



Novel techniques for biodiversity studies of gordiids and description of a new species of *Chordodes* (Gordiida, Nematomorpha) from Kenya, Africa

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Abstract

We review recent advances in the use of non-adult gordiid cyst stages to locate gordiids over large geographical regions and new culturing techniques which can help overcome current difficulties in nematomorph biodiversity studies. Using these techniques, we collected a new species of gordiid as cysts in aquatic snails (*Biomphalaria pfeifferi*) from the Lake Victoria Basin, western Kenya, Africa and cultured them in the laboratory. We describe the adult free-living male and female worms using morphological (light and scanning electron microscopy) and molecular data as well as the life cycle, mating and oviposition behavior, egg strings, eggs, larvae, and cysts of this new species. *Chordodes kenyaensis* n. sp. belongs to a large group of African *Chordodes* in which simple areoles are smooth or superficially structured less so than “blackberry” areoles but contain filamentous projections. Present among the simple areoles are clusters of bulging areoles, crowned and circumcluster areoles along with thorn and tubercle areoles. In the laboratory, worms developed and emerged within 53–78 days from three species of laboratory-reared crickets exposed to cysts of this species. Adult male and female *C. kenyaensis* n. sp. initiated typical Gordian knots within hours to days of being placed together and males deposited masses of sperm on the cloacal region of females. Females began oviposition within a week of copulating and attached egg strings in a continuous zigzag pattern on small branches or air-hoses but never free in the water column. Larvae hatched within two to three weeks, and cysts developed in laboratory-reared and exposed snails within 14–24 days. Morphological characteristics of egg strings, eggs, larvae and cysts of *C. kenyaensis* were most similar to other gordiids in the genus *Chordodes* but differed morphologically from other gordiid genera for which similar information is available.

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

Introduction

Our lack of knowledge of the biodiversity of gordiids stems from the fact that most hairworm species have been described based on random collections of single worms for which life cycles are unknown. In fact, it has been estimated that only 18% of hairworm species have been described globally (Poinar 2008). However, recent studies from our laboratories indicate that non-adult stages of gordiids, such as cysts, are the most commonly encountered life stages of hairworms in the environment and may be useful for discovering the hidden biodiversity of this group (Hanelt *et al.* 2001; Szymgiel 2012). In a 3-year study of 50 streams in a 2,000 km² prairie ecosystem in Nebraska (USA), free-living adult gordiids were found at one stream, whereas cysts were recovered from aquatic snails at 35 streams (Hanelt *et al.* 2001). In a similar study, Szymgiel (2012) examined 46 streams in a 1,810 km² central Great Plains ecosystem of Oklahoma (USA). Over a one year sampling period no adult worms were found at any streams but cysts of three different types were recovered from aquatic snails from 32 of those streams. Additional studies

by our group (Hanelt & Janovy 2002; Bolek & Coggins 2002; Bolek *et al.* 2010; Hanelt *et al.* 2012; Szmygiel 2012) on cyst morphology of six North American and African species of gordiids from three genera indicate that gordioid cysts can be useful in generic separation. Based on these observations, Hanelt *et al.* (2012) collected *Biomphalaria pfeifferi* (Krauss 1848) snails infected with cysts of an unidentified species of *Paragordius* Camerano 1897 from Kenya, a country for which no gordioid records existed. After identification of the cyst to genus, and exposing the appropriate group of laboratory-reared arthropods, the first parthenogenetic species of gordioid, *Paragordius obamai* Hanelt, Bolek and Schmidt-Rhaesa 2012, was discovered.

In this article, a new species of *Chordodes* Creplin 1847 is described from the Lake Victoria Basin, western Kenya, Africa. It was collected in the cyst stage in snail paratenic hosts and established in the laboratory. Morphological (light and/or scanning electron microscopy) and molecular data from adult worms are reported and the life cycle, mating and oviposition behavior, egg strings, eggs, larvae, and cysts of this new species are described. Additionally, we discuss new techniques for nematomorph biodiversity studies and additional difficulties with these techniques which must be overcome.

Methods

Collection and establishment of the life cycle of *Chordodes kenyaensis* n. sp. in the laboratory. Approximately 200 *Biomphalaria pfeifferi* snails were collected with a dip-net from a small stream in western Kenya (Kasabong stream, Nyanza province, Kenya, (-0.1519, 34.4455, approx. 1,170 m altitude) and exported live to our laboratories at the University of New Mexico and Oklahoma State University (U.S.A.). Snails were examined for the presence of cysts as previously described by Hanelt *et al.* (2001). Briefly, shells were removed from each snail and the soft tissue was crushed between 2 slides. Each snail was infected naturally with 0–78 cysts (average = 16), determined by dissecting 10 snails. Cysts were identified to genus based on size, folding pattern, and the presence or absence of visible spines. Based on morphology, most of these cysts were identified as belonging to the genus *Paragordius* (see Hanelt *et al.* 2012) but we observed a few that morphologically resembled cyst descriptions of *Chordodes morgani* Montgomery 1898 and *Chordodes janovyi* Bolek, Schmidt-Rhaesa, Hanelt, & Richardson 2010 (see Hanelt & Janovy 2002; Bolek *et al.* 2010). To isolate gordioid cysts from snails for cricket infections, the soft tissue was macerated with a razor blade. Small portions of the soft tissue from a single snail containing gordioid cysts were fed to 24 h starved six week-old commercially reared crickets *Acheta domesticus* Linnaeus 1758 (N = 25) and six week-old laboratory-reared crickets *Gryllus texensis* Cade and Otte 2000 (N = 25). Post-exposure, crickets were maintained in groups of 12–13 individuals per species in covered plastic shoe boxes (35 cm x 25 cm x 15 cm) with a paper-towel-substrate and a 4 cm² egg carton for a hiding place. Crickets were watered and fed by placing a 50 mm plastic centrifuge tube filled with aged tap-water with a cotton ball at the end, and an *ad libitum* supply of Purina Puppy Chow dog food. Starting at four weeks post exposure (WPE), crickets were placed in water daily to allow matured worms to exit. Adult worms were allowed to mate and lay eggs; and these eggs were used to establish the life cycle of this species in laboratory-reared freshwater snails *Physa (Physella) gyrina* (Say 1821) and three species of crickets (*A. domesticus*, *Gryllus firmus* Scudder 1902 and *G. texensis*).

Adult morphology of *Chordodes kenyaensis* n. sp. The length of newly emerged male (N = 15) and female (N = 15) worms was measured in mm with a ruler without stretching the worms; worms were then placed in a Petri dish with aged tap water and the diameter was measured with a calibrated ocular micrometer using a Wild Heerbrugg M5 stereomicroscope at 500x magnification. The color of each worm was recorded and some were fixed in 95% ethanol for molecular work; others were fixed in 10% neutral buffered formalin for morphological studies. Species identification was based on comparisons to published species description and re-descriptions in Schmidt-Rhaesa *et al.* (2008), Bolek *et al.* 2010, and Chiu *et al.* (2011). For light microscopy (LM) studies, the anterior and posterior ends of male and female worms were examined with a Wild Heerbrugg M5 stereomicroscope at 60x to 500x magnification. Five to ten mm sections of cuticle from male and female worms were cut from the midbody, removed from the remaining tissue and placed in glycerol prior to examination with an Olympus BX-51 upright research microscope configured for bright field and Nomarski differential interference contrast (DIC) microscopy with plain fluorite objectives at 400x to 1,000x total magnification and a Wild Heerbrugg M20 compound microscope configured for bright field and phase contrast microscopy with phase fluorite objectives at 100x to 400x total magnification. Digital images were recorded using a 5 megapixel Olympus digital camera and Q

Capture image analysis software. For scanning electron microscopy (SEM) studies, male and female worms were fixed within a day of emergence from crickets, and 5–10 mm sections of the anterior, mid-body and posterior regions of male and female worms were cut, critical point dried, mounted on aluminum stubs, coated with gold palladium, and examined with a FEI Quanta 600 field emission gun ESEM with Evex EDS and HKL EBSD. All terminology of areoles follows Schmidt-Rhaesa *et al.* (2008).

Cyst morphology, worm emergence, mating and oviposition behavior of *Chordodes kenyaensis* n. sp. *Physa gyrina* snails were obtained from established laboratory colonies maintained at Oklahoma State University as described in Bolek & Janovy (2007). Laboratory-reared *P. gyrina* snails were exposed to freshly hatched (2–5 day old) larvae of the new species of *Chordodes*. Snails were exposed for 48 hrs. Approximately 200–500 larvae were pipetted into 48 1.5 ml well plates filled with 1 mm of aged tap water. To each well a single laboratory-reared *P. gyrina* snail was added. Forty-eight hrs after exposure, snails were maintained in a 3.78 L jar with aerated aged tap water on a diet of frozen lettuce and TetraMin fish food. A few snails were examined for the location of larvae and cyst development within 1–5 days post exposure (DPE). However, most snails were examined for the presence of cysts 14–24 DPE as previously described. Four measurements were taken on live, fully formed cysts: 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.

Three species of laboratory-reared six to eight week-old crickets (*A. domesticus* N = 24, *G. firmus* N = 24 and *G. texensis* N = 12) were exposed to snail tissue containing fully developed cysts as previously described. Crickets were placed in water daily at 50 DPE to allow matured worms to exit as previously described. Once worms began to emerge, each cricket was isolated in an individual shoe box to keep track of the number of worms emerging from individual crickets. All crickets, which were not releasing worms after 80 DPE, were dissected and examined for the presence of immature worms. Worms emerging from hosts were immediately separated (if needed) and maintained in individual 250 ml Stender dishes partially filled with aerated aged tap water. A single male and single female worm were then placed together in a Stender dish and observed for mating. Sticks 5–10 cm long and 0.5 cm wide from unknown species of trees were collected from the campus of Oklahoma State University and soaked overnight in aged tap water. Mated females were placed individually in Stender dishes partially filled with aerated aged tap water with a small stick and allowed to oviposit egg strings.

Morphology of egg strings, eggs and larvae of *Chordodes kenyaensis* 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 sticks and air hoses 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 = 30) were measured within days of hatching (3–10). 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, the length and width of the three stylets, and pseudointestine length and width. Some larvae were fixed in 10% buffered formalin or 95% ethanol and adhered to Poly-L-Lysine coated cover-slips for SEM. The cover-slips were then dehydrated in a graded series of ethanol, dried using hexamethyldisilazane (HMDS), and mounted on aluminum stubs, coated with gold palladium, and examined with SEM as previously described for adults. Morphological characteristics for egg strings, eggs and larvae for SEM followed terminology in Bolek *et al.* (2010).

DNA extraction, amplification, and sequencing of *Chordodes kenyaensis* n. sp. Molecular work was conducted on two individuals. From each worm, a 0.5cm section was cut, dried at room temperature, and used for DNA extraction using the E.Z.N.A. Mollusc DNA Kit (Omega Bio-tek, Norcross, Georgia) following the manufacturer's instructions. Extracted DNA was stored at -70 C. A complete sequence of *cox1* was amplified using GoTaq Flexi DNA Polymerase (Promega Corp. Madison, Wisconsin). The primers used were the universal *cox1* primers (Folmer *et al.*, 1994) LCO1490: GGT CAA CAA ATC ATA AAG ATA TTG G and HCO2198: TAA ACT TCA GGG TGA CCA AAA AAT CA, using standard PCR protocols. 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) on an ABI 31303 sequence analyzer (Applied Biosystems). Both strands of the amplified DNA fragments were sequenced, edited in Sequencer version 4.10.1 (Gene Codes, Ann Arbor, Michigan), and manually corrected for ambiguous base calls.

Sequence analysis. DNA sequences were aligned to other sequences in the GenBank database by eye using Sequencer. The evolutionary history of these sequences was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980), using MEGA 5.0 (Tamura *et al.* 2011). Third codon positions were excluded from analysis due to signal saturation. A total of 1,000 bootstrap replicates were run.

Results

Establishing the life cycle of *Chordodes kenyaensis* n. sp. in the laboratory. Sixty-seven days post exposure of crickets to cysts from field collected snails a single male *Chordodes* emerged from a *G. texensis* at Oklahoma State University; and 63 DPE a single female *Chordodes* emerged from an infected *A. domesticus* at the University of New Mexico. Three additional crickets were infected with *C. kenyaensis* n. sp. at Oklahoma State University but died 71 or 72 DPE before worms emerged. These crickets contained double infections of the recently described *P. obamai* (see Hanelt *et al.* 2012) and *C. kenyaensis* n. sp. All individuals of *C. kenyaensis* n. sp. (N = 5) from crickets infected with both gordiid species appeared deformed. We define these worms as deformed because they were smaller and thicker in size than worms from single species infections, had difficulty emerging from their cricket hosts, and could not swim or mate. Therefore, the single *C. kenyaensis* n. sp. female with normal appearance which emerged at the University of New Mexico was shipped overnight in a bottle of aged tap water to Oklahoma State University, where both worms were combined and maintained with a stick in a 110 x 35 mm Stender dish partially filled with aerated aged tap water and allowed to mate. The female worm was allowed to deposit egg strings directly onto the stick. The white egg strings began turning a brown color over a period of two weeks and larvae began hatching three weeks post oviposition.

All 48 *P. gyrina* snails exposed to laboratory-reared larvae of *C. kenyaensis* n. sp. became infected with cysts with a mean intensity of 131 ± 91 (range 6–345). No snail defense response was noted to any *C. kenyaensis* n. sp. larvae or cysts. Most larvae developed into mature cysts by 24 DPE (see description of cysts). Twenty *G. firmus*, 13 *A. domesticus* and six *G. texensis* exposed to laboratory-reared cysts of *C. kenyaensis* n. sp. survived long enough for adult worm emergence. Adult *C. kenyaensis* n. sp. emerged from infected crickets 53–78 DPE. Fully mature worms emerged from crickets within 30 seconds of placing the cricket into a Stender dish partially filled with water. In addition, 12 males emerged from crickets in their cages sometime after the cricket was submerged in water and re-submerged the following day. Thirteen of 20 (65%) *G. firmus* were infected with *C. kenyaensis* n. sp. with a mean abundance of 2.3 ± 1.4 (0–5). Eight of 13 (61.5%) *A. domesticus* crickets were infected with *C. kenyaensis* n. sp. with a mean abundance of 1 ± 1 (0–3) and three of six (50%) *G. texensis* were infected with a mean abundance of 1.2 ± 1.3 (0–3) worms. A total of 51 adult worms were recovered including 24 males and 27 females. No other worms were found in any surviving and dissected crickets 80 DPE.

Description of *Chordodes kenyaensis* n. sp.

(Figs 1–6)

Holotype: 1 partial male from a population originally collected as a cyst stage in a *Biomphalaria pfeifferi* snail from Kasabong stream, Nyanza province, Kenya Africa and reared in an experimentally infected snail *Physa gyrina* and cricket *Gryllus texensis*, deposited at the Museum of Southwestern Biology-Parasitology Division, accession number MSB Para 18595.

Allotype: 1 partial female from a population originally collected as a cyst in *Biomphalaria pfeifferi* snail from Kasabong stream, Nyanza province, Kenya Africa and reared in an experimentally infected snail *Physa gyrina* and cricket *Gryllus texensis*, deposited at the Museum of Southwestern Biology-Parasitology Division, accession number MSB Para 18596.

Other material deposited: Larvae fixed in 95% ethanol from laboratory cultures, Museum of Southwestern Biology-Parasitology Division, accession number MSB Para 18597; 2 free-living male paratypes and 2 free-living

female paratypes fixed in 95% ethanol, Museum of Southwestern Biology-Parasitology Division, accession number MSB Para 18598; 1 deformed worm paratype fixed in 100% ethanol, Museum of Southwestern Biology-Parasitology Division, accession number MSB Para 18599; 1 deformed worm paratype fixed in 10% formalin, Museum of Southwestern Biology-Parasitology Division, accession number MSB Para 18600; and 1 deformed worm paratype fixed in 95% ethanol, Museum of Southwestern Biology-Parasitology Division, accession number MSB Para 18601.

Type locality: Kasabong stream, Nyanza province, Kenya (-0.1519°, 34.4455°, approx. 1,170 m altitude).

Other localities: Cysts were also collected from Asao stream, Nyanza province, Kenya (-0.3176°, 35.0065°, approx. 1250 m altitude). Samples MSB Para 18599 and 18600 were derived from cysts from this site.

Hosts: Definitive: unknown in nature but *Acheta domesticus*, *Gryllus firmus* and *Gryllus texensis* in the laboratory; paratenic: *Biomphalaria pfeifferi* in nature and *Physa gyrina* in the laboratory. Note that in the natural system, snails most likely represent a dead-end host since they are an unlikely part of the normal cricket diet.

Material examined: Free-living adult male holotype and female allotype, additional male and female adult worms including deformed individuals, egg strings, eggs, larvae and cysts. SEM of mid-body, anterior and posterior ends of free-living adult male and female, and larvae, and LM of mid-body, anterior and posterior ends of free-living adult male and female; egg strings, eggs, larvae, and cysts from laboratory infected snails.

Etymology: The species epithet is named for the country of origin (Kenya) of this newly discovered species.

Description of male: Body color tan to dark brown with anterior end lighter in color, no dark collar present. The lighter coloration blends into the normal coloration of the remaining body. Body length 114.3 ± 32.0 (38–162; $N = 15$) mm, with mid-body diameter 577.5 ± 111.6 (432–800) μm . Anterior end distinctly tapering (Fig. 1A) with a degenerate mouth; posterior end round with indication of two lobes (Fig. 1E). Cloacal opening located ventrally, 120–130 μm from posterior end, oval in shape and surrounded by circumcloacal spines (Figs. 1E, 1F). Additionally, short bristles, (10–20 μm by 1–2 μm in length and width), distributed laterally and medially on the non-areolated ventral region surrounding cloacal opening (Figs. 1E, 1F). Ventral region posterior and anterior to the cloacal opening lacks distinct areoles, is smooth, and contains canals (furrows) running laterally separated by 4–5 μm .

The body cuticle contains six types of areoles (Fig. 1D). Simple areoles are the most abundant and except for the ventral region surrounding the cloacal opening and the anterior region surrounding the degenerate mouth, they are distributed on the entire cuticle. On the anterior region they are 3–5 μm high, oval in shape (7–8 by 6–7 μm in length and width), and their surface contains short terminal bristles 1–3 μm in length and less than 1 μm in width (Fig. 1B). Simple areoles on the anterior region are separated by interareolar furrows, 3–4 μm apart and contain canals running laterally across the cuticle (Fig. 1B). In the mid-body and posterior regions simple areoles are 5–6 μm high and 12–13 by 6–7 μm in length and width, and their surface is also covered with short bristles 1–3 μm in length and less than 1 μm in width (Fig. 1D). Scattered among the simple areoles are tubercle, thorn, bulging, and crowned areoles surrounded by circumcluster areoles (Figs. 1A–D). Bulging areoles common in the mid-body region (Fig. 1D). Occur in groups of 2–4, have surface covered with short bristles (Fig. 1D), 8–10 μm high and 12–15 by 10–12 μm in length and width respectively. Crowned areoles more common in the mid-body region, absent from approximately 0.5–0.7 mm of the anterior and posterior regions of the cuticle. Crowned areoles occur in pairs, 8–10 μm high and 12–15 μm in diameter, and contain filaments 15–30 μm in length on their apical end (Figs. 1C, 1D). Each pair is surrounded by 6–12 circumcluster areoles (Fig. 1D). These are similar in size to bulging areoles being 8–10 μm long and 12–15 μm in diameter with small bristles on top (Fig. 1D). Thorn areoles are 10–14 μm in length and 3.5–4 μm in width at their base. Two types are present; most have typical thorn-like shape, but some have a round apical end (Fig. 1B). Tubercle areoles are most like simple areoles in size and shape. They contain filaments 7–10 μm in length and approximately 1–2 μm wide and some contain short bristles 1–3 μm in length and less than 1 μm wide surrounding the filament (Figs. 1B, 1D).

Description of female: Body color light tan to dark brown with anterior and posterior ends lighter in color; no dark collar on the anterior end. The lighter coloration of the anterior and posterior ends blends into the normal coloration of the remaining body. Body length 118.3 ± 28.1 (77–180; $N = 15$) mm with mid-body diameter 818.7 ± 70.3 (600–880) μm . Anterior end distinctly tapered (Fig. 2A) with a degenerate mouth; posterior end distinctly swollen in most but not all individuals, and cloacal opening terminal (Fig. 2G).

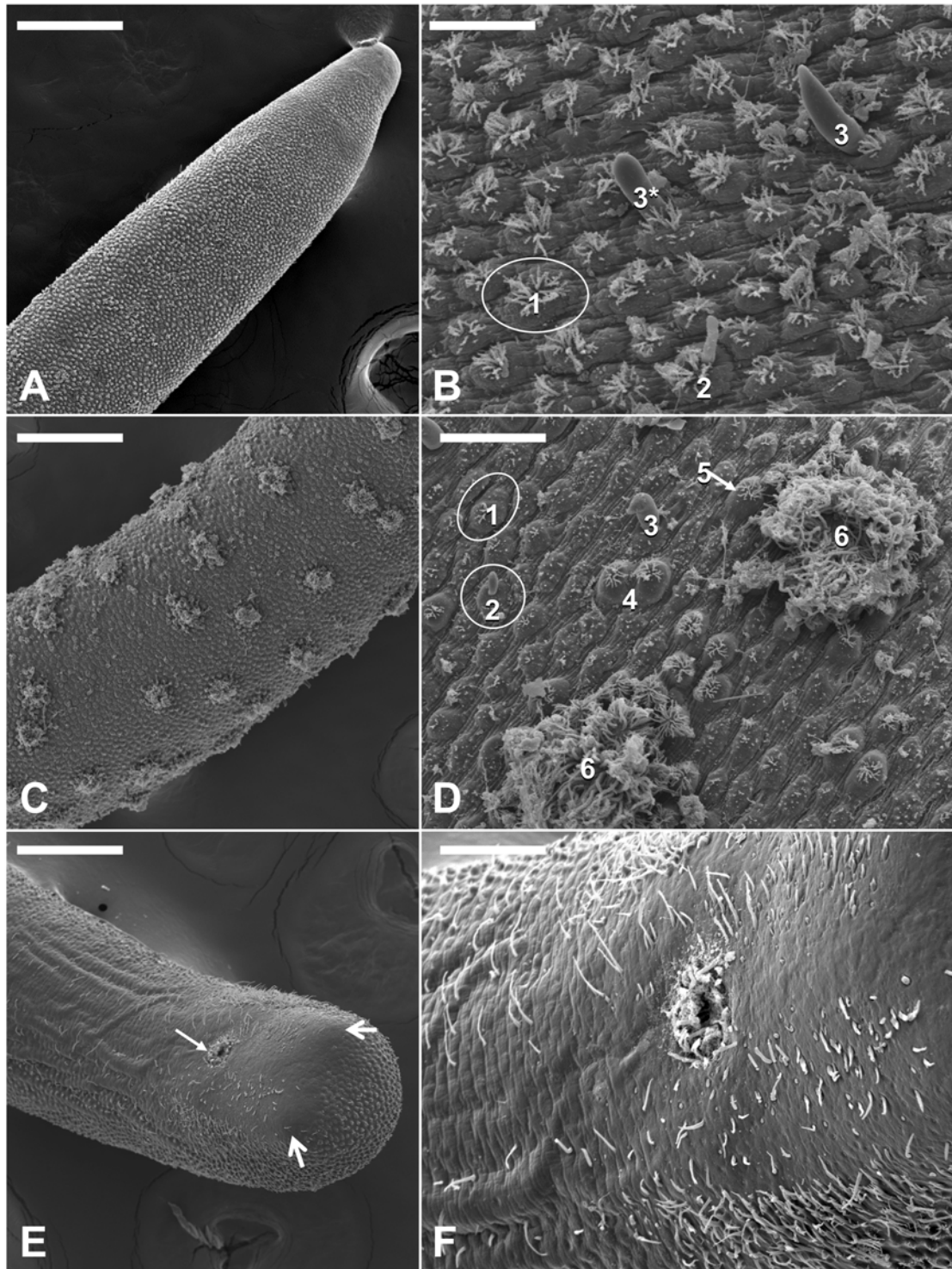


FIGURE 1. Holotype of male *Chordodes kenyaensis* n. sp. (A) SEM photomicrograph showing the tapered anterior end and areole pattern. Scale bar = 170 μm . (B) SEM photomicrograph showing higher magnification of the anterior region of the cuticle with simple (1), tubercle (2), and two types of thorn (3 and 3*) areoles. Note that interareolar furrows are arranged laterally across the cuticle. Scale bar = 15 μm . (C) SEM photomicrograph showing an overview of the mid-body cuticle. Note the pattern of crowned areoles. Scale bar = 190 μm . (D) SEM photomicrograph showing a higher magnification of the mid-body cuticle containing simple (1), tubercle (2), thorn (3), bulging (4), circumcluster (5) and crowned (6) areoles. Interareolar furrows are arranged laterally across the cuticle. Note the long filaments on crowned areoles and short bristles on top of simple smooth areoles which are separated between neighbors by interareolar furrows. Scale bar = 25 μm . (E) SEM photomicrograph showing the posterior end and cloacal opening (arrow). Note the indistinct lobes (thick arrows). Scale bar = 115 μm . (F) SEM photomicrograph showing a higher magnification of the posterior region of the cloacal opening surrounded by circumcloacal spines. Note the non-areolated region and bristles on the lateral regions of the non-areolated field. Scale bar = 40 μm .

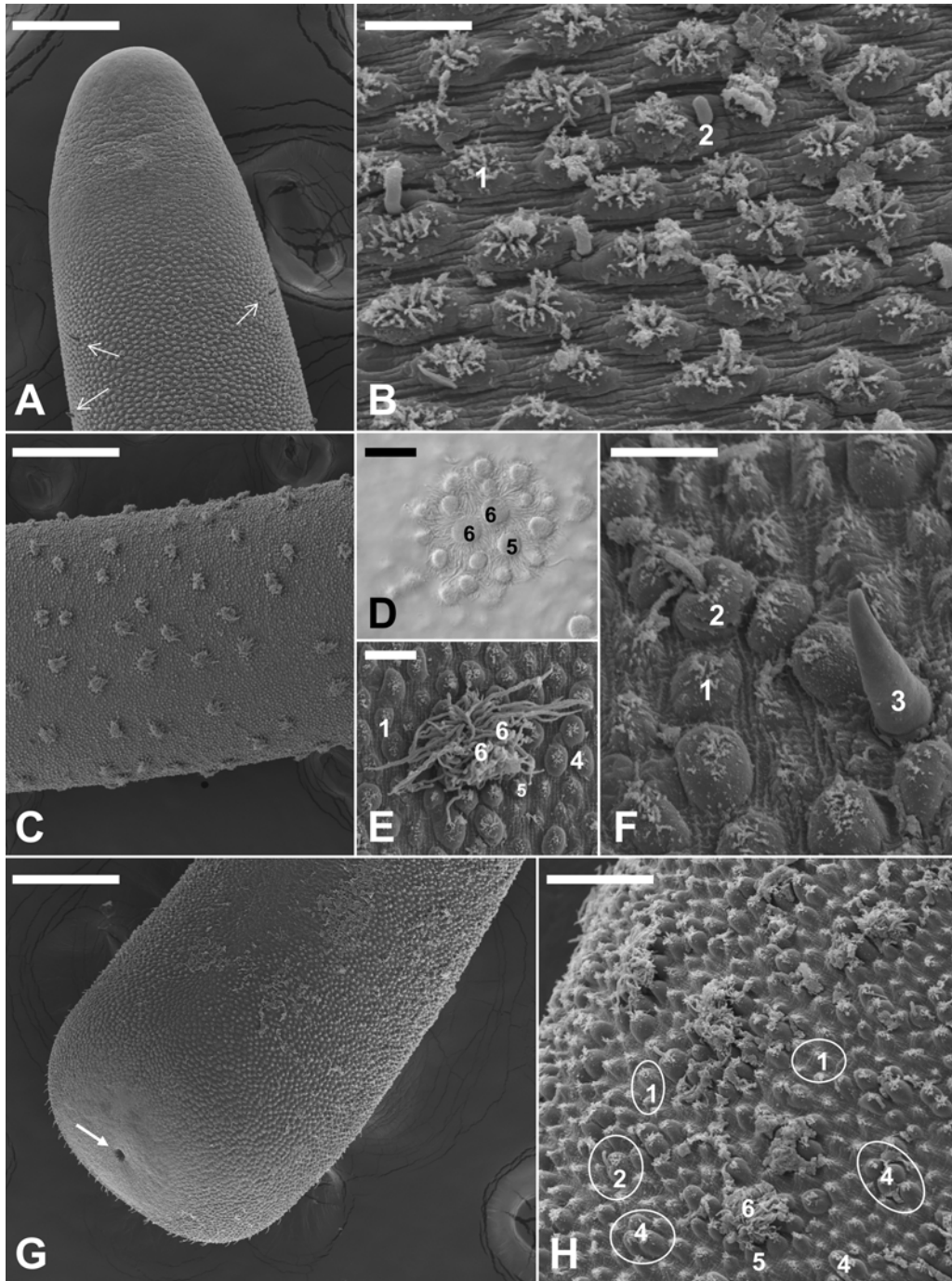


FIGURE 2. Allotype of female *Chordodes kenyaensis* n. sp. (A) SEM photomicrograph showing the tapered anterior end and areole pattern. Note distinct thorn areoles (arrows) among simple areoles. Scale bar = 115 μ m. (B) SEM photomicrograph showing higher magnification of the anterior region of the cuticle with simple (1) and tubercle (2) areoles. Note that interareolar furrows are arranged laterally across the cuticle. Scale bar = 10 μ m. (C) SEM photomicrograph showing overview of the mid-body cuticle. Note the pattern of crowned areoles. Scale bar = 300 μ m. (D) DIC photomicrograph showing two crowned (6) areoles surrounded by circumcluster (5) areoles in the mid-body region. Scale bar = 20 μ m. (E) SEM photomicrograph showing simple (1), bulging (4), circumcluster (5) and crown (6) areoles in the mid-body region. Note the long filaments on crown areoles. Scale bar = 20 μ m. (F) SEM photomicrograph showing higher magnification of simple (1), tubercle (2), and thorn (3) areoles in the mid-body region. Interareolar furrows are arranged laterally across the cuticle. Note the fine terminal bristles on the simple and tubercle areoles. Scale bar = 10 μ m. (G) SEM photomicrograph showing posterior end and cloacal opening (arrow). Note the non-areolated region around the cloacal opening and bristles on the lateral sides of the non-areolated field. Scale bar = 150 μ m. (H) SEM photomicrograph showing higher magnification of the posterior region of the cuticle with simple (1), tubercle (2), bulging (4), circumcluster (5) and crowned (6) areoles. Note the variation in simple areoles in this body region compared to the anterior and mid-body region of the cuticle. Scale bar = 40 μ m.

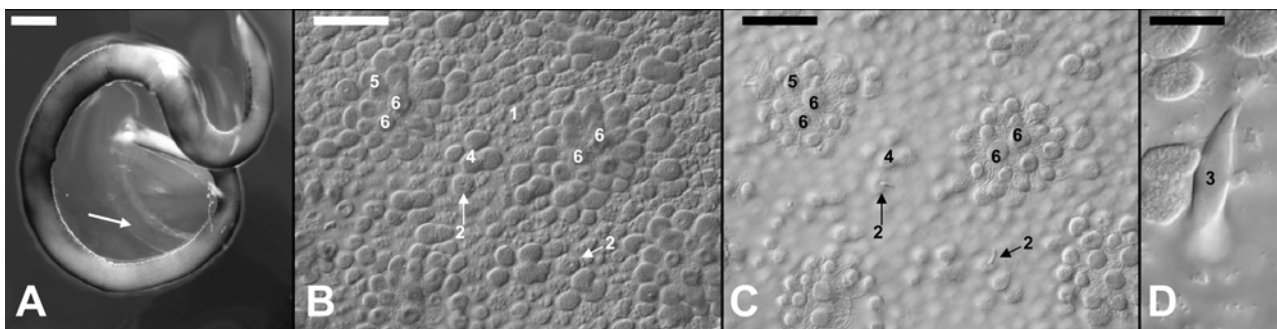


FIGURE 3. Adult and areole pattern of deformed *Chordodes kenyaensis* n. sp. (A) Adult male removed from a cricket while trying to emerge. Note the larval cuticle (arrow). Scale bar = 1.0 mm. (B) DIC photomicrograph of the mid-body cuticle of a female showing tubercle (2), bulging (4), circumcluster (5) and crowned (6) areoles scattered among simple (1) areoles. Note that simple areoles are smooth but contain short bristles making them appear bumpy. Scale bar = 30 μm . (C) DIC photomicrograph of the same region of the cuticle in a higher focal plane of the mid-body cuticle showing tubercle (2), bulging (4), circumcluster (5) and crowned (6) areoles scattered among simple areoles. Scale bar = 30 μm . (D) DIC photomicrograph of a thorn (3) areole among simple areoles of the mid-body region of an adult deformed female worm. Scale bar = 7 μm .

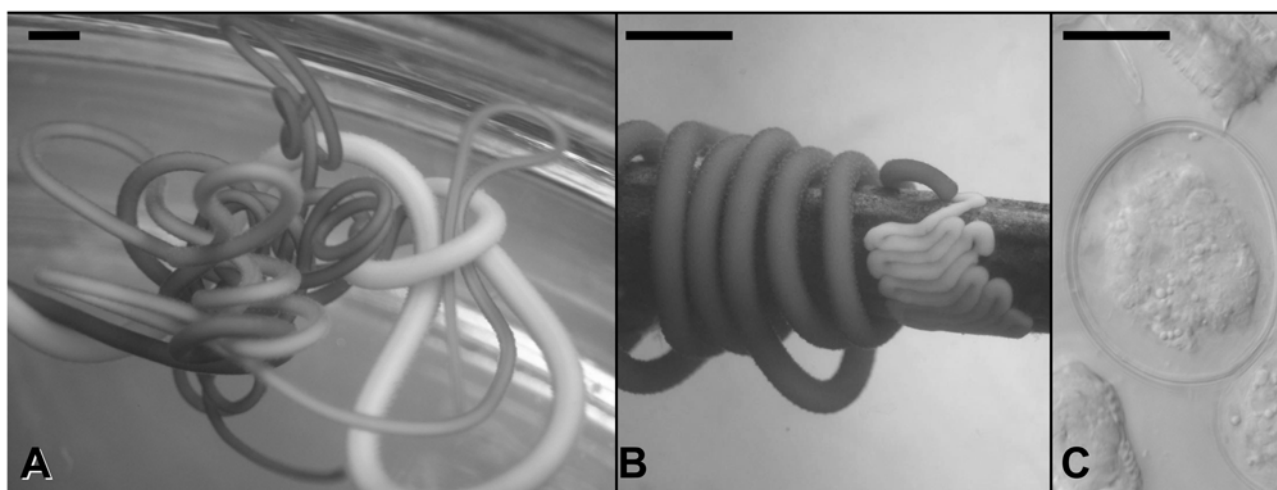


FIGURE 4. Mating, oviposition behavior, egg strings, and eggs of *Chordodes kenyaensis* n. sp. (A) Male and female in a Gordian knot. Note the lighter color and thicker size of the female worm compared to the male. Scale bar = 2.0 mm. (B) Female in the process of depositing egg strings in a zigzag pattern on a stick. Scale bar = 5 mm. (C) Undeveloped egg. Scale bar = 15 μm .

As in males, the body cuticle of females has six types of areoles. Simple areoles are most abundant, found on most areas of the cuticle, but lacking in regions surrounding the terminal cloacal opening and the degenerate mouth (Figs. 2A, 2G). Separated by interareolar furrows, 3–5 μm apart and running laterally across the cuticle (Figs. 2A, 2B, 2F). Unlike in males, in females simple areoles are morphologically distinct in the posterior region of the cuticle from those on the anterior and mid-body regions of the cuticle (Figs. 2B, 2F, 2H). In the anterior and mid-body regions, they are 3–4 μm high, oval to round in shape (5–8 by 4–7 μm) with a smooth surface. On the anterior region of the cuticle, simple areoles contain terminal bristles 1–3 μm in length and less than 1 μm in width; but the bristles are much smaller in the mid-body region (Figs. 2B, 2F). In contrast, on the posterior region of the cuticle simple areoles are not as high, less well developed and nested among groups of bulging areoles (Fig. 2H). Bulging areoles occur in higher densities on the posterior than the anterior and mid-body regions (Fig. 2H). They are scattered among simple areoles and most common on the mid-body and posterior regions of the cuticle. Occur in groups of three on the mid-body cuticle and groups of two to four on the posterior region of the cuticle (Figs. 2E, 2H). Height 7.6–10 μm high, oval to round in shape (6–8 μm) with a smooth surface containing small terminal bristles 1–3 μm in length and less than 1 μm in width (Figs. 2E, 2H). Scattered among the simple areoles are tubercle and thorn areoles (Figs. 2A, 2F, 2H). Thorn areoles 7–14 μm in length and 3–4 μm wide at their base with

typical thorn shape. The filaments of tubercle areoles are 5–7 μm in length and approximately 1–2 μm in width. Crowned areoles occur on mid-body and posterior regions of the cuticle, much denser in distribution than in males (see Figs. 1C, 2C). As in males, crowned areoles in females occur in pairs with long filaments on top 20–70 μm long (Figs. 2D, 2E, 2H). Surrounded by 8–12 circumcluster areoles with small bristles on top and morphological similar to bulging areoles (Figs. 2D, 2E, 2H).

Description of deformed individuals: All individuals of *C. kenyaensis* recovered from crickets infected with *P. obamai* were much smaller in length than *C. kenyaensis* recovered from crickets with single infections. Females (N = 3) were light tan or white in color and 16 \pm 3.6 (13–20) mm in length and 986.7 \pm 162.9 (800–1100) μm in mid-body diameter; whereas males (N = 2) were tan in color and 12–16 mm in length and 800–1000 μm in mid-body diameter (Fig. 3A). Deformed individuals of *C. kenyaensis* contained six types of areoles on the cuticle including simple areoles with terminal bristles, bulging, tubercle, thorn and crown areoles surrounded by 8–12 circumcluster areoles (Figs. 3B, 3C, 3D). Areole patterns in deformed male and female *C. kenyaensis* were indistinguishable from normal individuals recovered from crickets with single species infections of *C. kenyaensis*.

Description of mating, oviposition, egg strings, and eggs: Individual male and female worms initiated typical Gordian knots within hours to days of being placed together in Stender dishes (Fig. 4A). Males were observed to move up and down the female's body with their coiled posterior end. Once a male's cloaca was in proximity with the female cloaca, the male deposited a mass of sperm that remained on the female for up to a week. Females began oviposition within a week of copulating. During oviposition female *C. kenyaensis* attached egg strings in a continuous zigzag pattern on small branches or air-hoses (Fig. 4B) but never deposited free egg strings in the water column. Egg string width was 449 (220–580) μm ; whereas individual undeveloped eggs (Fig. 4C) were 39 (32–45) μm in length and 30 (24–35) μm in width (Fig. 4C). Larvae developed in eggs and began hatching within 3–7 weeks at room temperature.

Description of larvae: Larvae of *C. kenyaensis* possessed a cylindrical body divided by a septum into two regions, the preseptum and a postseptum (Figs. 5A, 5C). The preseptum was 21.0 (17–24) μm in length and 15.7 (14–17) μm in width and contained an eversible proboscis supported by three internal stylets which were 14.9 (12–18) μm in length and 3.6 (3–4) μm in width (Figs. 5A, 3B). The postseptum was 23.0 (14–26) μm in length and 13.0 (12–15) μm in width and contained a clearly visible pseudointestine. The pseudointestine was v-shaped with one smaller and one larger branch both positioned anteriorly (Fig. 5A). The pseudointestine was 12.2 (12–14) μm in length and 9.7 (8–12) μm in width (Figs. 5D, 5E). Three to four weeks after hatching free-living larvae secreted thread like projections by emptying their pseudointestine and stopped moving.

Externally, larvae were superficially annulated and the postseptum contained two pairs of terminal spines located ventrally (Fig. 5D). The pseudointestine exterior opening was centrally located above the pair of anterior terminal spines (Fig. 5D). The preseptum contained three sets of cuticular hooks (Figs. 5C, 5E, 5F). The outer ring of hooks contained seven hooks, two of which are very close together and ventrally positioned, and there were six hooks in the middle and inner rings (Figs. 5E, 5F). The dorsal and ventral side of the anterior end of the laterally flattened and eversible proboscis each contained five spines (two aligned pairs and one single spine above); whereas the left lateral side of the proboscis contained four spines (Figs. 5E, 5F, 5G).

Development and descriptions of cysts: After being ingested by snails, larvae penetrated snail intestines and became distributed throughout the snail tissues. Once inside snail tissue, larvae emptied the content of their pseudointestine and began the process of cyst formation (Fig. 6A). Fully formed cysts of *C. kenyaensis* were recovered from laboratory-reared and exposed snails 14–24 DPE. Fully formed cysts of *C. kenyaensis* possessed a clear cyst wall of unknown composition 11.4 (5.5–18.5) μm in length and 11.9 (7–16.5) μm in width (Fig. 6B). Folded larvae inside of the cyst were folded only once and were 28.2 (22–35) μm in length and 20.2 (15–25) μm in width (Fig. 6B).

Diagnosis and comments: Male and female *C. kenyaensis* contain six type of areoles and exhibit minor differences in cuticular morphology (number of circumcluster areoles, length of filaments on crowned areoles, density and distribution of bulging and crowned areoles); thus *C. kenyaensis* is sexually dimorphic. *Chordodes kenyaensis* belongs to a large group of African *Chordodes* in which simple areoles are smooth or superficially structured less so than “blackberry” areoles but contain filamentous projections. Among the simple areoles are clusters of bulging areoles, crowned and circumcluster areoles along with thorn and tubercle areoles. Our observations on deformed individuals of *C. kenyaensis* clearly indicate that this areal pattern is consistent within this species but worm length appears not to be a good character for species differentiation within the genus

Chordodes. Of the 19 other sufficiently described African *Chordodes* species, 14 species contain simple areoles that are not of the “blackberry” type (Zanca *et al.* 2006a; 2006b; De Villalobos *et al.* 2007; Schmidt-Rhaesa *et al.* 2008; De Villalobos *et al.* 2009; Bolek *et al.* 2010). Of those, seven species contain smooth simple areoles with minute bristles or projections on their apical surface (*Chordodes capensis* Camerano 1895, *Chordodes digitatus* Linstow 1901, *C. janovyi*, *Chordodes kolensis* Sciacchitano 1933, *Chordodes madagascariensis* (Camerano 1897), *Chordodes sandoensis* Sciacchitano 1937, and *Chordodes tuberculatus* Linstow 1901). However, they can be differentiated from *C. kenyaensis* by the arrangement and types of areoles on the cuticle. Additionally, simple areoles of these seven species never contain bristles or projections on their apical surface which are as pronounced as in *C. kenyaensis*. Finally, males of two species, *C. digitatus* and *C. tuberculatus*, are superficially similar to males of *C. kenyaensis* because they possess a smooth cuticle structured with fine canals (furrows) surrounding the cloacal opening similar to the cuticular region in males of *C. kenyaensis*. However, both these species differ from *C. kenyaensis* by the following characteristics: crown areoles of *C. digitatus* are arranged in groups of three versus groups of two in *C. kenyaensis*; whereas *C. tuberculatus* only contains three types of areoles (simple, tubercle and crowned) versus six types of areoles in *C. kenyaensis*.

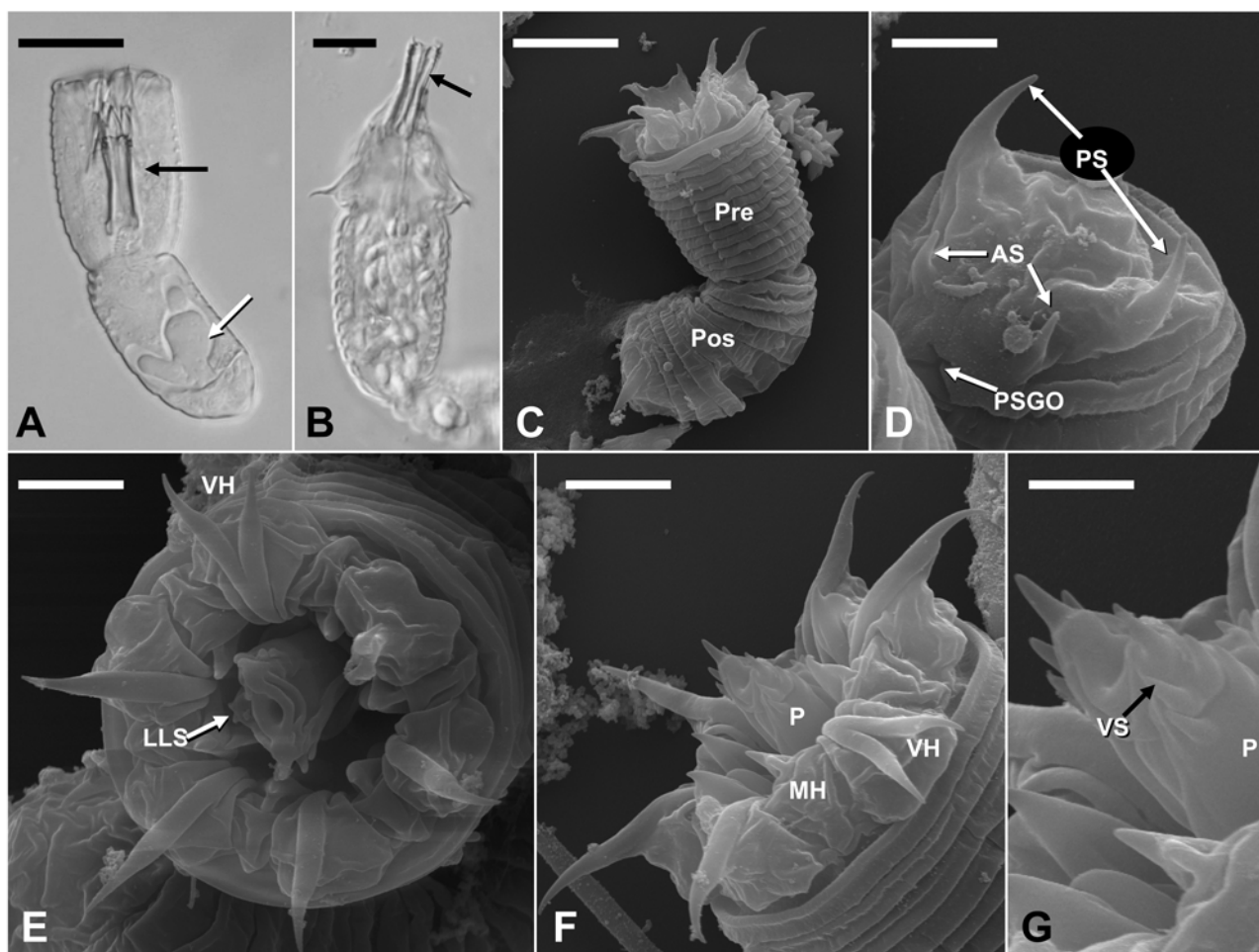


FIGURE 5. Larval characteristics of *Chordodes kenyaensis* n. sp. (A) DIC photomicrograph of a live larva (lateral view). Note two of the three stylets (black arrow) and v shaped pseudointestine (white arrow). Scale bar = 15 µm. (B) DIC photomicrograph of anterior end of larva (lateral view), note the everted proboscis (arrow) with three stylets. Scale bar = 13 µm. (C) SEM photomicrograph of an entire larva (lateral view). Note the distinct preseptum (Pre) and postseptum (Pos). Scale bar = 8.0 µm. (D) SEM photomicrograph of the posterior end (ventral side). Note the two anterior spines (AS), two posterior spines (PS) and pseudointestine gland opening (PSGO). Scale bar = 3.0 µm. (E) SEM photomicrograph of the anterior end (en face view). Note the orientation of the proboscis (P) with left lateral spines (LLS) to the ventral hooks (VH). Scale bar = 3.0 µm. (F) SEM photomicrograph of the preseptum (ventral view). Note the orientation of the proboscis (P) to the ventral hooks (VH); median hooks (MH). Scale bar = 3.3 µm. (G) Close up SEM photomicrograph of a ventral view of the proboscis (P). Note the arrangement of the five ventral spines (VS). Scale bar = 1.5 µm.



FIGURE 6. Developing and fully formed cysts of *Chordodes kenyaensis* n. sp. recovered from an experimentally infected *Physa gyrina* snail. (A) Larva in snail tissue 14 DPE in the process of folding (lateral view). Note the empty pseudointestine (black arrow). Scale bar = 12 µm. (B) Mature cysts (lateral view); note the clear halo surrounding the encysted larvae and single folding pattern. Scale bar = 15 µm.

Observations on the oviposition behavior, egg strings, eggs, larvae and cysts of *C. kenyaensis* indicate that non-adult characteristics of this species are most similar to other species in the genus *Chordodes*, and are distinct from other genera and species such as *Gordius* Linnaeus 1758 and *Paragordius* 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; Bolek *et al.* 2010; Chiu *et al.* 2011). Females of both the African *C. janovyi* and the North American *C. morgani* deposit egg strings in the water on twigs and detritus in a zigzag pattern, whereas females of the Asian *Chordodes formosanus* Chiu 2011 deposit their egg strings on rocks in a similar zigzag pattern. In contrast, the attachment of egg strings to substrate has never been reported for other gordiid genera such as *Gordius* and *Paragordius*, which simply release egg strings into water (Hanelt & Janovy 2002; Hanelt *et al.* 2012). Larvae and cysts of *C. kenyaensis* are also similar in morphology to other *Chordodes* species (*C. formosanus*, *C. janovyi*, *C. morgani*, and *Chordodes japonensis* Inoue 1952), but differ morphologically from larval and cyst stages of species of *Paragordius* and *Gordius* (see Schmidt-Rhaesa 1997; Bohall *et al.* 1997; Bolek & Coggins 2002; Hanelt & Janovy 2002; Marchiori, *et al.* 2009; Bolek *et al.* 2010; Chiu *et al.* 2011; Hanelt *et al.* 2012). This is particularly true for the arrangement of spines on the proboscis of larvae. As in our study, both Bolek *et al.* (2010) and Chiu *et al.* (2011) clearly showed that the proboscis in the African *C. janovyi* and Taiwanese *C. formosanus* was laterally compressed and contained spines on the dorsal, ventral and left lateral side, whereas Marchiori *et al.* (2009) indicated that the proboscis in the South American *Gordius dimorphus* Poinar 1991 was dorso-ventrally compressed with spines positioned on the left and right lateral sides and the dorsal side of the proboscis.

Phylogenetic analysis of *Chordodes kenyaensis*. Both fragments of 658 base pairs were amplified from the *cox1* region and sequenced. Sequences derived from the worms were identical. These sequences were placed into

GenBank (KF381359; KF381360). After addition of samples from GenBank, sequences were trimmed to 526 bp, and aligned. Addition of gaps was not required. The phylogenetic tree produced was based on 17 nucleotide sequences (Fig. 7). After removal of the third base codon positions, the final dataset contained 351 positions.

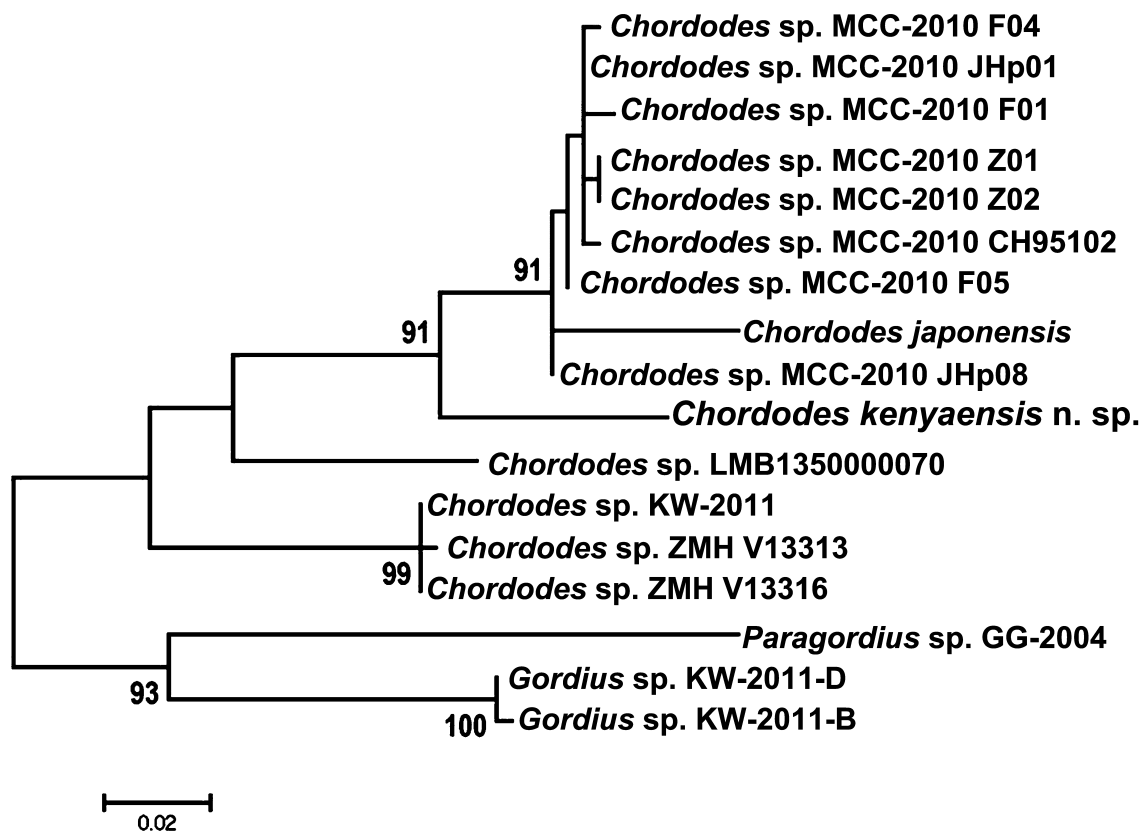


FIGURE 7. Molecular phylogenetic analysis by maximum likelihood method based on partial *cox1* gene from *Chordodes kenyaensis* n. sp. and 16 other nucleotide sequences from Genbank. *Paragordius* and *Gordius* were used as outgroups. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Only bootstrap values with a value of 85% or higher are shown. Note that *Chordodes kenyaensis* n. sp. is placed within other species of *Chordodes*.

Discussion

With few exceptions, the genus *Chordodes* has a tropical and subtropical distribution and is one of the most speciose of the 19 extant and two extinct nematomorph genera with approximately 100 reported species (Poinar 1999; Poinar & Buckley 2006; Zanca *et al.* 2006a; 2006b; De Villalobos *et al.* 2007; Schmidt-Rhaesa *et al.* 2008; Schmidt-Rhaesa, 2012). However, limited morphological data is available for many species descriptions in the genus *Chordodes*. This has led some investigators to re-evaluate the genus, and currently only 56 of the approximately 100 species are sufficiently described to be recognized; whereas 36 species are considered *species inquirenda* and 22 species are considered *incertae sedis* (see Schmidt-Rhaesa *et al.* 2008; Bolek *et al.* 2010; Chiu *et al.* 2011). More problematic is the fact that of the 56 currently recognized species of *Chordodes* no type hosts exist, and/or the exact type localities are not known for a majority of these species. The lack of data on hosts, type localities and knowledge of nematomorph species distribution, confound subsequent identification of species and make identification of new species problematic in this poorly studied group of parasites. 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.

Our description of *C. kenyaensis* indicates that it is a distinct species, with six types of areoles in both sexes. Additionally, our observations on the oviposition behavior and non-adult life stages of *C. kenyaensis* confirm that

egg deposition and non-adult life stages are similar to these features in other species of *Chordodes* (see Inoue 1958; Bolek & Coggins 2002; Hanelt & Janovy 2002). Clearly detailed studies on the oviposition behavior and morphological investigations using modern techniques such as SEM and DIC microscopy are needed on the egg strings, eggs, larval and cyst stages of other genera and species of gordiids to evaluate the importance of these observations for systematics and biodiversity studies of gordiids.

Our molecular data, based on the *cox1* region, supports a close association of *C. kenyaensis* with other species within the genus *Chordodes*. This result is consistent with the morphological data. However, due to a paucity of genetic data available for this phylum, and for Gordiids in general, our phylogenetic analysis cannot place this species with much certainty. The 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.

More importantly, our ability to collect hairworms in the cyst stage, identify those cysts to genus level, and culture adult worms from field collected cysts in the laboratory have several implications for hairworm systematics and biodiversity studies. First, genus level identification may be possible, for some genera, from features of the cyst stage (Schmidt-Rhaesa 1997; Hanelt & Janovy 2002; Bolek *et al.* 2010; Hanelt *et al.* 2012), which is the most encountered gordiid life stage in nature (Hanelt *et al.* 2001; Hanelt *et al.* 2012; Szmygiel 2012). Second, collecting cysts in aquatic snails over large geographical areas is a useful tool for studies on the distribution and biodiversity of gordiids over large geographical regions because free-living adult gordiids are difficult to locate (De Villalobos & Voglino 2000; Inoue 1958; Reutter 1972). Third, recent advances in culturing gordiids in the laboratory (Hanelt & Janovy 1999; Hanelt & Janovy 2004; Hanelt *et al.* 2012) have provided a framework for completing life cycles of gordiids and these new techniques will undoubtedly allow others to culture other known and new species of gordiids from around the world.

Although culturing gordiids in the laboratory from around the world is one solution for obtaining difficult-to-find, free-living adult worms, it raises a number of difficulties that must be overcome. First, there are logistical and timing issues of returning live cysts for arthropod infections to the laboratory, and second most species of definitive arthropod hosts for gordiids such as roaches, mantids and millipedes are not commonly available for laboratory infections of gordiids. However, we propose a number of solutions for these issues. Our recent work on North American and African gordiids suggests that gordiid cysts have the ability to survive super cooling and/or freezing (-20 to -80 C) for up to 7 months (Bolek *et al.* 2013). The ability of gordiid cysts from field collected snails to survive rapid cooling and/or freezing temperatures and produce viable adult worms when fed to laboratory reared hosts suggests that this technique will allow researchers to establish novel nematomorph life cycles in the laboratory. This technique will only be possible if the appropriate definitive arthropod hosts are available for gordiid infections. Currently there is a lack of knowledge on host use and host specificity for most gordiid species is unknown (see Schmidt-Rhaesa 1997; Schmidt-Rhaesa *et al.* 2003; Schmidt-Rhaesa *et al.* 2008). Recent field studies indicate that some, but not all, gordiids appear to be host specific at the arthropod definitive host level (Poinar, 1991; Bolek & Coggins 2002; Schmidt-Rhaesa *et al.* 2005; Chiu *et al.* 2011; Looney *et al.* 2012). For example, most *Chordodes*, for which definitive arthropod hosts are known, infect mantids or roaches and only a hand full of species are known to infect orthopteran hosts (Schmidt-Rhaesa *et al.* 2008). Of these potential host species, only a few can be easily cultured in the laboratory (Schmidt-Rhaesa *et al.* 2003; Schmidt-Rhaesa *et al.* 2008; Bolek *et al.* 2010; Chiu *et al.* 2011). However, over the last few years cultures of a large number of species of beetles, roaches, mantids, crickets and millipedes have been established for the exotic arthropods pet trade (McMonigle & Willis 2000; McMonigle 2008; 2011; 2012a; 2012b). This recent commercial availability of diverse arthropod host species will hopefully allow researchers to establish other novel life cycles of nematomorph species in the laboratory. We hope that our work provides an incentive for comprehensive morphological descriptions of multiple life stages of gordiids using DIC and SEM accompanied by molecular analyses and life cycle studies, which will aid in alleviating the current problems in nematomorph identification, systematics, and our understanding of gordiid distribution and biogeography.

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