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Redescription of the African *Chordodes albibarbatus* Montgomery 1898, and description of *Chordodes janovyi* n. sp. (Gordiida, Nematomorpha) and its non-adult stages from Cameroon, Africa

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Abstract

We redescribe *Chordodes albibarbatus* Montgomery 1898 from the original holotype male and the originally described female specimen using Nomarski interference contrast microscopy. Our reinvestigation indicates that *C. albibarbatus* is sexually dimorphic and contains five types of areoles in the male and six types of areoles in the female. Our reinvestigation of *C. albibarbatus* indicates that it is a distinct species, and is most similar to the African *Chordodes gariazzi* Camerano 1902 and *Chordodes heinzei* Sciacchitano 1937, all of which share simple "blackberry", bulging, tubercles, and thorn areoles. In addition, we describe adult free-living male and female *Chordodes janovyi* **n. sp.** collected from West Province, Cameroon, Africa using both morphological (light and scanning electron microscopy) and molecular data, and designate types for this species. *Chordodes janovyi* belongs to a large group of *Chordodes* in which simple areoles are smooth or superficially structured less so than "blackberry" areoles. Present among the simple areoles are clusters of crowned and circumcluster areoles along with thorn and tubercle areoles, whereas bulging areoles are absent. We also describe the egg strings, eggs, larvae, cysts, and oviposition behavior of *C. janovyi* and compare these non-adult life stages to other nematomorph genera and species for which such life cycle stages are known, and we discuss the use of non-adult stages and the use of molecular tools in future studies of nematomorph systematics and biodiversity.

Key words: Gordiida, gordiid, hairworm, Gordian worm, Nematomorpha, Africa, scanning electron microscopy, Nomarski interference contrast microscopy, molecular data, oviposition behavior, non-adult life stages

Introduction

With few exceptions, the genus *Chordodes* Creplin, 1874 has a tropical and subtropical distribution and is the most specious of the 19 known nematomorph genera with around 100 reported species (Zanca *et al.* 2006a; 2006b; De Villalobos *et al.* 2007; Schmidt-Rhaesa *et al.* 2008). However, the identification and taxonomic status of many of these species is questionable. Many original species descriptions are based solely on bright field light microscopy, and limited morphological data is available from a single or a few adult worms collected from a single location. This has led some investigators to re-evaluate the genus, and currently only 54 of the species are sufficiently described to be recognized; whereas 36 species are considered *species inquirenda* and 22 species are considered *incertae sedis* (Schmidt-Rhaesa *et al.* 2008). More problematic is the fact that of the 54 currently recognized species of *Chordodes* no morphological data is known on non-

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adult life cycle stages, no type specimens or type hosts exist, and/or the exact type localities are not known for a majority of these species. The lack of data on hosts, non-adult gordiid life stages, which are more common in the environment than the adults (Hanelt *et al.* 2001), and the lack of type localities and knowledge of nematomorph species distribution, confound subsequent identification of species and non-adult life cycle stages, and make identification of new species, and locating populations of known species problematic. However, comprehensive morphological descriptions of multiple life stages using light and scanning electron microscopy accompanied by molecular analyses are likely to alleviate these problems in nematomorph identification, systematics, and knowledge of gordiid distribution.

The African *Chordodes albibarbatus* Montgomery 1898a is one such species with limited or no morphological, type locality and non-adult life cycle stage data available. *Chordodes albibarbatus* was originally described by Montgomery (1898a) using only bright field microscopy from a single free-living male collected from an unknown location in the Ogove River in Africa. Later Montgomery (1898b) provided a description for a female *C. albibarbatus* from a single individual removed from a jar containing preserved orthopterans collected from an unknown location around the Gabon River in West Africa, and it is assumed that both individuals of *C. albibarbatus* were collected in Gabon. However, both these rivers originate in neighboring countries, Equatorial Guinea and the Democratic Republic of the Congo, and the exact type locality of this species is uncertain. Taken together, the limited morphological data on this species, the questionable identity of the type host and the lack of type locality for *C. albibarbatus* warrants a redescription of *C. albibarbatus*.

In this article, we redescribe the adult male and female *Chordodes albibarbatus* from the original male holotype specimen and originally described female specimen deposited by Montgomery (1898a, 1898b) in the invertebrate collection at the Philadelphia Academy of Natural Sciences using Nomarski interference contrast microscopy. Additionally, we describe a new species of *Chordodes* similar to *C. albibarbatus*, from new collections from West Province, Cameroon, using both morphological (light and/or scanning electron microscopy) and molecular data. Finally, we describe the egg strings, eggs, larvae, cysts, and oviposition behavior of this new species. We then compare these non-adult life stages to other nematomorph genera and species for which such life cycle stages are available, and discuss the use of these stages in future studies of nematomorph systematics and biodiversity.

Methods

Redescription of *Chordodes albibarbatus.* We investigated a partial male holotype and a partial female specimen of *C. albibarbatus* borrowed from the invertebrate collection at the Philadelphia Academy of Natural Sciences (WM4525, and WM 4526) on which Montgomery (1898a, 1898b) based his original descriptions. In order to preserve the holotype and original female material, we removed a tangential section of the mid-body cuticle with a razor blade, and placed it in glycerol prior to examination with Nomarski interference contrast microscopy (NICM). Additionally, we examined the anterior, midbody, and posterior regions of worms under a dissecting microscope. Digital images were recorded with a Nikon Coolpix S4 digital camera (Tokyo, Japan) equipped with a Martin Microscope MM99-S4 adaptor (Easley, South Carolina). We report the morphological characteristics of this species from our redescription and compare our observations to the original descriptions by Montgomery (1898a, 1898b).

Collection and adult morphology of *Chordodes janovyi* n. sp. A single adult male and a single adult female worm were collected from a small un-named river within the Menoua River Drainage in the village of Bawa, department of Menoua, West Province, Cameroon (5°24’N, 10°03’E) during June 2006. Worms were discovered in the river wrapped around a stick in the process of mating and were placed with the stick in a plastic bottle with river water and brought to the University of Nebraska-Lincoln Cedar Point Biological Station, Ogallala, Nebraska, USA.

Both worms were maintained with the stick in a 110 x 35 mm stender dish partially filled with aged tap water and aerated. The female worm was allowed to deposit egg strings directly onto the stick. Once egg

string deposition stopped, the length of both male and female worms were measured in mm with a ruler without stretching the worms; worms were then placed between two slides and the diameter was measured with an ocular micrometer under a compound microscope. The color of each worm was recorded and worms were fixed in 95% ethanol. Initial species identification was based on Schmidt-Rhaesa *et al*. (2008) and comparisons to our redescription of *C. albibarbatus*. For light microscopy (LM) studies, the anterior and posterior ends of male and female worms were placed between two slides in alcohol and examined with a compound microscope, whereas the middle 5–10 mm sections of cuticle from the male and female worms were cut, removed from the remaining tissue and placed in glycerol prior to examination. Digital images were recorded as previously described. For scanning electron microscopy (SEM) studies, 5–10 mm sections of the mid-body cuticle of the male and female worm were cut, critically point dried, mounted on aluminum stubs, coated with gold palladium, and examined with a Hitachi SEM 450 (Tokyo, Japan) at 15 kV. The remaining tissues were saved for molecular analysis (see below). All terminology of areoles follows Schmidt-Rhaesa *et al*. (2008).

Morphology of egg strings, eggs, and larvae of *Chordodes janovyi* n. sp. Egg strings (n = 30) and undeveloped eggs (n = 30) were measured within 2–5 days of oviposition. Five mm sections of individual egg strings were removed from the stick with a scalpel, placed on microscope slides with water, gently covered with a cover slip without crushing, and examined under a compound microscope. Length and width measurements were taken for each undeveloped egg, whereas only width measurements were taken for egg strings with an ocular micrometer. The remaining eggs were allowed to develop. Egg maturity was judged using the color of the egg strings; strings became darker as the larvae developed. Larvae (n = 32) were measured immediately after hatching. Eight measurements were taken on relaxed living larvae following the protocol of Hanelt & Janovy (2002). These measurements included the preseptum length and width, postseptum length and width, stylet length and width, and pseudointestine length and width. Some larvae were fixed in hot formalin and processed for SEM as previously described.

Physa (*Physella*) *gyrina* (Say, 1821) snails were obtained from established laboratory colonies as described in Bolek & Janovy (2007). Laboratory reared *P. gyrina* snails were exposed to freshly hatched (2–5 day old) *C. janovyi* larvae. Fifty snails were exposed for 48 hrs to approximately 2,500 larvae in a plastic shoe box (35 cm X 25 cm X 15 cm) partially filled with aerated aged tap water and TetraMin fish food (Tetra, Blacksburg, Virginia). Snails were allowed to feed on the larvae/TetraMin fish food mixture for 48 hr. Exposed surviving snails were maintained in a 3.78 L jar with aerated age tap water on a diet of frozen lettuce and TetraMin fish food. Snails were examined for the presence of cysts 14–20 days post exposure as previously described by Hanelt *et al*. (2001). Briefly, shells were removed from the snails, and the soft tissue was crushed between two slides. Only those cysts, which lay perfectly flat, were measured following the protocol of Hanelt & Janovy (2002). Four measurements were taken on live, fully formed cysts; these included cyst larval length and width, and cyst wall length and width. Cyst wall length and width were calculated according to Hanelt & Janovy (2002) by subtracting the length and width of the larva from the length and width of cyst and dividing by two, respectively.

DNA extraction, amplification, and sequencing of *Chordodes janovyi* n. sp. Molecular work was conducted on both male and female worms collected from Cameroon, preserved in 95% ethanol. From each, a 0.5 cm section was cut, dried at room temperature and used for DNA extraction using a modification of the HotSHOT method (Truett *et al.* 2000). Briefly, the dried material was minced with a razor blade, placed into 50µl of lysis buffer for 30 min at 96 °C. Samples were moved to ice, and an equal volume (50µl) of neutralization buffer was added and mixed by inversion. Samples were spun at 10,000xg at room temperature for 5 min, and the supernatant was moved to a fresh tube, and stored at -70 °C.

Partial sequences of 18S, and 28S rDNA were amplified using TaKaRa Ex Taq (Takara Bio Inc., Otsu, Japan) following manufacturer's instructions and using modified universal primers: 28SF1, GTC TTG AAA CAC GGA CCA AGG AGT; 28SR1, CCC CGA GAC CTC TAA TCA TTC; 18SF1, CAT GCA TGT GTC AGT ATG AAC; 18SR1, CAT TCC AAT TAC AGG GTC TCG. PCR reactions were analyzed by agarose gel electrophoresis, with the use of 1.0% agarose gels, stained with 0.5% GelRed Nucleic Acid Gel Stain (Biotium, Hayward, California), and visualized on a UV transilluminator. Amplicons were purified by ethanol precipitation and sequenced using the BigDye version 3.1 kit (Applied Biosystems, Foster City, California) in

an ABI 3130x sequence analyzer (Applied Biosystems). Both strands of the amplified DNA fragments were sequenced, edited in Sequencer version 4.9 (Gene Codes, Ann Arbor, Michigan), and manually corrected for ambiguous base calls.

Sequence analysis. DNA sequences were compared to other sequences in the GenBank database using BLAST (Altschul *et al.*, 1990), applying the distance tree of results utility employing the Fast Minimum Evolution model (Jukes and Cantor, 1969) associated with BLASTN to infer phylogenetic relationships.

Results

Redescription

Chordodes albibarbatus Montgomery, 1898

(Figures 1–3)

1898. *Chordodes albibarbatus* Zool. Jahrb. Syst. 11: p 22 & p 29

Holotype: 1 partial male

Other material: 1 partial female

Material examined: NICM mid-body, and anterior and posterior ends of a partial specimen of adult male holotype WM4525 and partial specimen of adult female WM4526 deposited by Montgomery in the Philadelphia Academy of Natural Sciences.

Type locality: Unknown location in the Ogoou River in Gabon, west central Africa, with tributaries reaching into the Republic of the Congo, Cameroon and Equatorial Guinea.

Other localities: Unknown location around the Gabon River in West Africa.

Host: Undetermined orthopteran reported by Montgomery (1898b).

Redescription of holotype male: The body color is brown with mottled darker spots and prominent white tufts of filaments corresponding to crowned areoles cover most of the body (Figs. 1A & C). The anterior end is lighter in color and no dark collar is present (Fig. 1A). Lighter coloration blends into the normal coloration of the remaining body. Body length according to Montgomery (1898a) is 223 mm and the midbody diameter is 1.25 mm. The anterior end is distinctly tapering (Fig. 1A); the posterior end is round with the indication of two lobes and the cloacal opening is subterminal and oval (Fig. 1B).

The body cuticle contains five types of areoles. Simple areoles are the most abundant, they are low (6–7 µm), oval and 10–14 x 7–9 µm in length and width, with a warty surface (blackberry type) (Fig. 1D). Simple areoles are separated by interareolar furrows, 2–5 µm apart. Scattered among the simple areoles are bulging, tubercle, and pairs of crowned areoles surrounded by circumcluster areoles (Figs. 1E, 1F & Figs. 2A, 2B, 2C, 2D). Bulging areoles form clusters of two, three or four areoles (Figs. 1E, 1F). Bulging areoles are taller (11–18 µm) then simple areoles and are 13–17 x 10–14 µm in length and width (Figs. 1E, 1F). Filaments on tubercle areoles are club shaped 14 µm (13–15) in length and approximately 1–2 µm in width at the base and become wider 3–4 µm distally (Fig. 2D). Crowned areoles occur in pairs (Figs. 1E, 1F & Figs. 2A, 2B). Crowned areoles are 15–25 µm tall, 17–22 µm in length and 15–17 µm wide and contain apical filaments that are 35–60 µm in length on top (Figs. 2A & 2C). Crowned areoles are surrounded by 19–22 circumcluster areoles (Figs. 1E, 1F & Figs. 2A, 2B, 2C). These areoles are taller (11–25 µm) than simple areoles, with small bristles on top; the tallest and thickest in diameter circumcluster areoles are positioned next to the crowned areoles, and decrease in length and diameter away from the central crowned areoles (Figs. 1E, 1F & Figs. 2A, 2B, 2C).

Redescription of female: The body color is dark brown with the anterior and posterior ends being lighter in color; with no dark collar present on the anterior end (Figs. 3A, 3B). The lighter coloration blends into the normal coloration of the remaining body. Body length according to Montgomery (1898a) is 215 mm, and the diameter is 2 mm midbody. The anterior end is distinctly tapered (Fig. 3A); the posterior end is distinctly swollen, and the cloacal opening is terminal (Fig. 3B).

Unlike the males, the body cuticle of the female contains six types of areoles. Simple areoles are the most abundant, they are low (4–7 µm), oval and 10–14 x 7–8 µm in length and width; with a warty surface (blackberry type; Fig. 3D). Simple areoles are separated by interareolar furrows, 2–5 µm apart. Scattered

among the simple areoles are bulging, tubercle, thorn, and crowned areoles surrounded by circumcluster areoles (Figs. 3E, 3F, 3G, 3H). Bulging areoles are single or form clusters of two, three or four areoles (Fig. 3E). Bulging areoles are taller (14–17 µm) then simple areoles and are 13–28 x 8–10 µm in length and width. Filaments on tubercle areoles of the female are less club-shaped than in the male; they are 10 µm (9–12) in length and approximately 2 µm in width at the base and become wider 3–4 µm distally (Fig. 3H). Thorn areoles are 28–30 µm tall and 9–10 µm in diameter at the base (Fig. 3G). Crowned areoles occur in pairs (Fig. 3F). Crowned areoles are 21–23 µm tall, 15–23 µm in length and 11–17 µm wide and contain apical filaments that are 60–120 µm in length on top. Crowned areoles are surrounded by 14–22 circumcluster areoles. These areoles are taller (15–20 µm) than simple areoles, with small bristles on top; circumcluster areoles are thinner than in the male being 4–8 µm in length and 3–7 µm in width.

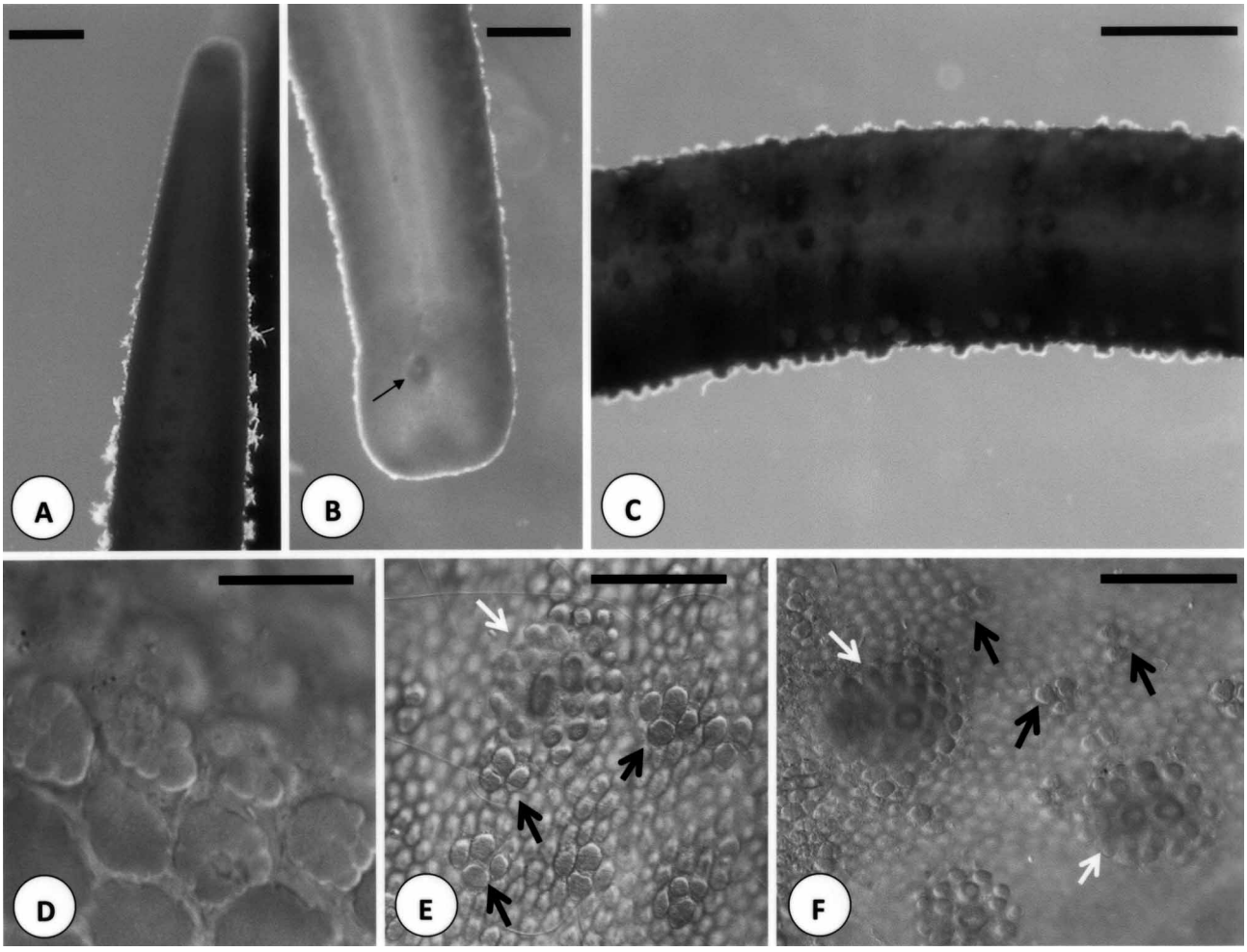

FIGURE 1. Holotype of male *Chordodes albibarbatus*. (A) Tapering anterior end with a white cap, and no dark collar. Note the white tufts representing crowned areoles. Scale bar = 500 µm. (B) Posterior end. Note the posterior end is round with the indication of two lobes; oval cloaca (arrow). Scale bar = 500 µm. (C) Steriomicroscope, overview of midbody region showing the spotted pattern and distribution of crowned areoles. Scale bar = 750 µm. (D) NICM showing close up of simple "blackberry" areoles. Scale bar = 14 µm. (E & F) NICM overview of cuticle showing simple areoles and clusters of bulging areoles (black arrows) and crown areoles surrounded by circumcluster areoles (white arrows). Scale bar = 90 µm.

Comments: Our reinvestigation of *C. albibarbatus* indicates that it is distinct from other known species of African *Chordodes*. Montgomery (1898a, b) originally described areoles that correspond to simple, crowned, circumcluster, tubercle, and bulging areoles on the male and simple, crowned, circumcluster, tubercle and thorn areoles on female *C. albibarbatus*, and later Montgomery (1898b) indicated that thorn areoles were present on the male, but they were less numerous than on the female. One major difference in our

redescription of *C. albibarbatus* from the original deiscription of Montgomery (1898a, b) was our discovery of bulging areoles on the female and our inability to find thorn areoles on the male. Additionally, we confirm that the simple areoles are of the blackberry type.

Of the 33 described species of *Chordodes* from Africa, 15 species are considered *species inquirenda* (Schmidt-Rhaesa *et al.* 2008). However, of the remaining 18 African species of *Chordodes*, *C. albibarbatus* is most similar to *Chordodes gariazzi* Camerano, 1902a and *Chordodes heinzei* Sciacchitano, 1937 both described from the Democratic Republic of the Congo. *Chordodes gariazzi* contains six types of areoles including simple "blackberry", groups of two to four bulging, tubercle, thorn, and pairs of crowned areoles surrounded by 12–20 circumcluster areoles, but differs from *C. albibarbatus* in the presence of two rows of bristle fields anterolateral to the cloacal opening in male *C. gariazzi*. *Chordodes heinzei* has five types of areoles including simple "blackberry", isolated or clusters of two to four bulging areoles, club shaped tubercles, and pairs of crowned areoles surrounded by 17–24 circumcluster areoles (Zanca *et al.* 2006). The only apparent difference between male *C. albibarbatus* and *C. heinzei*, is based on the original description of thorn areoles on male *C. albibarbatus* by Montgomery (1898b). Unfortunately, Montgomery's permanent slide of the cuticle of a male *C. albibarbatus* is lost and we cannot refute or confirm his observation of thorn areoles on the male of *C. albibarbatus*. It is premature to synonymize *C. heinzei* with *C. albibarbatus* until a female *C. heinzei* is described, more specimens are available for comparison, and/or molecular sequences are available to differentiate or synonymize these species.

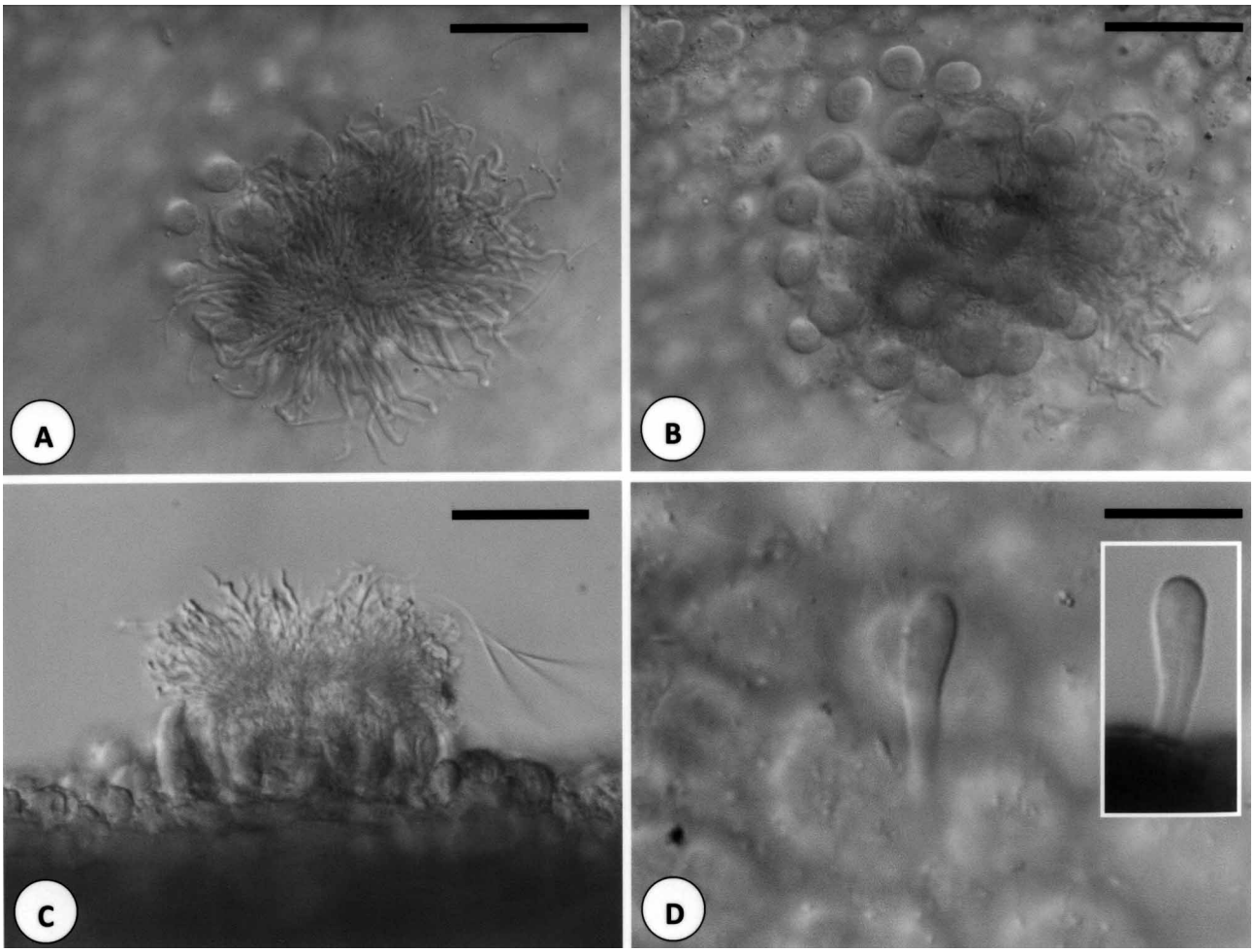

FIGURE 2. Cuticular structures of male *Chordodes albibarbatus*. (A) NICM surface view of pair of crowned areoles showing filaments. Scale bar = 30 μm. (B) NICM pair of crown areoles surrounded by circumcluster areoles. Scale bar = 30 μm. (C) NICM lateral view of crown areoles surrounded by circumcluster areoles. Scale bar = 30 μm. (D & Inset) NICM club shaped tubercle areole among simple areoles. Scale bar = 10 μm.

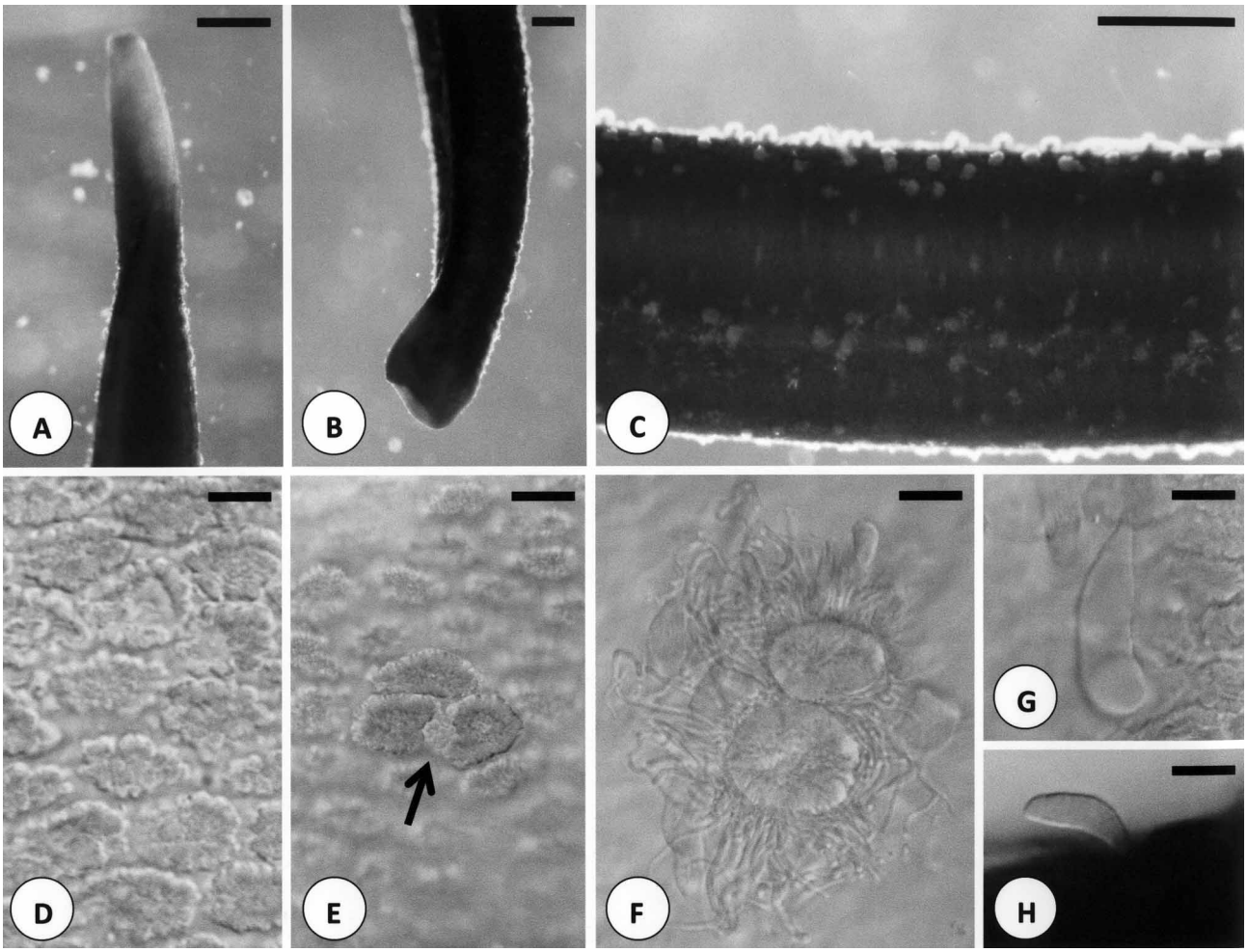

FIGURE 3. Cuticular structures of female *Chordodes albibarbatus*. (A) Tapering anterior end with a white cap, and no dark collar. Scale bar = 500 μm. (B) Steriomicroscope, posterior end. Scale bar = 500 μm. (C) Steriomicroscope, overview of midbody region showing the distribution of crowned areoles. Scale bar = 750 μm. (D) NICM showing close up of simple "blackberry" areoles. Scale bar = 5 μm. (E) NICM showing a close up of a cluster of three bulging areoles (black arrows) among simple areoles. Scale bar = 10 μm. (F) NICM showing a pair of crowned areoles surrounded by circumcluster areoles. Scale bar = 10 μm. (G) NICM showing a close up of a thorn areole. Scale bar = 10 μm. (H) NICM showing a close up of a tubercle areole. Scale bar = 5 μm.

***Chordodes janovyi* n. sp.**
(Figures 4–8)

Holotype: 1 male collected from Menoua River Drainage in the village of Bawa in the West Province of Cameroon, Zoological Museum Hamburg, accession number V13291.

Paratype: 1 female collected from, Menoua River Drainage in the village of Bawa in the West Province of Cameroon, Zoological Museum Hamburg, accession number V13292.

Other material deposited: Larvae from laboratory cultures, Zoological Museum Hamburg, accession number V13293.

Type locality: Menoua River Drainage in the village of Bawa approximately 17 km southwest of Dschang in the West Province of Cameroon (5°24'N, 10°03'E).

Other localities: None.

Hosts: Definitive: unknown, paratenic: *Physa gyrina* in the laboratory.

Material examined: Free-living adult male holotype and female paratype, egg strings, eggs, larvae and cysts. SEM midbody of free-living adult male and female, and larvae, and LM anterior, posterior ends and

midbody of free-living adult male and female; egg strings, eggs, larvae, and cysts from laboratory infected snails.

Etymology: The species epithet is named in honor of John Janovy Jr., who has supported our work on nematomorphs over the years.

Description of male: The body color is dark brown with the anterior end whitish, no dark collar present. Whitish coloration blends into the normal coloration of the remaining body. Body length 86 mm and the midbody diameter is 500 µm. The anterior end is distinctly tapering with a degenerate mouth (Fig. 4A); the posterior end is round with the indication of two lobes (Fig. 4B). The cloacal opening is oval.

The body cuticle contains five types of areoles. Simple areoles are the most abundant, they are low (3µm), oval to round, 3–5 µm in diameter, and their surface is smooth or structured into denticles and canals (Fig. 4C). Simple areoles are separated by interareolar furrows, 2–5 µm apart. Interareolar furrows contain canals running laterally across the cuticle (Fig. 4C). Scattered among the simple areoles are tubercles, thorn, and crowned areoles surrounded by circumcluster areoles (Figs. 4C, 4D, 4E, 4F). Thorn areoles are rare, and they are 22 µm (18–26) in length and 5 µm (3–6) in width at their base; whereas the filaments on tubercle areoles are 11 µm (9–12) in length and approximately 1–2 µm in width. Crowned areoles occur in pairs and have filaments that are 15–30 µm in length on top (Figs. 4C, 4D). Crowned areoles are surrounded by 8–16 circumcluster areoles (Figs. 4C, 4D). These areoles are taller (4–6 µm) than simple areoles, with small bristles on top (Figs. 4C & 4E, 4F).

Description of female: The body color is light tan with the anterior and posterior ends whitish in color; with no dark collar present on the anterior end. The white coloration blends into the normal coloration of the remaining body. Body length is 80 mm, and the diameter is 800 µm midbody. The anterior end is distinctly tapered with a degenerate mouth (Fig. 5A); the posterior end is distinctly swollen (Fig. 5B), and the cloacal opening is terminal.

As in males, the body cuticle of females contains five types of areoles. Simple areoles are the most abundant, they are low (3 µm), oval to round, 8–15 µm in diameter, with a smooth surface (Figs. 5C, 5D). Simple areoles are separated by interareolar furrows, 1–5 µm apart. Unlike the male, interareolar furrows do not contain canals running laterally across the cuticle (Fig. 5D). Scattered among the simple areoles there are tubercle and thorn areoles (Figs. 5D, 5E, 5F). Thorn areoles are more common than on the male specimen, and they are 20 µm (17–22) in length and 5 µm (4–6) in width at their base; whereas the filaments on tubercle areoles are 10 µm (8–12) in length and approximately 1–2 µm in width. Crowned areoles occur in pairs and have longer filaments on top than in the males being 30–70 µm in length (Figs. 5C, 5D). Crowned areoles are surrounded by 7–12 circumcluster areoles with small bristles on top (Fig. 5D).

Description of oviposition, egg strings, and eggs: During oviposition the female *C. janovyi* attached egg strings in a continuous zigzag pattern around a small branch (Fig. 6A). Egg string width was 385 µm (250–600); whereas individual undeveloped eggs were 36.2 µm (30–40) in length and 30.3 µm (27–35) in width (Fig. 6B). Larvae developed in eggs within 2–5 weeks at room temperature (Fig. 6C).

Description of larvae: Larvae of *C. janovyi* possessed a cylindrical body divided by a septum into two regions, the preseptum and a postseptum (Fig. 6D). The preseptum was 22.8 µm (18–30) in length and 15.0 µm (14–17) in width and contained an evertable stylet 13.8 µm (11–15) in length and 4.2 µm (3.5–5) in width; whereas the postseptum was 25.7 µm (21–32) in length and 12.5 µm (12–17) in width and contained a clearly visible pseudointestine. The pseudointestine was v-shaped with unequal branches positioned anteriorly and was 13.3 µm (10–17) in length and 8.3 µm (7–10) in width (Figs. 6D, 6E). Three weeks after hatching free–living larvae secreted thread like projections by empting their pseudointestine and stopped moving (Fig. 6F).

Externally, larvae were superficially annulated and the postseptum contained two pairs of terminal spines located ventrally (Figs. 6G & 7A); the pseudointestine exterior opening was centrally located between pairs of anterior and posterior terminal spines (Fig. 7B). The preseptum contained three sets of cuticular hooks (Figs. 6H & 7C, 7D). These contained seven hooks in the outer ring, two of which are very close together and ventrally positioned (Fig. 6H), and six hooks in the second (middle) and third (inner) rings (Figs. 6G, 6H & 7C). The dorsal and ventral side of the anterior tip of the laterally flattened stylet each contained five spines (two aligned pairs and one single spine above); whereas the left lateral side of the stylet contained four papillae like structures (Figs. 6H & 7D).

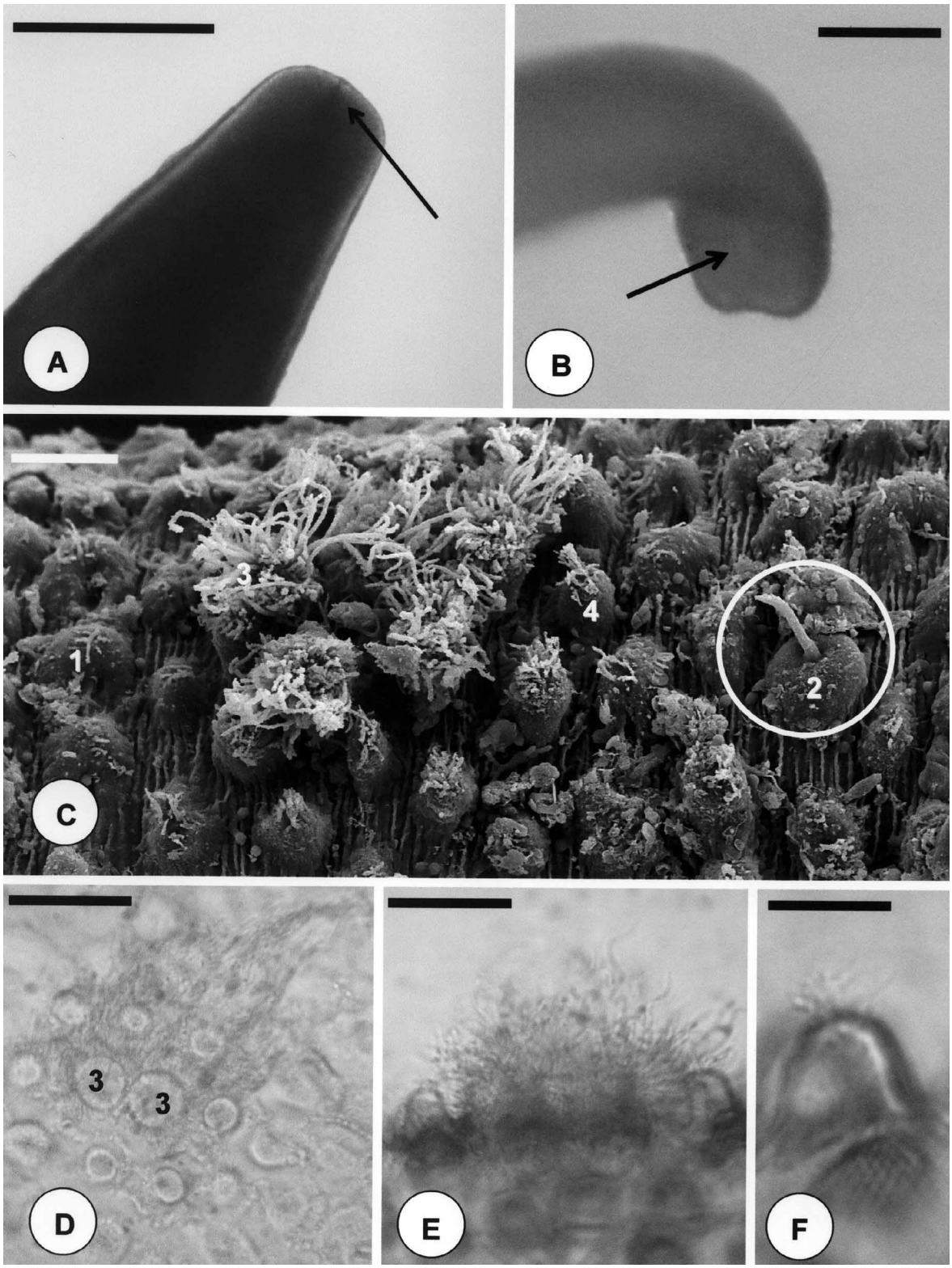

FIGURE 4. Holotype of male *Chordodes janovyi* **n. sp.** (A) Anterior end showing the degenerate mouth (black arrow); tapered anterior end with a white cap, and no dark collar. Scale bar = 300 μm. (B) Posterior end. Note the posterior end is round with the indication of two lobes; oval cloaca (arrow). Scale bar = 300 μm. (C) SEM overview of cuticle showing simple areoles (1), tubercle areoles (2), and crowned (3) and circumcluster areoles (4). Note simple areoles are separated between neighbors by interareolar furrows by a distance of 2–5 μm apart. Scale bar = 30 μm. (D) Light microscopy showing pair of crowned areoles (3) surrounded by smaller circumcluster areoles. Scale bar = 30 μm. (E) Light microscopy lateral view of two crowned areole with long filaments surrounded by circumcluster areoles. Scale bar = 30 μm. (F) Circumcluster areole with small bristles on top. Scale bar = 10 μm.

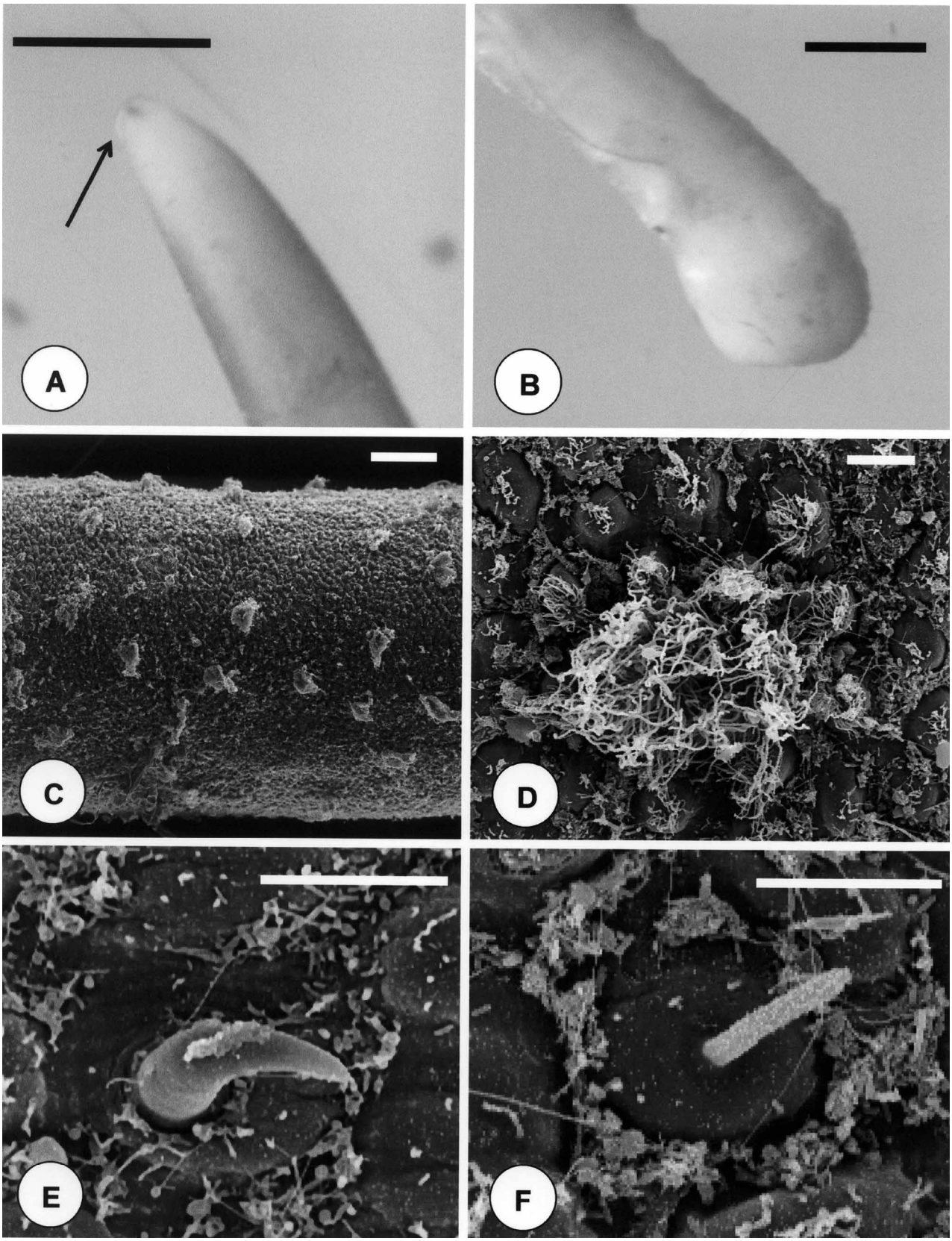

FIGURE 5. Paratype of female *Chordodes janovyi* **n. sp.** (A) Anterior end; note the degenerate mouth (black arrow). Scale bar = 1 mm. (B) Posterior end; cloaca is terminal. Scale bar = 500 μm. (C) Overview of cuticle showing: groups of crowned and circumcluster areole with long filaments among simple areoles. Scale bar = 100 μm. (D) Close up of crowned areole with long filaments surrounded by circumcluster areoles. Scale bar = 10 μm. (E) Thorn areole. Scale bar = 10 μm. (F) Tubercle areoles. Scale bar = 10 μm.

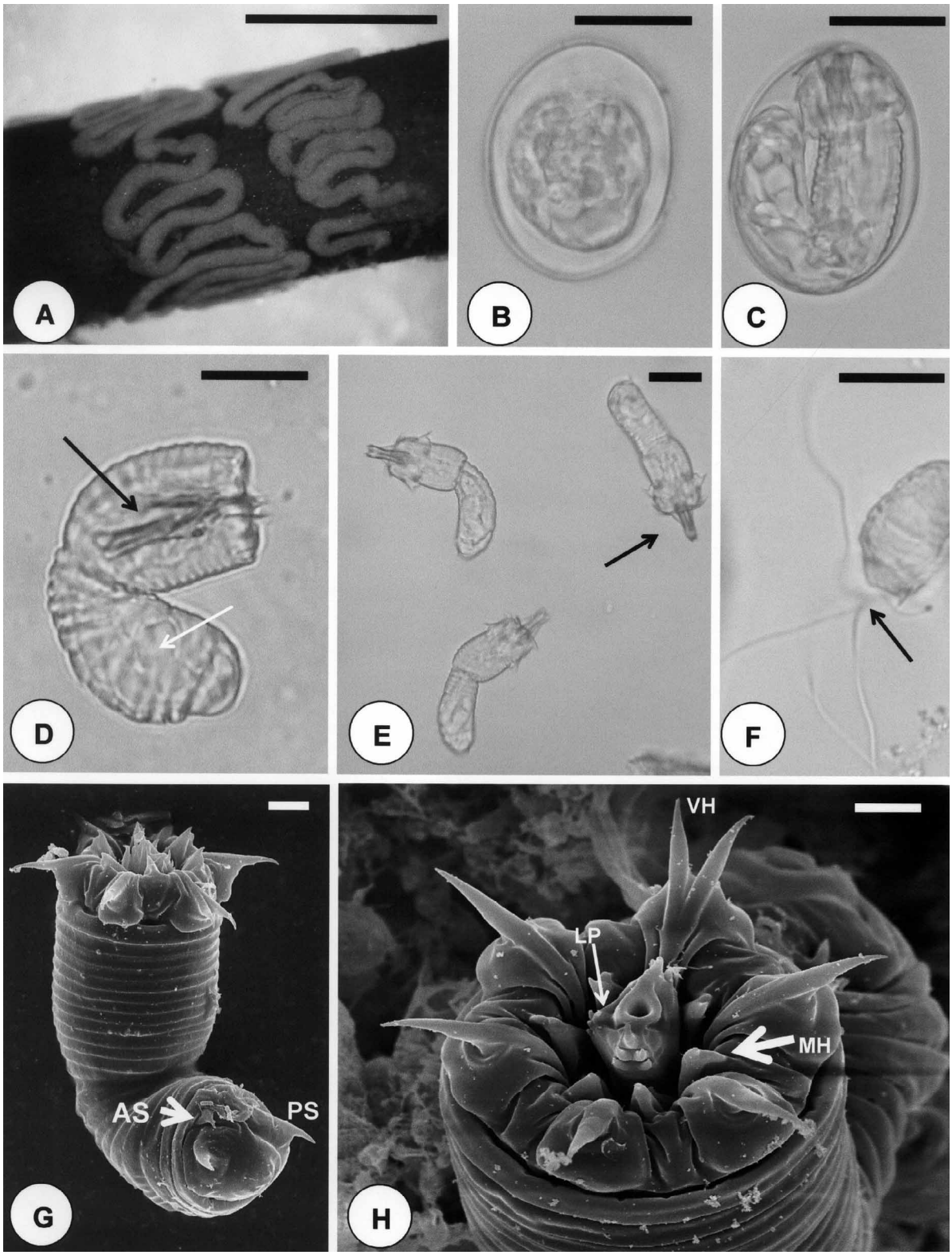

FIGURE 6. Eggs strings, eggs, and larvae of *Chordodes janovyi* **n. sp.** (A) Egg strings deposited on a stick. Scale bar = 5 mm. (B) Undeveloped egg. Scale bar = 20 μm. (C) Egg with larva. Scale bar = 20 μm. (D) Hatched larva; note the stylet black arrow, and pseudointestine white arrow (lateral view). Scale bar = 10 μm. (E) Larvae with everted stylets. Scale bar = 12 μm. (F) Larva secreting thin threads from the pseudo intestine. Scale bar = 20 μm. (G) SEM ventral view of larva showing superficial annulation and the postseptum containing two pairs of terminal spines located ventrally. AS anterior spines; PA posterior spines. Scale bar = 2 μm. (H) SEM anterior view of larva showing stylet and hook arrangement. LP lateral papillae, MH middle hook, VH ventral hooks. Scale bar = 2 μm.

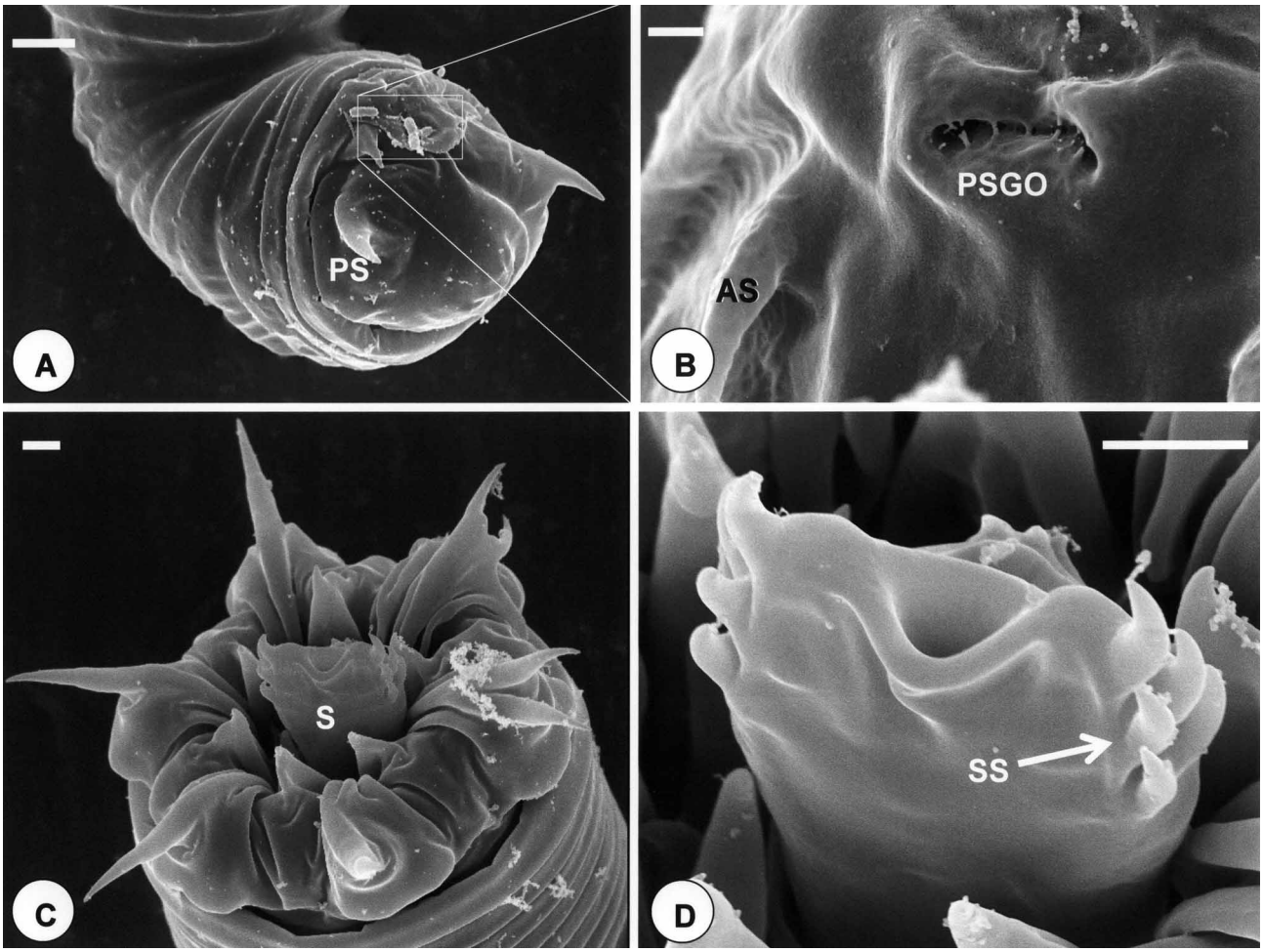

FIGURE 7. SEM of larvae of *Chordodes janovyi* **n. sp.** (A) Ventral side; note two posterior spines (PS). Scale bar = 2 μm. (B) Close up of ventral posterior side showing the pseudointestine gland opening (PSGO); AS anterior spine. Scale bar = 400 nm. (C) Larva anterior end; note stylet (S). Scale bar = 1 μm. (D) Close up of stylet from (C). SS stylet spines; note arrangement of five spines (two aligned pairs and one single spine above) on the dorsal and ventral sides of the style. Scale bar = 1 μm.

Descriptions of cysts: Cysts of *C. janovyi* possessed a clear cyst wall 7.1 μm (3.5–14) in length and 6.9 μm (3.5–12) in width (Fig. 8A & B). Fully folded larvae inside of the cyst were folded only once and were 27.6 μm (24–30) in length and 20.5 μm (17–24) in width (Fig. 8B).

Comments: Male and female *C. janovyi* contain five types of areoles and exhibit minor differences in cuticular morphology (number of circumcluster areoles, length of filaments on crowned areoles, distribution of thorn areoles, and presence or absence of interareolar furrows canals); thus *C. janovyi* is sexually dimorphic. *Chordodes janovyi* belongs to a large group of *Chordodes* in which simple areoles are smooth or superficially structured, less so than "blackberry" areoles and clearly differ from *C. albibarbatus.* Among the simple areoles are clusters of crowned and circumcluster areoles along with thorn and tubercle areoles, whereas bulging areoles are absent (Zanca *et al.* 2006a; 2006b; De Villalobos *et al.* 2007; Schmidt-Rhaesa *et al.* 2008). Of the 18 other sufficiently described African *Chordodes* species, 13 contain simple areoles that are not of the "blackberry" type and of those species only *Chordodes digitatus* Linnstow 1901, *Chordodes hawkeri* Camerano 1902b, and *Chordodes mülleri* Sciacchitano 1937 contain five types of areoles. These species differ from *C. janovyi* by the following characteristics: *C. digitatus* contains crowned areoles in groups of three, *C. hawkeri* contains bulging areoles, whereas *C. mülleri* does not contain thorn areoles.

Observations on the oviposition behavior, egg strings, eggs, larvae and cysts of *C. janovyi* suggests that non adult characteristics of this species are most similar to other species in the genus *Chordodes*, and are

distinct from genera and species of *Gordius* Linn 1758 and *Paragordius* Camerano 1897 for which such characteristics are available (Inoue 1958; Bohall *et al.* 1997; Schmidt-Rhaesa 1997; Bolek & Coggins 2002; Hanelt & Janovy 2002; Marchiori, *et al*. 2009). Both *C. janovyi* and the North American *Chordodes morgani* Montgomery 1898c deposit egg strings on twigs and detritus in a zigzag pattern, whereas European, and North and South American *Gordius* and *Paragoridus* species deposit free 3–10 mm and up to 50 cm long egg strings into the water, respectively. Larvae of *C. janovyi*, *C. morgani* and *Chordodes japonensis* Inoue 1952 are also similar in the structure of their pseudointestine (v-shaped, with unequal branches positioned anteriorly), whereas the North American *Paragordius varius* (Leidy 1851) has an elongate oval pseudointestine with a pair of anterior granules and North American and European *Gordius* species, have a single elongated oval pseudointestine subdivided into unequal portions anteriorly (Inoue 1958; Schmidt-Rhaesa 1997; Hanelt & Janovy 2002). Additionally, the stylet of *C. janovyi* is flattened laterally as in *C. morgani* with spines and/or papillae on the dorsal, ventral and left lateral sides but differs from the dorsoventrally flattened stylet with three aligned pairs of spines on the left and right lateral sides of *Gordius dimorphus* Poinar 1991 (Bohall *et al.* 1997; Marchiori, *et al*. 2009). Finally, the cysts of *C. janovyi* and *C. morgani* are also more similar in morphology to each other than to cysts of other genera and species of *Gordius* and *Paragordius*. Cysts of *C. janovyi* and *C. morgani* are folded only once, and differ from cysts of *P. varius*, which are also folded once but have hooks protruding from their preseptum, whereas cysts of North American and European *Gordius* species are all folded three times (see Schmidt-Rhaesa 1997; Bolek & Coggins 2002; Hanelt & Janovy 2002).

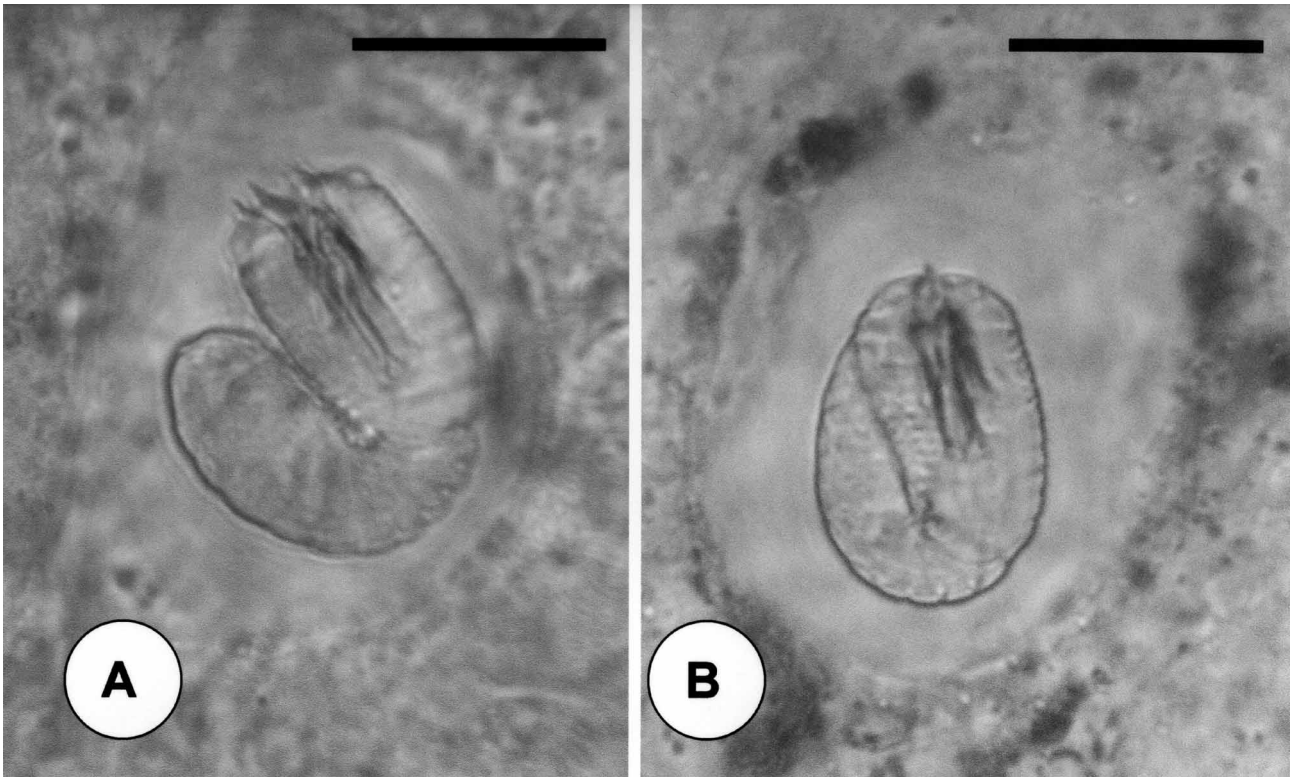

FIGURE 8. *Chordodes janovyi* **n. sp.** cysts recovered from an experimentally infected *Physa gyrina* snail. (A) An immature cyst. Note the single folding pattern. Scale bar = 20 μm. (B) Mature cysts; note the clear halo surrounding the encysted larvae. Scale bar = 20 μm.

Phylogenetic analysis of *Chordodes janovyi*

Two fragments of 456 and 421 base pairs were amplified from 18S and 28S regions respectively, and sequenced. These sequences were placed into GenBank (HM770080-HM770083). Presently, GenBank

contains very little information for the phylum Nematomorpha (19 sequences for 18S and 4 sequences for 28S). This data limitation, especially for 28S, will not allow our analysis to place *C. janovyi* into a phylogenetic context, but it will allow us to check our genetic data against contamination and broadly place this species into the phylum. For 28S, the BLAST search and distance tree revealed that the sequence obtained was most closely related to *Paragordius* sp. (AY428827; max score: 492; max identity 88%; e-value: $1x10^{-141}$), but did not place it as a sister group to *Chordodes morgani*; again, due to issues of taxon sampling, caution is warranted in interpretation of these results. For 18S, the closest match was to *Chordodes* sp. (AF421763; max score: 824; max identity 98%; e-value: 0), and both of these taxa were distantly clustered from *C. morgani*.

Discussion

Our reinvestigation of *C. albibarbatus* and description of *C. janovyi* confirm that these two African species of *Chordodes* are distinct species, with five types of areoles in the male and six types of areoles in the female *C. albibarbatus* and five types of areoles in both male and female *C. janovyi*. Additionally, our observations on the ovipositioning behavior and non-adult life stages of *C. janovyi* confirm that egg deposition and non-adult life states are more similar to egg deposition behavior and non-adult life stages of *C. morgani* and *C. japonensis* than other nematomorph genera (*Gordius*, *Paragordius*, and *Neochordodes*) for which such data are available (see Inoue 1958; Poinar & Doelman 1974; Bolek & Coggins 2002; Hanelt & Janovy 2002; Marchiori *et al*. 2009). These observations have several implications. First, non-adult life stages may be useful in generic separation and may be phylogenetically conserved. Second, genus level identification may be possible by features other than adult characters, especially those of the cyst stage, which is the most encountered gordiid life stage in nature, and cysts may be useful as a tool for studies on the distribution and biodiversity of gordiids which are difficult to locate in their free-living adult stage. Clearly, in order to test these hypotheses data will have to be collected on other species and genera of gordiids.

Although our molecular data, based on 18S, supports a close association of *C. janovyi* with another *Chordodes* species, both 18S and 28S datasets suggest that *C. janovyi* is not closely related to *C. morgani*, thus suggesting that the genus *Chordodes* is polyphyletic. This result is consistent with the only other previous molecular study of this phylum (Bleidorn *et al.* 2002). However, as stated above, due to a paucity of genetic data available for this phylum, phylogenetic data need to be interpreted with caution. This lack of gordiid DNA data highlights two critical needs: 1) to expand molecular study of this group, and 2) to begin a gordiid DNA barcoding library capable of identifying existing and new species.

We hope that the present study provides an incentive for comprehensive morphological redescriptions and descriptions of multiple life stages of nematomorphs using LM and SEM accompanied by molecular analyses, which will aid in alleviating the current problems in nematomorph identification, systematics, and our understanding of gordiid distribution and biogeography.

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