each papillae pair composed of one small and one large one. Amphids subterminal on latero-dorsal part of cephalic end (Fig. 3 C and 3D). Corpus, 134 (109–154) long, 8.9 (8.1–10) wide; leads into narrow isthmus, 53 (43–63) long, 6.4 (5.5–6.8) wide; that enlarges into pyriform glandular basal bulb (Figs. 2B, 3A), 29 (21–34) long, 15 (13–17) wide. Nerve ring, 151 (137–169) and excretory pore, 164 (142–184) from anterior extremity. Uterus contains 8 (3–12) eggs, 4 (1–7) eggs with coiled developed larvae, larvated eggs 66 (60–77) long and 23 (19–28) wide. Uterus with short post-vulvar sac, 37 (32–49) long. Vulva slightly protruded (Fig. 4B and D), 1.09 (0.80–1.28) mm from cephalic end. Anus, 74 (64–87) from posterior extremity, cuticular knob-like protrusion immediately posterior to anal opening (Fig. 4C and E). Tail conically attenuated, curved 87–110° dorsally.

Males (n = 11): Length 1.13 (1.02–1.27) mm, maximum width 28 (26–32). Stoma, pharyngeal lobes, lips and cephalic papillae similar to females; amphids inconspicuous (Fig. 3F). Corpus, 111 (99–128) long, 8.1 (6.8–9.3) wide; isthmus, 37 (34–40) long, 5.7 (5.3–6.8) wide; basal bulb, 28 (25–30) long, 12 (11–14) wide (Figs. 2C and 3B). Nerve ring 123 (104–138) and excretory pore 134 (111–144) from anterior extremity. Testis, 396 (302–561) from anterior extremity, reflexed (Fig. 5A); reflexed part 105 (95–117) long. Genital papillae: six latero-ventral pairs (five pre-cloacal, one post-cloacal) (Figs. 2F and 5D), and single, median ventral papilla just anterior to cloacal aperture (Fig. 5E). Spicule arcuate, 33 (32–35) long, with cylindrical manubrium 3.9 (3.5–4.2) long, 3.6 (3.3–3.9) wide; lamina curved ventrad, 29 (28–31) long, maximum width 5.6 (5.2–6.1), tapers gradually to distal end; lamina with lateral longitudinal groove that extends posteriorly towards distal tip; distal tip recurved (Fig. 5B, C and E). Ratio of spicule length/spicule width, 5.9 (5.5–6.3). Gubernaculum, 21 (20–23) long, maximum width 3.5 (3.1–4.0), capitulum broad and slightly curved ventrad, enlarged mid-length on ventral edge and distal end terminates in triangular plate (Figs. 2G & 5B). Ratio of gubernaculum length/gubernaculum width, 5.9 (5.4–6.5). Tail, 33 (30–37) long; tail tip pointed, 5.1 (3.6–7.1) long.

3.1.3. Taxonomic remarks

3.1.3.1. Diagnostic features of the current species. In both sexes, the oesophagus is characterised by a short isthmus and a small glandular basal bulb. The corpus is 2.6–3.4 times longer than isthmus, and 1.5–2.0 times longer than the isthmus and basal bulb combined (Fig. 2B, C, 3A and 3B). In other species of the genus, the post-corpus part of their oesophagi is either longer or nearly the same length as the corpi. The female has a cuticular knob-like protrusion immediately posterior to the anus; a feature that is not reported in the other species of *Daubaylia*. The male has a short tail, hence, a larger ratio of body length/tail length, compared to other species (Table 2). The spicules are longer, compared to those in males of species that are of comparable overall body size (Table 2) and differ in shape (Fig. 6). The current study is the first report of pharyngeal lobes in a species of the family Daubayliidae. However, the pharyngeal lobes were observable only through scanning electron microscopy.

Based on the number and arrangement of latero-ventral genital papillae in males and larvated eggs in the uterus, the current species resembles *D. malayanum*. However, both the females and males of the current specimens have shorter tails and oesophagi, reflected in the larger values of ratios b and c (Tables 1 and 2). Also, even though *D. malayanum* is shorter than *D. burnupiae* n. sp., the former has a larger basal bulb (Sullivan and Palmieri, 1978), at least 1.5 times longer than in the latter. Moreover, the spicules of *D. malayanum* are smaller

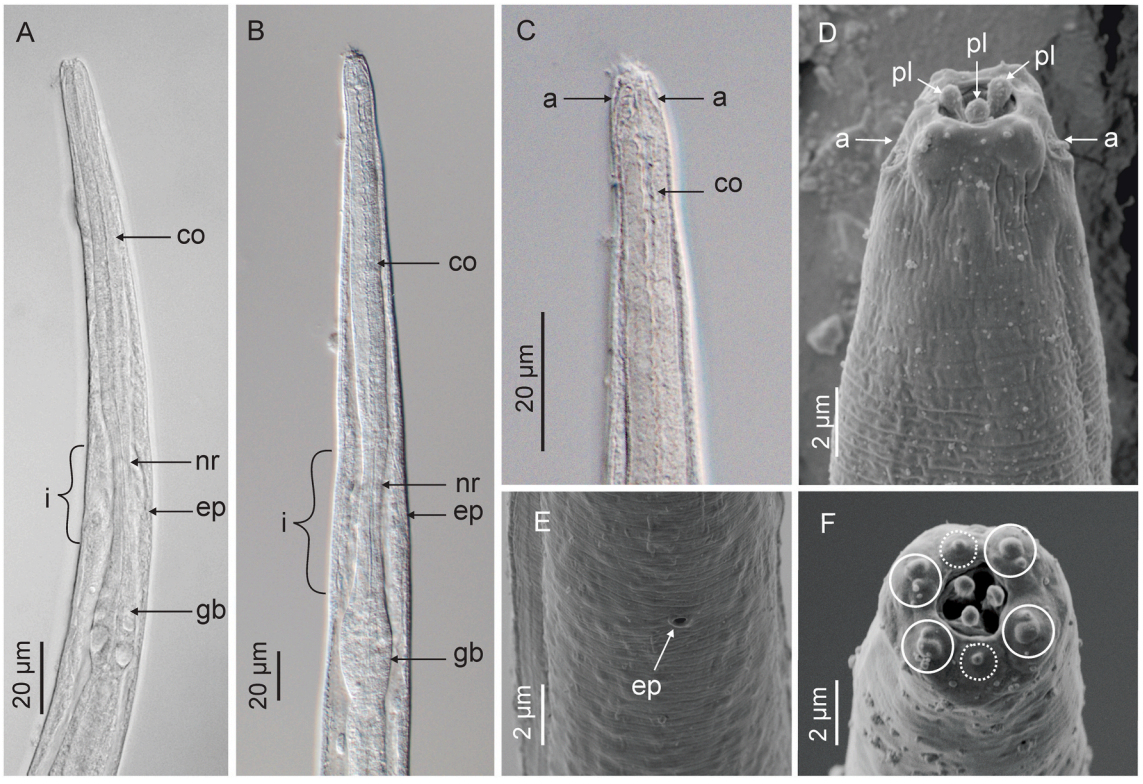

Fig. 3. Light (A–C) and scanning electron (D–F) micrographs of *Daubaylia burnupiae* n. sp. anterior end. A, oesophagus male; B, oesophagus female; C, anterior region of corpus female; D, dorsal view of cephalic end female; E, subventral view showing excretory pore; F, apical view cephalic end male, showing papillae on lateral lips (broken line circles), and on the dorso-ventral lips (solid line circles). Abbreviations: a, amphids; co, corpus; ep, excretory pore; gb, glandular basal bulb; i, isthmus; nr, nerve ring and pl, pharyngeal lobes.

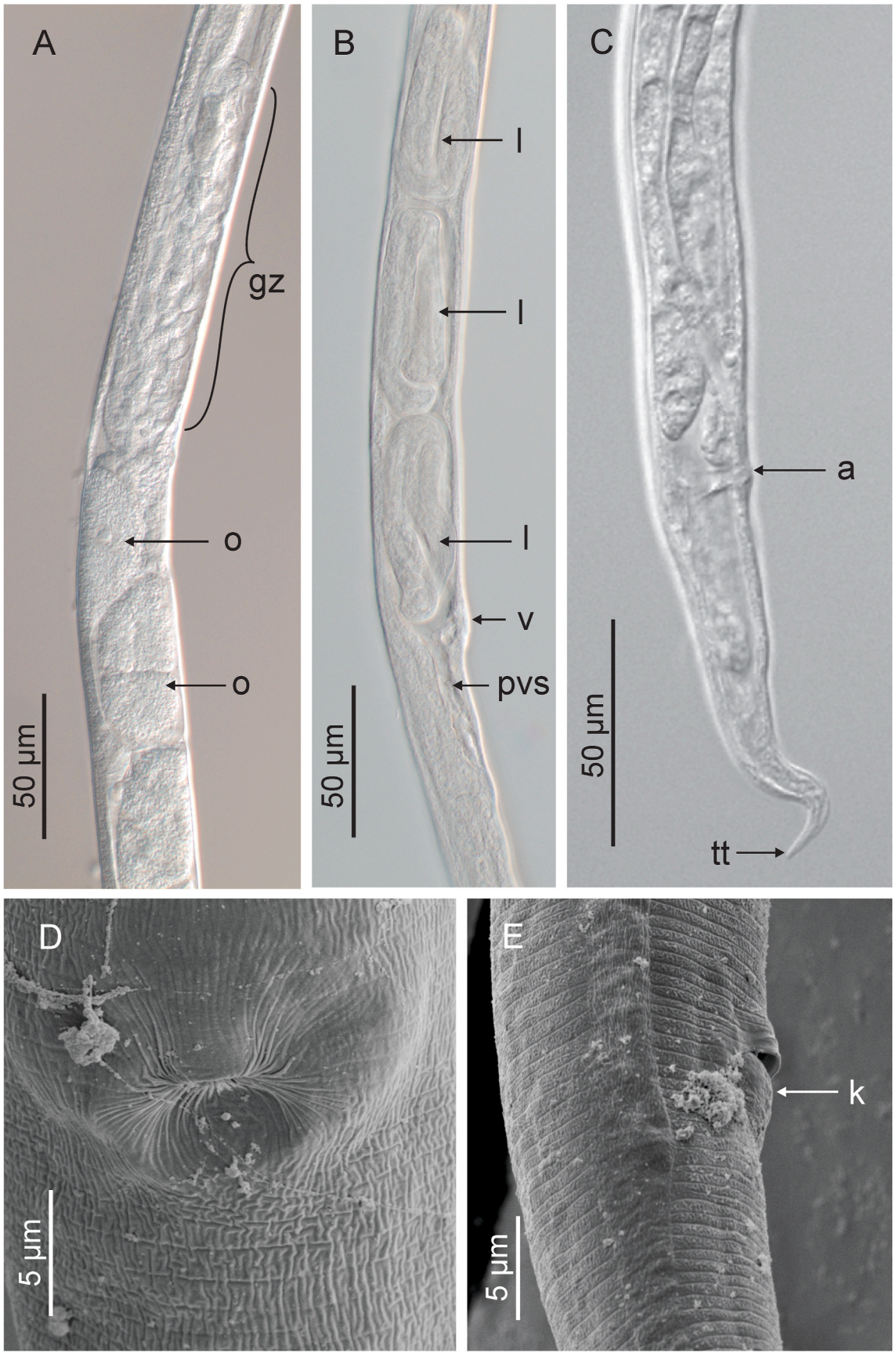

Fig. 4. Light (A–C) and scanning electron (D,E) micrographs of *Daubaylia burnupiae* n. sp. female. A, ovary anterior end; B, uterus and vulvular region; C, caudal region; D, ventral view of vulva and D, lateral view of anus. Abbreviations: a, anus; gz, germinal zone; k, knob-like protrusion; l, larva; o, oocyte; pvs, post-vulvular sac; tt, tail tip and v, vulva.

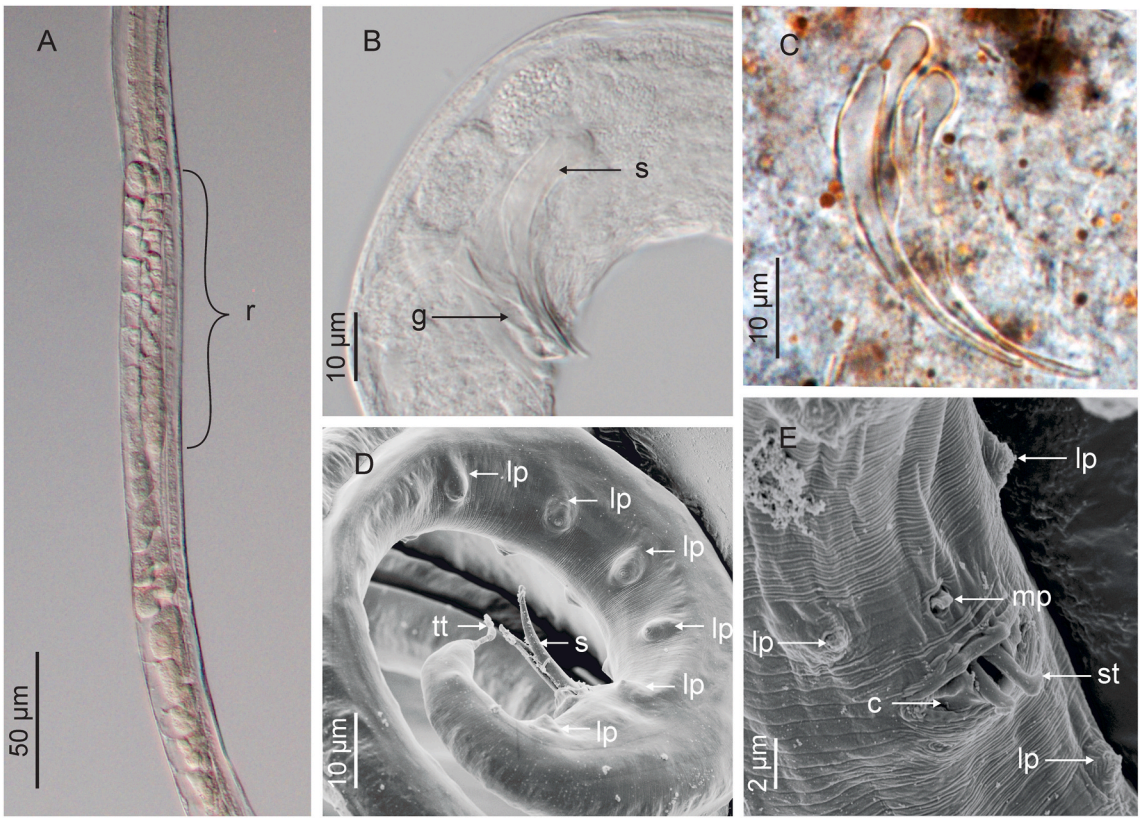

Fig. 5. Light (A–C) and scanning electron (D,E) micrographs of *Daubaylia burnupiae* n. sp. male. A, testis anterior end; B, genital armature; C, paired spicules; D, caudal region, subventral view; E, cloacal region, subventral. Abbreviations: c, cloaca; g, gubernaculum; lp, latero-ventral papilla; mp, median papilla; r, reflexed part of testis; s, spicules; st, spicule tip and tt, tail tip.

(Table 2) and differ in shape. Unlike the spicule in the current species, which is arcuate and has a cylindrical manubrium, the spicule of *D. malayanum* is almost straight and has a broad manubrium (Fig. 6).

Like *D. burnupiae* n. sp., *D. pearsoni* is characterised by the presence of more than one egg (usually larvated), in the uterus. The female of *D. pearsoni* is distinguished by a pair of prominent caudal toothlike appendages, near the dorsally curved extremity of the tail (Anderson and Bartlett, 1993). Also, unlike the current species whose spicule is arcuate, with a cylindrical manubrium and recurved tip, the spicule of *D. pearsoni* is almost straight, and has a broad manubrium (Fig. 6). The presence of a posteriorly directed spine on the tail of male *D. pearsoni*, further sets it apart from the current specimen. What is more, *D. pearsoni* has only four pairs of genital papillae: three pre-cloacal pairs and one post-cloacal pair (Anderson and Bartlett, 1993). In contrast, the males of the current specimens possess six pairs of latero-ventral papillae (five pre-cloacal pairs and one post-cloacal pair), and a single median papilla.

The number of lateroventral genital papillae in males of *D. olsoni*, correspond with the current species. However, in *D. olsoni*, the fifth pair of genital papillae is adanal (Poinar, 1984), contrary to the present species in which the fifth pair is pre-anal. Also, the spicules of *D. olsoni* differ in shape from the current species (Fig. 6). The female of *D. olsoni* is distinguished from the current species by the presence of an elongated saclike spermatheca, at the junction of the oviduct and uterus, and rarely contains eggs in the uterus (Poinar, 1984). Moreover, *D. olsoni* have longer tails in both sexes; reflected in the smaller values of ratio c (Tables 1 and 2).

The male of the present species resembles *D. helicophilus*, based on the number of lateroventral genital papillae. However, the spicules and gubernaculum of *D. helicophilus* are smaller (Table 2) and differ in shape from those of the current species (Fig. 6). The gubernaculum of *D. helicophilus* is pointed on both ends (Poinar and Richards, 1979), unlike in the current species. Compared with *D. helicophilus*, the vulva of the current species is located further away from the anterior extremity, reflected in the larger value of V (Table 1).

Daubaylia seistanensis differs from the current species on the values of ratios b and c, and sizes of spicules and gubernaculum (Tables 1 and 2). What is more, Baylis and Daubney (1922) reported that gravid *D. seistanensis* contained only one, undeveloped ovum: the current species has up to 12 eggs, often larvated in the uterus. Also, the males have only two pairs of genital papillae (Baylis and Daubney, 1922), which is at least three times fewer than the current specimen.

Unlike *D. burnupiae* n. sp. whose males are smaller than the females, for *D. potomaca*, they are nearly equal in size (Chitwood and Chitwood, 1934; Chernin et al., 1960). Secondly, according to Chernin et al. (1960), mature females of *D. potomaca* contained a single undeveloped egg in the uterus. In the present species, gravid females contained up to 12 eggs, often with developed larvae. Thirdly, *D. potomaca* has three pairs of pre-cloacal and two pairs of post-cloacal genital papillae (Chitwood and Chitwood, 1934), while the current species has five precloacal and one postcloacal pairs of papillae. What is more, the shapes of the manubrium and lamina of the spicule in *D. potomaca* separates it from *D. burnupiae* n. sp. (Fig. 6).

Data from Honer and Jansen (1961) shows that *D. elegans* is comparable in length to the current specimens. However, *D. elegans* has longer tails in both sexes, hence smaller values of ratio c, compared with the current specimens (Tables 1 and 2). In addition, the female of *D. elegans* has a cuticular knob-like process, located near the posterior extremity, 16–28 µm from the tail tip. In contrast, the female of the current species possesses a cuticular knob immediately after the anus, 60–83 µm from the tail tip. Also, the uterus of *D. elegans* is about half the size of the current species (Table 1) and lacks embryonated eggs (Honer and Jansen, 1961). Moreover, the female of *D. elegans* is thinner (larger ratio a) and the vulva is situated closer to the anterior, compared with the current species (Table 1). Finally, the males of *D. elegans* are

Table 1
Measurements (in μm unless stated), ratios a, b, c and index V, of adult female specimens of the current species (in bold) and the previously described species of *Daubaylia*.

Measurement	[a]*D. seistanensis*	[b]*D. potomaca*	[c]*D. dewiti*	[d]*D. elegans*	[e]*D. malayanum*	[f]*D. helicophilus*	[g]*D. olsoni*	[h]*D. pearsoni*	[i]*D. bonaerensis*	*D. burnupiae* sp. n.
Body length (mm)	1.30–1.43	1.74–1.84	2.13	1.85–2.07	0.96–1.11	1.11–1.36	1.02–1.63	2.50–2.70	5.48	**1.61 (1.20–1.91)**
Body width	35	30–40	28	23–26	22–32	16–25	20–29	40–46	165	**32 (27–37)**
Oesophagus length	200–270	213–239	300	238–258	151–233	190–245	179–237	330–350	365	**214 (184–252)**
Nerve ring, from anterior end		125–180	273	135–160		100–130	104–133	205–235	205	**151 (137–169)**
Excretory pore, from anterior end		150–172		155–175		113–138	120–149	250–270		**164 (142–184)**
Tail length	80	100–106	68	110–133	71–83	60–80	78–107	87–115	517	**74 (64–87)**
Vulva, from anterior end (mm)	0. 815	1.00–1.06	1.26	1.03–1.23	0.59–0.73					**1.09 (0.80–1.28)**
Gonad, from anterior end		650–740	700	825–838	269–418					**546 (460–662)**
Gonad, from vulva		340–450		203–225						**554 (401–796)**
Ratios										
a	38–41	46–58	76	75–86	31.7–49.5	60	53.8	61.9	33.2	**51 (41–65)**
b	4.8–5.3	7.0–8.5	7.1	7.6–8.6	4.6–5.7	6.35	6.26	7.67	15	**7.4 (6.6–8.3)**
c	17.8	17–18	31.3	14.6–17.8	12.6–15.3	18	13.3	26.3	10.6	**21 (18–24)**
v		56	59.2	54.9–62	62–68	57–63	45–60		44.97	**68 (66–70)**

[a] Baylis and Daubney (1922).
[b] Chitwood and Chitwood (1934).
[c] Schuurmans-Stekhoven (1956).
[d] Honer and Jansen (1961).
[e] Sullivan and Palmieri (1978).
[f] Poinar and Richards (1979).
[g] Poinar (1984).
[h] Anderson and Bartlett (1993) and.
[i] Camino and Gonzalez (2011).

Table 2
Measurements (in μm unless stated) and ratios a, b and c, of adult male specimens of the current species (in bold) and the previously described species of *Daubaylia*.

Measurement	[a]*D. seistanensis*	[b]*D. potomaca*	[c]*D. dewiti*	[d]*D. elegans*	[e]*D. malayanum*	[f]*D. helicophilus*	[g]*D. olsoni*	[h]*D. pearsoni*	[i]*D. bonaerensis*	*D. burnupiae* sp. n.
Body length (mm)	0.95–1.08	1.52–1.82	1.25–1.52	1.17–1.42	0.84–1.05	0.970–1.05	1.20–1.42	1.60–1.80	4.34	**1.13 (1.02–1.27)**
Body width	25	24–30	24	21–28	19–26	20–24	20–22	24–38	116	**28 (26–32)**
Oesophagus length	200–270	187–212	142–220	185–200	156–233	165–175	203–224	208–255	285.3	**178 (164–193)**
Nerve ring, from anterior end		116–125	120–136	108–118	109–118	95–105	118–131	134–175	151.32	**123 (114–138)**
Excretory pore, from anterior end		160–175		118–135	115–129	113–120	131–147	175–208		**134 (122–144)**
Tail length	50	52–56	48–80	43–68	52–62	35–40	48–50	40–59	320	**33 (30–37)**
Spicule length	26	28–29	28	22–25	24–30	23–25	26–32	30–36	224	**33 (32–35)**
Gubernaculum length	15	16	19	12–15	14–16	14–16	17–20	18–21	62	**21 (20–23)**
Gonad, from anterior end		640–660	620–740	521–625	350–470					**396 (302–561)**
Ratios										
a	38–40.9		58–63	47–58	34–47	45.5	61.9	51.5	37.4	**41 (38–49)**
b	4.75–5.30	7.0–8.5	6.3–7.0	7.0–7.6	4.2–5.5	5.88	5.99	7.2	15.2	**6.3 (5.8–6.8)**
c	19	28	15.7–18.3	20.5–28	14–19	27	26.5		13.6	**35 (33–38)**

[a] Baylis and Daubney (1922).
[b] Chitwood and Chitwood (1934).
[c] Schuurmans-Stekhoven (1956).
[d] Honer and Jansen (1961).
[e] Sullivan and Palmieri (1978).
[f] Poinar and Richards (1979).
[g] Poinar (1984).
[h] Anderson and Bartlett (1993) and.
[i] Camino and Gonzalez (2011).

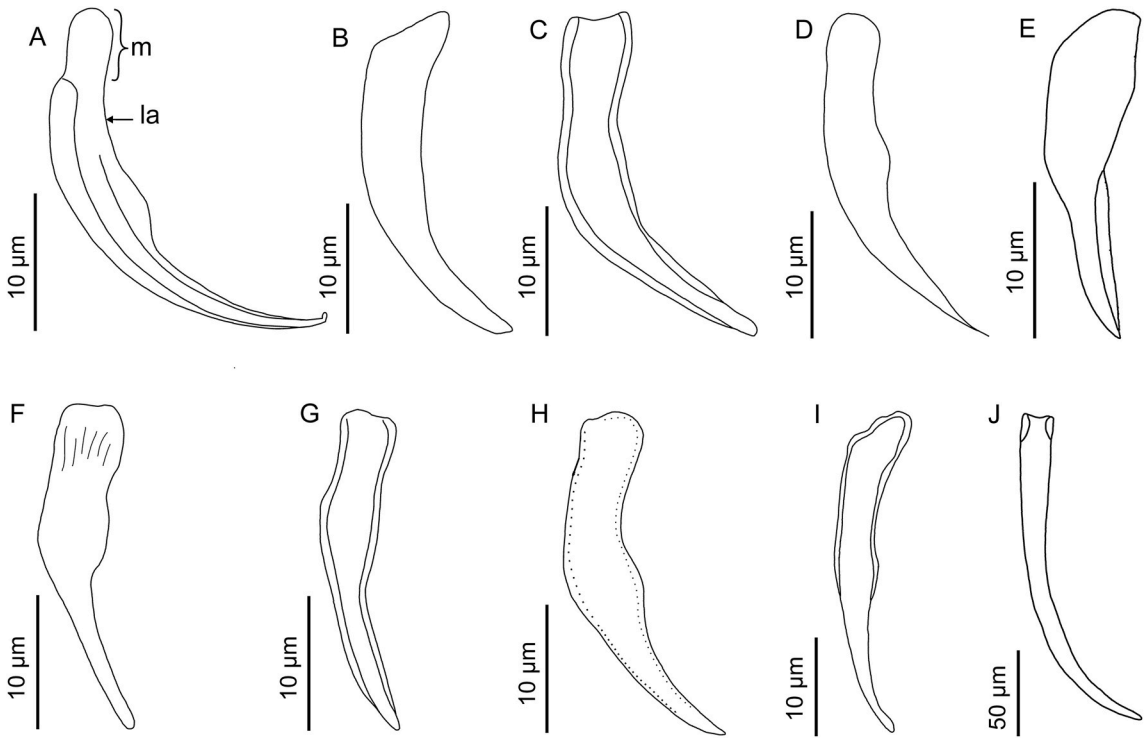

Fig. 6. Spicules of *Daubaylia* spp. A, *D. burnupiae* n. sp.; B, *D. seistanensis*; C, *D. potomaca*; D, *D. dewiti*; E, *D. elegans*; F, *D. malayanum*; G, *D. helicophilus*; H, *D. olsoni*; I, *D. pearsoni* and J, *D. bonaerensis*. Abbreviations: m, manubrium; la, lamina. B-J, redrawn from Baylis and Daubney (1922), Chitwood and Chitwood (1934), Schuurmans-Stekhoven (1956), Honer and Jansen (1961), Sullivan and Palmieri (1978), Poinar and Richards (1979), Poinar (1984), Anderson and Bartlett (1993), Camino and Gonzalez (2011), respectively.

distinguished by their minute spicules and gubernaculum (Table 2).

Daubaylia dewiti is thinner than the present species; evident from the larger value of ratio a in both sexes (Tables 1 and 2). Secondly, in *D. dewiti*, the location of the vulva is almost equidistant between the anterior and posterior ends of the body, while in *D. burnupiae* n. sp., the vulva is in the posterior third. Thirdly, the male of *D. dewiti* has a longer tail, hence, a smaller value for ratio c (Table 2). Also, the gubernaculum of *D. dewiti* is characterised by longitudinal striations (Schuurmans--Stekhoven, 1956), which were not observed in the present species. What is more, the male of *D. dewiti* differs from *D. burnupiae* n. sp. on the arrangement of lateroventral genital papillae. The former has four precloacal pairs and two post-cloacal pairs (Schuurmans-Stekhoven, 1956); the latter has five precloacal pairs and one post-cloacal pair.

Daubaylia bonaerensis is clearly distinguished from the current species based on size: being at least three times larger (Tables 1 and 2). According to Camino and Gonzalez (2011), *D. bonaerensis* has six cephalic papillae, while *D. burnupiae* n. sp. has ten. Also, the male of *D. bonaerensis* has eight pairs lateroventral genital papillae: two pairs more than the present species. Finally, *D. bonaerensis* has a valvated basal bulb (Camino and Gonzalez, 2011); the basal bulb is non-valvated in the current species.

3.1.4. Molecular data

Partial sequences for 28S and 18S rDNA genes were successfully obtained from eight *Daubaylia burnupiae* n. sp. specimens. Analyses of the generated sequences showed that the specimens were entirely identical for each set of the genes. The representative 28S rDNA sequence (1041 bp) of *D. burnupiae* n. sp. was aligned with the two available daubayliid sequences from GenBank. Based on 28S rDNA *p*-distances, the sequence showed 2.7% difference with *Daubaylia potomaca* (Accession no. KU180680.1) and 2.5% with an unidentified species of *Daubaylia* (Accession no. KY319365.1). Correspondingly, *D. burnupiae* n. sp. had 27 and 26 base pair differences with *D. potomaca* and *Daubaylia* sp., respectively. For 18S rDNA, two fragments (886 and 893 bp) overlapping by 195 bp were assembled into a single sequence (1584 bp). A comparison of the representative sequence with the available sequence for this gene on GenBank, showed 0.7% difference with *D. potomaca* (Accession no. KU180669.1), corresponding to 9 nucleotide substitutions. Sequence divergence (%), based on average uncorrected *p*-distance and the number of base differences between *Daubaylia* spp. are presented in Table 3.

3.1.5. Prevalence and infection intensities

Overall, 41.2% of the snails were infected with nematodes. The nematodes were found in the visceral tissues, body cavities and on the body wall of the snails, with infection intensities ranging from one to seventy-two. Table 4 shows nematode prevalence, mean abundance and mean intensities, in the snails, and the corresponding physico-chemical parameters of water at the sampling sites. Prevalence of nematodes in snails from S1, was significantly higher compared with those from S2, X^2 (1, N = 746) = 48.58, $p < 0.0001$. Also, snails from S1 had a higher nematode MA, compared with those from S2 (U = 48968, $p < 0.0001$). In contrast, MI of the nematodes did not differ significantly between the two sites (U = 8456, $p = 0.258$). The proportion of immature nematodes in snails from S1 (23.1%) was significantly higher than in snails from S2

Table 3
The number of base pair differences (above the diagonal) and sequence divergence (%) (below the diagonal), of *Daubaylia burnupiae* n. sp. (in bold) and other *Daubaylia* spp.

28S rDNA	Species	Accession number	1	2	3
	***D. burnupiae* n. sp.**	OQ269618	_-	**26**	**27**
	Daubaylia sp.	KY319365.1	**2.5**	–	5
	D. potomaca	KU180680.1	**2.8**	0.5	–
18S rDNA	***D. burnupiae* n. sp.**	OQ269617	_-	9	
	D. potomaca	KU180669.1	0.7	–	

Table 4
Physicochemical characteristics of water and the corresponding nematode infection parameters in the snails from the sampling sites. Except for EC (μS/cm), the units for water characteristics are mg/L; prevalence is given as percentages and confidence intervals of prevalence in parentheses.

Sampling site	Water quality parameters							Nematode nfection parameters		
	EC	Salinity	TDS	Sulphates	Ammonia-N	Nitrate-N	Chlorides	Prevalence	MI±SE	MA±SE
Site 1	225	0.1	0.1	21	0.3	0.4	21.3	52.3 (47.5–57.0)	12.7 ± 0.75	6.65 ± 0.50
Site 2	832	0.4	0.6	144	1.6	2.3	144.3	26.6 (21.5–31.6)	11.8 ± 1.40	3.11 ± 0. 48

Note: EC, electrical conductivity; TDS, total dissolved solids; MI, mean intensity; MA, mean abundance; SE, standard error. Water quality data: Pretorius and Avenant-Oldewage (2022).

(13.6%), X^2 (1, N = 3952) = 39.47, $p < 0.0001$.

4. Discussion

4.1. Taxonomy

Distinctions between species of *Daubaylia* have been based primarily on overall body size and morphological features observed using light microscopy (Chitwood and Chitwood, 1934; Schuurmans-Stekhoven, 1956; Honer and Jansen, 1961; Sullivan and Palmieri, 1978; Poinar, 1984; Anderson and Bartlett, 1993). However, it should be noted that species distinction based on size is challenging, due to the overlap in lengths of different species. Indeed, *D. burnupiae* n. sp. cannot be distinguished from the females of *D. dewiti*, *D. elegans*, *D. helicophilus*, *D. olsoni*, *D. potomaca* and *D. seistanensis*, based on total body size. Therefore, features such as a big uterus, absence of a spermatheca, *in utero* egg and larval development, presence of a cuticular anal knob-like protrusion, absence of caudal appendages, and the values of ratios a, b and c, and index V, are necessary for delineating *D. burnupiae* n. sp. Except for *D. potomaca*, *D. pearsoni* and *D. bonaerensis*, the males are generally small (<1.5 mm). Thus, clearer distinctions between the males of different species in the genus are seen in genital features and the values of ratios a, b and c. The identity of *Daubaylia burnupiae* n. sp. as a separate species is confirmed by the shape of the spicules, and the short tail of the male (larger value for ratio c), compared to its congeners. In both sexes, *D. burnupiae* n. sp. is characterised by an oesophagus with a short isthmus and a short glandular basal bulb. In contrast, in other *Daubaylia* spp., the post-corpi of the oesophagi are nearly equal in length to the corpi, or even longer.

The current study is the first to characterise a daubayliid by integrating morphological examination using light and scanning electron microscopy, and DNA data. Using scanning electron microscopy, we have highlighted features which have not been previously reported, for *Daubaylia*. They include, the arrangement of cephalic papillae, the occurrence of three club-shaped pharyngeal lobes, in both sexes and a median genital papilla anterior to the cloacal opening of the male. Regarding molecular data, only three daubayliid genetic sequences (two for 28S and one for 18S rDNA), had been deposited on GenBank before the present study. The sequences analysed for the current specimens, showed no variations, but differed significantly with those from GenBank. Therefore, morphological and DNA characterisation support that this species is distinct from the known species of *Daubaylia*.

Daubaylia bonaerensis, described by Camino and Gonzalez (2011), seems to deviate from the diagnostic characteristics for the genus. For instance, *D. bonaerensis* lacks a clear distinction between the corpus and isthmus of the oesophagus, the basal bulb is valvated and it is the only species in the genus that is longer than 2.7 mm, double the length of the other species. What is more, all the other species of *Daubaylia* have purely aquatic lifecycles, while *D. bonaerensis* is a parasite of *Thelidomus aspera* (Férussac, 1821) (Pleurodontidae), a terrestrial snail. We suggest that the inclusion of *D. bonaerensis* into the genus, should be confirmed by DNA characterisation.

4.2. Life cycle strategies

Simultaneous occurrence of larvae and adults of the current nematode within the host tissues, confirms that it is monoxenous, just like the other species of *Daubaylia*. However, some aspects of the life histories and infection mechanisms of most *Daubaylia* spp. remain unresolved. Experimental studies on *D. potomaca* and *D. malayanum* showed that after infection, the worms require at least 11 days to complete the developmental cycle (Chernin, 1962; Sullivan and Palmieri, 1978). Poinar and Richards (1979) and Zimmermann et al. (2011a), observed that snails infected by *D. potomaca* and *D. helicophilus* respectively, had reduced lifespans, and gravid nematode females exited the snails before they died. According to Zimmermann et al. (2013), detection of the host's impending death, competition for space and resources, and maturity of the female worm, might be cues for *D. potomaca* to exit their hosts. Thus, the gravid female is the stage that initially infects the snail (Sullivan and Palmieri, 1978; Poinar and Richards, 1979; Zimmermann et al., 2011a). However, the mode of transmission for daubayliids, whether active or passive, is still unclear. Poinar and Richards (1979), suggested that the transmission of *D. helicophilus* is via ingestion of gravid females by the snails, after which the nematode passed through the intestine into the haemocoel, where proliferation occurs. In contrast, Zimmermann et al. (2011b) suggested that *D. potomaca* transmission occurs through direct penetration of the snail foot, since the nematodes were not observed in the hosts' intestinal tracts.

Egg production and larval development vary among daubayliids. The uteri of *D. seistanensis* and *D. potomaca* contained single undeveloped eggs, hence, after deposition in the snail tissues, further egg development was required before the release of larvae (Baylis and Daubney, 1922; Chernin et al., 1960). The time required for the released eggs to complete development before hatching is about 5 days for *D. potomaca* (Chernin, 1962), and unknown for *D. seistanensis*. Other species, such as *D. elegans* and *D. olsoni*, exhibited rapid oviposition and eggs were rarely observed in the uterus (Poinar, 1984). In contrast, the uteri of *D. malayanum* and *D. pearsoni* had well-developed eggs, containing first stage larvae. According to Sullivan and Palmieri (1978), the eggs of *D. malayanum* hatched within 12 h after leaving the uterus. Based on the presence of *in utero* larvated eggs, it appears that the reproductive pattern of *D. burnupiae* n. sp., might correspond with *D. malayanum* and *D. pearsoni*. However, the life history patterns, infection dynamics and pathology of the current nematode on the snail host, are still unknown, and will be the subject of a separate study.

4.3. Host preference and geographic distribution of daubayliids

Daubaylia spp. have been reported from the Americas, Europe, Africa, Asia and Australia. Except for one report of infections in a leech, the nematodes predominantly parasitise freshwater pulmonate snails. Poinar (1984) reported *D. olsoni* in co-occurring populations of the leech *Dina anoculata* Moore, 1898 and the snail *Planorbella tenuis* (Dunker, 1850) (Planorbidae), from a pond in California, USA. Notably, *Daubaylia potomaca* has been reported from snails of three different families, in two continents. In the USA, planorbids *Planorbella trivolvis* (Say, 1817), *Biomphalaria glabrata* (Say, 1818) and *Helisoma anceps* (Menke, 1830), are the known hosts of *D. potomaca* (Chitwood and Chitwood, 1934;

Chernin et al., 1960; Zimmermann et al., 2011a). *Daubaylia potomaca* also occurred in *Bulinus globosus* (Morelet, 1886) (Bulinidae) in Nigeria (Okeke and Ubachukwu, 2017), and *Radix natalensis* (Krauss, 1848) (Lymnaeidae) in Kenya (Outa et al., 2022). In Europe, there are two species of *Daubaylia*, both from planorbid snails in the Netherlands: *Daubaylia dewiti* from *Bathyomphalus contortus* (Linnaeus, 1758) and *D. elegans* from *Planorbis carinatus* Müller, 1774 (Schuurmans-Stekhoven, 1956; Honer and Jansen, 1961). Three species of *Daubaylia* were described from Asia, all from planorbid snails. *Daubaylia seistanensis* was reported from *Gyraulus convexiusculus* (Hutton, 1849) in Iran (Baylis and Daubney, 1922), *D. malayanum* from *G. convexiusculus* in Malaysia (Sullivan and Palmieri, 1978) and *D. helicophilus* from *Gyraulus spirullus* (Gould, 1859) in Taiwan (Poinar and Richards, 1979). From Australia, *D. pearsoni* was reported to parasitise the planorbid *Glyptophysa gibbosa* (Gould, 1847) (Anderson and Bartlett, 1993). The current study is the first report of *Daubaylia* from southern Africa, and the first in a snail belonging to the family Burnupiidae. Apart from the reports from Africa, and the single case *D. olsoni* in a leech, it appears that planorbid snails are the preferred hosts for daubayliids. Also, while other *Daubaylia* spp. have restricted ranges, *D. potomaca* is the only species with a wide geographical distribution.

4.4. Nematode occurrence in relation to pollution

Low prevalence and fewer larvae of nematodes in *B. stenochorias*, corresponded with increased pollution in the river, while mean intensities did not differ between the sampling sites. These findings indicate that the establishment of infection might have been influenced by the conditions in the habitats of the snails. After successful infection, the nematodes are probably buffered from the effects of the host's environment, which allows for the maintenance of infection intensities. The sampling site where nematode prevalence was low, is under pollution pressure from point discharge of industrial and municipal wastewater and has elevated salinity, total dissolved solids, ammonia nitrogen, nitrates, chlorides, sulphates and metals (Pretorius and Avenant-Oldewage, 2022). Even though we had postulated that pollution might compromise the immune response of the snails, thereby allowing for increased infection of the snails by the nematodes, it appears that the nematodes are more vulnerable to pollution than the snails. Since daubayliids have a brief free-living stage, during the transmission of gravid females between individual snails (Sullivan and Palmieri, 1978), we suspect that exposure to pollutants either reduces the viability or kills the gravid nematode. Therefore, the nematode is a potential bioindicator of aquatic pollution. However, further studies are required, to determine the survival rate of this nematode species to different doses of individual or combinations of pollutants.

Ethical standards

This study was approved by the University of Johannesburg Ethics Committee (Reference Number: 2022-08-05/Outa_Oldewage), and the procedures undertaken in this study complied with the relevant institutional and national research guidelines.

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Declaration of competing interest

The authors declare that they have no competing interests.

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