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Comparative descriptions of non-adult stages of four genera of Gordiids (Phylum: Nematomorpha)

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Abstract

Freshwater hairworms infect terrestrial arthropods as larvae but are free-living in aquatic habitats as adults. Estimates suggest that only 18% of hairworm species have been described globally and biodiversity studies on this group have been hindered by unreliable ways of collecting adult free living worms over large geographical areas. However, recent work indicates that non-adult cyst stages of hairworms may be the most commonly encountered stages of gordiids in the environment, and can be used for discovering the hidden diversity of this group. Unfortunately, little information is available on the morphological characteristics of non-adult stages of hairworms. To address this problem, we describe and compare morphological characteristics of non-adult stages for nine species of African and North American gordiids from four genera (*Chordodes*, *Gordius*, *Paragordius*, and *Neochordodes*). Observations were made on the oviposition behavior of adult worms and morphological characteristics were recorded for egg strings, larvae and cysts using light and differential interference contrast microscopy and/or scanning electron microscopy. Our study indicates that three distinct types of oviposition behaviors and three distinct morphological types of egg string, larva, and cysts were present among the four genera of gordiids. Although species identification based on cyst characteristics was not always possible among different species of gordiids, cyst morphology was conserved among some genera and all clades of gordiids. More importantly, our work indicates that gordiid larval morphology can be used for predicting cyst morphology among other gordiid genera. The capability to identify and predict gordiid genera and/or clades based on cyst morphology will be useful for culturing gordiids in the laboratory from field collected cysts and these new techniques will undoubtedly allow others to discover new species of gordiids from around the world.

Key words: Gordiida, hairworm, Gordian worm, Africa, North America, scanning electron microscopy, differential interference contrast microscopy, life cycle, oviposition behavior, non-adult life stages, *Chordodes*, *Gordius*, *Paragordius*, *Neochordodes*

Introduction

The phylum Nematomorpha consists of two taxa, the freshwater Gordiida and the marine Nectonematida, and represents one of three entirely parasitic animal phyla (Hanelt *et al.* 2005). Gordiids have complex life-cycles which include multiple hosts and a free-living aquatic phase. At the end of their parasitic phase, gordiids manipulate the behavior of their terrestrial arthropod hosts to enter aquatic environments where adult worms emerge at the expense of the host committing suicide (Thomas *et al.* 2002; 2003). After emerging from their host, dioecious species form Gordian knots, mate, and females deposit egg strings as free-living forms; but females of parthenogenetic species deposit egg strings (Hanelt *et al.* 2012; Bolek *et al.* 2013a). Females produce up to eight million eggs during their short (2 weeks–2 months) free-living phase (Bolek & Coggins 2002; Hanelt 2009). Within weeks, larvae develop, infect, and encyst indiscriminately within a variety of aquatic vertebrate and

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invertebrate animals (Hanelt & Janovy 2003). Some of these infected animals (such as aquatic insect larvae) act as paratenic (transport) hosts by carrying cysts to land where they are consumed by omnivorous or predatory definitive hosts including millipedes, orthopterans (crickets, grasshoppers, etc.), beetles, cockroaches, and mantids. In many cases, gordiids penetrate and encyst in dead-end hosts (e.g. snails), which cannot link the cyst with the terrestrial hosts. Cysts are long-lived within paratenic hosts (including snails) and can survive for years (Hanelt *et al.* 2001; Hanelt & Janovy, 2003, 2004a).

Approximately 350 species of gordiids have been described worldwide from 19 extant and two extinct genera (Poinar 1999; Poinar & Buckley 2006). However, estimates suggest that only 18% of the hairworm diversity has been documented globally (Poinar 2008). Lack of knowledge on the diversity of hairworm species exists because previous studies were hindered by the lack of reliable ways to collect adult hairworms over large geographical areas and the relatively short life span of the free-living adults, making them difficult to collect. However, recent studies suggest that non-adult stages of gordiids, such as cysts, may be the most commonly encountered life stages of gordiids in the environment (Hanelt *et al*. 2001; Szmygiel 2012). Both Hanelt *et al.* (2001) and Szmygiel (2012) sampled for adult and cyst stages of gordiids in 50 and 46 streams in a single county in Nebraska and Oklahoma U.S.A. respectively. Free-living adult gordiids were found in only one stream in Nebraska. In contrast, cysts of gordiids were found in aquatic snails in 70% of the streams sampled from Nebraska and Oklahoma. These studies suggested that cysts are the most commonly encountered gordiid stages in nature and may be useful for sampling large geographical areas for nematomorph biodiversity studies (Hanelt *et al.* 2001; Hanelt *et al.* 2012; Szmygiel 2012; Bolek *et al.* 2013a).

One major difficulty in utilizing gordiid cyst stages for biodiversity studies is our lack of knowledge on the morphology of cysts and other non-adult stages for most hairworm species and genera. The lack of descriptions of non-adult stages of gordiids stems from the fact that until recently, the life-cycles for most species of gordiids were unknown. However, recent advances in our understanding of gordiid life-cycles in combination with new culturing and domestication techniques have made these life stages more accessible for morphological studies (Hanelt & Janovy 1999; 2002; 2004a; 2004b; Bolek & Coggins 2002; Hanelt *et al.* 2012; Bolek *et al.* 2010; 2013a; 2013b). Currently, a single comparative study exists on cyst morphology for three species of hairworms (Hanelt & Janovy 2002). Using this information, Hanelt *et al.* (2012) and Bolek *et al.* (2013a) collected snails infected with two types of gordiid cysts from Kenya, a country for which no gordiid records existed. After exposing the appropriate group of laboratory-reared arthropods to the cysts, two new species of gordiids were discovered. Importantly, their work highlighted a critical need for comprehensive morphological descriptions of non-adult stages of gordiids which can be used to discover the hidden biodiversity of this group.

In this study, we compare the oviposition behavior and non-adult stages (egg strings, larvae and cysts) for nine species of gordiids across four genera from two continents (Africa and North America) using light and differential interference contrast microscopy and/or scanning electron microscopy (SEM). Secondly, we provide the first SEM descriptions for larvae of two species of *Gordius* and for two genera (*Paragordius* and *Neochordodes* Montgomery, 1898) which have never been examined with SEM. Thirdly, we evaluate the usefulness of cyst morphometrics for gordiid identification. Finally, we review other studies on non-adult stages of gordiids that are available in the literature and discuss our findings on cyst morphology as a tool for nematomorph biodiversity studies.

Methods

Material examined, collection of non-adult stages and maintenance of gordiid species in the laboratory. The oviposition behavior and morphological characteristics of egg strings, larvae and cysts were examined for seven species of North American (NA) and African (AF) gordiids: *Chordodes morgani* Montgomery, 1898 (NA), *Gordius* cf. *robustus* #1 Leidy, 1851 (NA), *Gordius* cf. *robustus* #2 (NA), *Gordius difficilis* Montgomery, 1898 (NA), *Neochordodes occidentalis* Montgomery, 1898 (NA), *Paragordius varius* Leidy, 1851 (NA), and *Paragordius obamai* Hanelt, Bolek & Schmidt-Rhaesa, 2012 (AF). Additionally, data sets on oviposition behavior and non-adult characteristics for two additional species of hairworms including *Chordodes janovyi* Bolek, Schmidt-Rhaesa, Hanelt, & Richardson, 2010 (AF) and *Chordodes kenyaensis* Bolek, Szmygiel, Kubat, Schmidt-Rhaesa & Hanelt, 2013 (AF) were taken from previous publications (Bolek *et al.* 2010; Bolek *et al.* 2013a) and/or new material from laboratory infections (see below). We also provide new light microscopy (LM), differential

interference contrast microscopy (DIC) and/or scanning electron microscopy (SEM) images for egg strings, larvae and/or cysts for these species.

Observations on oviposition behavior and samples of non-adult stages for *C. kenyaensis*, *P. obamai* and *P. varius* were obtained from laboratory cultures of these species maintained at Oklahoma State University and the University of New Mexico. These species were originally isolated as cysts from field collected snails (see Table 1 for locations) and their life cycles established in the laboratory as described by Hanelt *et al.* (2012) and Bolek *et al.* (2013a). Briefly, laboratory cycles of these species were maintained in the following manner: the soft tissue of snails infected with cysts was macerated with a razor blade, placed on a microscope slide with a drop of water, covered with a cover slip and examined for cysts at 100x–400x magnification. A small portion of the soft tissue from a single infected snail containing 1–15 gordiid cysts was fed to 4–5 week old and 24 hr starved commercially reared crickets, *Acheta domesticus* (Linnaeus, 1758), or laboratory reared crickets, *Gryllus firmus* Scudder, 1902, in individual exposure cages. Exposed crickets were maintained for 24 hr in exposure cages until they ingested all snail tissue. Exposed crickets were maintained in groups of 15 in covered plastic shoe boxes (35 cm x 25 cm x15 cm) with a paper-towel-substrate and a 4 cm^2 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. Depending on the species of gordiid, 30–60 days post exposure (DPE) all exposed crickets were placed in 110 × 35 mm Stender dishes partially filled with aged tap water and allowed to release worms. Once worms emerged, male and female worms of dioecious species were isolated as pairs in Stender dishes with aerated aged tap-water and allowed to mate. Females of *P. obamai*, a parthenogenetic species, and mated females of *P. varius* and *C. kenyaensis* were isolated individually in Stender dishes with aerated aged tap-water and a small stick (5–10 cm long and 0.5 cm wide) as described in Bolek *et al.* (2013a). Observations were made on the oviposition behavior of each species and where egg strings were deposited. All deposited egg strings were rinsed in 0.5% bleach solution to prevent fungal growth and moved to new Stender dishes filled with aged tap-water and an aerator following the protocol of Hanelt & Janovy (1999). Egg strings were visually observed over a period of 2–4 weeks for larval maturation indicated by a color change from white to brown. Hatched larvae were collected with a Pasteur pipette and approximately 100–200 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 *Physa* (*Physella*) *gyrina* (Say, 1821) snail was added. Snails fed on the larva mixture for 48 hrs, were then removed and maintained in 3.75 L jars filled with aerated aged tap water with a calcium gravel substrate. Snails were fed on a diet of frozen lettuce and Tetra Min® fish food and examined for gordiid cysts over a period of four weeks post exposure as previously described.

TABLE 1. Collection locality and stage collected of North American and African gordiids.

Species	Stage	Locality	Latitude and Longitude
Chordodes morgani	Adults	Lancaster County, NE, USA	+40.8833, -96.8333
Chordodes kenyaensis	Cysts	Nyanza Province, Kenya	-0.1516 , +34.3352
Gordius difficilis	Adults and cysts	Waukesha County, WI, USA	+42.1833, -88.3500
Gordius cf. *robustus* #1	Adults	Bernalillo County, NM, USA	+35.2666, -106.3666
Gordius cf. *robustus*#2	Adults	Payne County, OK, USA	+36.1121, -97.0655
Paragordius obamai	Cysts	Nyanza Province, Kenya	-0.1516 , +34.3352
Paragordius varius	Cysts	Lancaster County, NE, USA	+41.8125, -101.8386
Neochordodes occidentalis	Adults	Pima County, AZ, USA	+31.8655, -109.1905

For the remaining five species of gordiids used in this study (*C. morgani*, *G.* cf. *robustus* #1, *G.* cf. *robustus* #2, *G. difficilis*, and *N. occidentalis*) free living adults were collected in the field (see Table 1 for locations). All field collected free living worms were brought back to the laboratory in 0.5 L plastic bottles with stream water, identified to species based on keys in Schmidt-Rhaesa *et al.* (2003) or molecular data for cryptic species of *Gordius* in the *Gordius robustus* complex (Hanelt unpublished data). Male and female worms of the appropriate species were paired in Stender dishes as previously described. Oviposition behavior was observed and egg strings, larvae and cysts were obtained as described above. Because larvae of *G. difficilis* never hatched in the laboratory, we collected *P. gyrina* snails naturally infected with *G. difficilis* cysts for observations on cyst morphology (see Table 1).

Preparation and morphological characteristics of egg string, larvae and cysts using light microscopy. The width of 20–30 egg strings for each species of gordiids was measured with a calibrated ocular micrometer using a Wild Heerbrugg M5 stereomicroscope at 500x magnification. All larvae were prepared as live wet mounts and observed using an Olympus BX-51 upright research microscope configured for bright field and DIC microscopy with plain fluorite objectives at 400x to 1000x total magnification. The length and width of the preseptum, postseptum, pseudointestine and stylets were measured for 20–30 larvae of each gordiid species following the protocols of Hanelt & Janovy (2002). Measurements on larval characteristics were taken using a calibrated ocular micrometer or by capturing digital images of live larvae using an Olympus 5 megapixel digital camera and Q Capture image analysis software to obtain measurements. In addition, digital photographs of larvae of each gordiid species were recorded in order to examine the shape of the psuedointestine. Cysts were examined by preparing wet mounts of the infected snail as previously described. Cysts were digitally photographed and the length and width of the cyst, cyst wall and encysted larvae were obtained using Q Capture image analysis software as previously described for larvae. Additionally, the folding pattern of encysted larvae was recorded for each species of gordiid.

Larval preparation for SEM and external larval characteristics. Poly-L-Lysine coated cover-slips were placed in 1.5 ml plastic well plates. Live larvae suspended in water were then pipetted onto the Poly-L-Lysine coated cover-slips and fixed in 10% neutral buffered formalin. Poly-L-Lysine coated cover-slips with fixed larvae were then dehydrated in a graded series of ethanol, dried using hexamethyldisilazane (Jirků *et al.* 2006), 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. The following morphological surface characteristics were recorded for at least 30 individual larvae of each gordiid species: number of terminal spines on the postseptum, the number and relative size of cuticular hooks on the preseptum, the proboscis orientation (dorso-ventrally or laterally compressed) and the number and orientation of spines on the proboscis. Morphological characteristics for larvae examined with SEM followed terminology with some modifications from Bolek *et al.* (2010) and Bolek *et al.* (2013a).

Material deposited: Larvae of *C. morgani*, *C. kenyaensis*, *G.* cf. *robustus* #1, *G.* cf. *robustus* #2, *P. obamai*, *P. varius*, and *N. occidentalis,* all mounted on SEM stubs, Museum of Southwestern Biology-Parasitology Division, accession numbers MSB Para 19014–19020.

Data analysis for cyst morphometrics: Because we found three distinct types of folding patterns among cysts within the four genera of gordiids, morphometric analyses were performed on three separate cyst data sets: (1) *Gordius*, (2) *Paragordius* and (3) *Chordodes* and *Neochordodes*. To avoid problems associated with character correlation, significant differences among groups were first determined using multivariate analyses. For each data set separately, a 1-way multivariate analysis (MANOVA) was performed to determine if significant differences exist among species. When a MANOVA was significant, a corresponding 1-way analysis of variance (ANOVA) was subsequently performed for each cyst character alone to evaluate its potential contribution to differences among species. Furthermore, species that were significantly different from each other were identified using Scheffe's post hoc test (Sokal & Rohlf 1981). Data on cysts were also analyzed using discriminant function analysis (DFA). Principal Component Analysis (PCA) was subsequently implemented to determine if separation could be based on morphological characteristics. The assumption of PCA is that the total variance of a variable reflects the sum of explained and error variance (Grimm & Yarnold 1995).

Results

Oviposition behavior and egg string morphology: Morphological characteristics and measurements for egg strings are presented in Table 2. All three *Gordius* species examined in this study deposited short pieces of egg string (3–10 mm) in the water column over a period of 2–3 weeks after copulation (Fig. 1A); but both *Paragordius* species deposited a single continuous unbroken egg string in the water column within hours or days of emerging and mating or emerging from the cricket hosts (Figs 1B, 1C). Egg strings of *Paragordius* species were 2–5 times the length of the female worm's body. In contrast, females of all *Chordodes* species and *N. occidentalis* attached their egg strings in a zig-zag pattern to air hoses or sticks during oviposition and egg strings were never deposited free in the water column (Figs 1D, 1E, 1F).

TABLE 2. Measurements and morphological characteristics of egg strings, larvae and cysts for nine species of gordiids from this study and for which data were available from the literature. Means and ranges (in parentheses) are given in micrometers.

	C. janovyi	*C. morgani*	*C. kenyaensis*	*G. difficilis*	*G.* cf. *robustus* #1	*G.* cf. *robustus* #2	*N. occidentalis*	*P. obamai*	*P. varius*
Egg String									
Egg string width	385.0 (250–600)	475.9 (317–633)	449.4 (220–580)	538.8 (314–754)	544.1 (346–754)	655.5 (276–966)	596.8 (327–784)	246.9 (170–430)	249.2 (188–282)
Egg string pattern	Zig-Zag	Zig-Zag	Zig-Zag	Short Pieces	Short Pieces	Short Pieces	Zig-Zag	Continues (not in short pieces)	Continues (not in short pieces)
Larva									
Preseptum length	22.8 (18–30)	22.8 (18–21)	21.0 (17–24)	?	38.4 (21–49)	24.9 (21–30)	33.0 (23–51)	23.0 (22–30)	22.3 (16–31)
Preseptum width	15.0 (14–17)	13.5 (12–17)	15.7 (14–17)	?	30.5 (16–39.5)	16.9 (14.3–19.3)	25.9 (17–33)	15.0 (12–17)	14.0 (11–18)
Postseptum length	25.7 (21–32)	25.7 (18–33)	23.1 (14–26)	?	101.8 (63–130)	85.1 (75–103)	37.8 (26–46)	31.0 (25–38)	30.2 (22–39)
Postseptum width	12.5 (12–17)	11.2 (10–13)	13.0 (12–15)	?	26.8 (17–33)	16.1 (13–19)	19.8 (15–26)	12.5 (10–14)	11.4 (10–16)
Average Postseptum Preseptum ratio	1.1/1.0	1.1/1.0	1.1/1.0	?	2.6/1.0	3.0/1.0	1.1/1.0	1.3/1.0	1.3/1.0
Stylet length	13.8 (11–15)	12.8 (9–15)	14.9 (12–18)	?	18.3 (12–22)	?	18.6 (16–26)	12.4 (11–13)	12.7 (10–15)
Stylet width	4.2 (4–5)	1.4 (1–3)	3.6 (3–4)	?	2.3 (1–4)	?	1.8 (1–3)	3.7 (3–5)	2.9 (2–4)
Pseudointestine length	13.3 (10–17)	9.1 (7–12)	12.2 (10–14)	?	75.5 (39–104)	64.5 (57–78)	12.3 (9–19)	14.8 (12–17)	15.9 (11–25)
Psuedointestine width	8.3 (7–10)	6.7 (5–10)	9.7 (8–12)	?	38.8 (22–57)	12.7 (10–16)	10.6 (7–19)	9.75 (8–11)	7.5 (5–11)
Pseudointestine shape	V-shaped	V-shaped	V-shaped	Subdivided oval	Subdivided oval	Subdivided oval	V-shaped	2 anterior granules and posterior mass	2 anterior granules and posterior mass
Proboscis orientation	LC	LC	LC	?	DVC	DVC	LC	DVC	DVC
Proboscis spine arrangement	DV 2:2:1 LL 2:2	DV 2:2:1 LL 2:2	DV 2:2:1 LL 2:2	?	LL; RL 2:2:2:2:1 D : 2:2:2:1	LL; RL 2:2:2:2:1 D 2:2:2:1	DV 2:2:1 LL 2:2	LL; RL ? V ?	LL; RL 2:2:2:2:1 V 2:2:1
Terminal spines on postseptum	4	4	4	?	1	1	4	4	4
Cyst									
Cyst wall length	7.1 (4–14)	7.3 (4–12)	11.4 (6–19)	11.1 (5–19)	7.4 (1–14)	22.8 (16–33)	7.9 (3–19)	14.3 (6–25)	12.8 (9–20)
Cyst wall width	6.9 (4–12)	5.8 (3–11)	11.9 (7–17)	10.7 (4–17)	7.2 (2–16)	19.9 (14–28)	7.0 (3–13)	9.7 (5–14)	9.2 (5–17)
Cyst larva length	27.6 (24–30)	25.9 (24–31)	28.2 (22–35)	31.6 (25–34)	37.3 (33–46)	36.1 (15–48)	27.5 (23–37)	29.7 (20–33)	27.5 (23–31)
Cyst larva width	20.5 (17–24)	21.3 (18–25)	20.2 (15–25)	19.2 (18–22)	26.0 (23–29)	27.8 (15–38)	20.8 (16–25)	18.5 (16–21)	18.5 (15–22)
Cyst total length	41.8 (33–55)	40.5 (32–56)	50.9 (40–67)	53.9 (36–72)	52.0 (38–65)	81.8 (55–100)	43.2 (32–68)	58.2 (42–70)	53.0 (43–65)
Cyst total width	34.3 (28–46)	33.0 (26–44)	43.9 (35–53)	40.7 (28–52)	40.3 (31–58)	67.6 (50–82)	34.7 (25–50)	37.9 (30–45)	36.9 (31–52)
Cyst larva folding pattern	1	1	1	2	2	2	1	2	2

D = dorsal, DV = dorsal-ventral, DVC = dorso-ventrally compressed, LC = laterally compressed, LL = left lateral, RL = right lateral, V = ventral. Data for *C. janovyi* and *C. kenyaensis* ar egg data and pseudointestine shape for *G. difficilis* obtained from Bolek *et al.* (2010), Bolek *et al.* (2013), and Bolek & Coggins (2002), respectively.

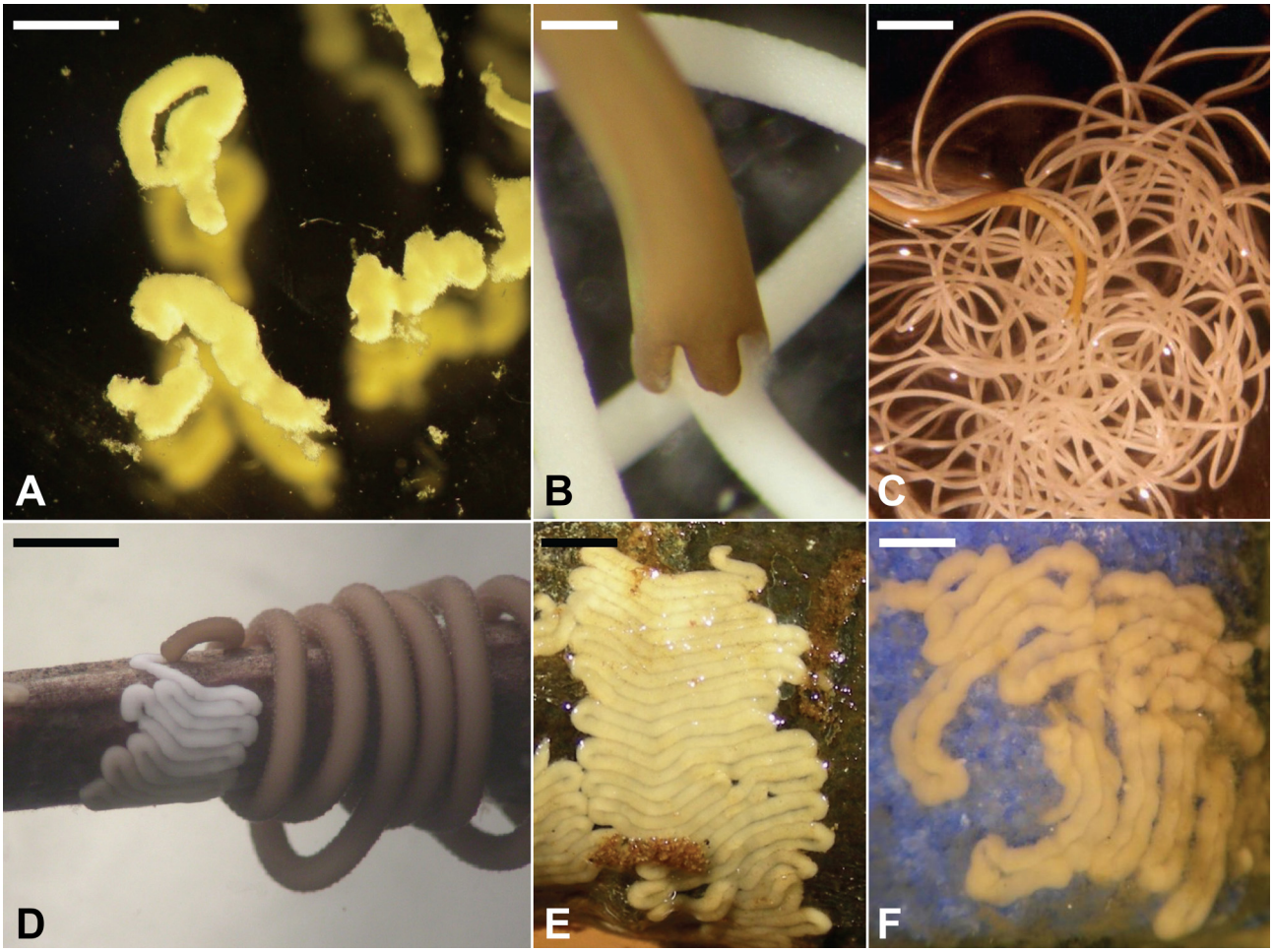

FIGURE 1. Egg strings of four genera of gordiids. (**A**) Egg strings of *G.* cf. *robustus* #1. Scale bar = 1.9 mm. (**B**) Posterior end of female *P. obamai* in the process of depositing an egg string. Scale bar = 625 µm. (**C**) Female *P. varius* (brown) in the process of depositing an egg string (white). Scale bar = 1.2 mm. (**D**) Female *C. kenyaensis* (brown) depositing an egg string (white) in a zig-zag pattern on a branch. Scale bar = 3.1 mm. (**E**) Egg string of *C. morgani* deposited in a zig-zag pattern on a branch. Scale bar = 5.8 mm. (**F**) Egg string of *N. occidentalis* deposited in a zig-zag pattern on an air-stone. Scale bar = 1.9 mm.

Larval morphology: Morphological characteristics and measurements for larvae are presented in Table 2. Larvae of both species of *Gordius* were cylindrical in shape and their body was divided by a septum into two regions, the preseptum and a postseptum (Figs 2A, 2C). The preseptum contained an eversible proboscis supported by three internal stylets (Fig. 2B). The pseudointestine was an elongated oval structure, subdivided into unequal portions (Fig. 2A). Externally, larvae were superficially annulated and the postseptum contained a single posterior spine (Figs 2A, 2C, 2G). The preseptum had three sets of cuticular hooks. The outer ring of hooks contained seven hooks, two of which were very close together and ventrally positioned; while the middle and inner rings contained six hooks each (Figs 2D, E). The left and right lateral side on the distal end of the dorsoventrally compressed and eversible proboscis each contained nine spines (four pairs arranged in tandem and one single spine above); whereas the distal end of the dorsal side of the proboscis contained seven spines (three pairs arranged in tandem and one single spine above; Figs 2E, 2F).

As in *Gordius* species, larvae of *P. obamai* and *P. varius* were superficially annulated, cylindrical in shape, the body was divided by a septum into two regions and the eversible proboscis was supported by three internal stylets (Figs 3A, 3B). However, in contrast to *Gordius* species, the pseudointestine contained two anterior granules and a large posterior mass; pairs of anterior and posterior terminal spines were present on the ventral side of the postseptum (Figs 3A, 3C, 3D). As with *Gordius* species three rings of seven outer, six middle and six inner cuticular hooks were present on the preseptum (Figs 3B, 3E, 3F). However, the length of the cuticular hooks on the outer ring was noticeably longer than in any other species or genera of gordiid examined in this study (Figs 3B, 3E, 3F). Clearly visible spines on the proboscis could only be observed in larvae of *P. varius* (Figs 3E, 3F, 3G). The left

and right side of the distal end of the dorsoventrally compressed and eversible proboscis each contained nine spines (four pairs arranged in tandem and one single spine above); whereas the distal end of the ventral side of the proboscis contained five spines (two pairs arranged in tandem and one single spine above; Figs 3F, 3G). Larvae of *P. obamai* also contained pairs of spines on the right, left and the ventral sides of the proboscis. However, because all proboscis of *P. obamai* were retracted during fixation the exact number of spines on each side could not be determined.

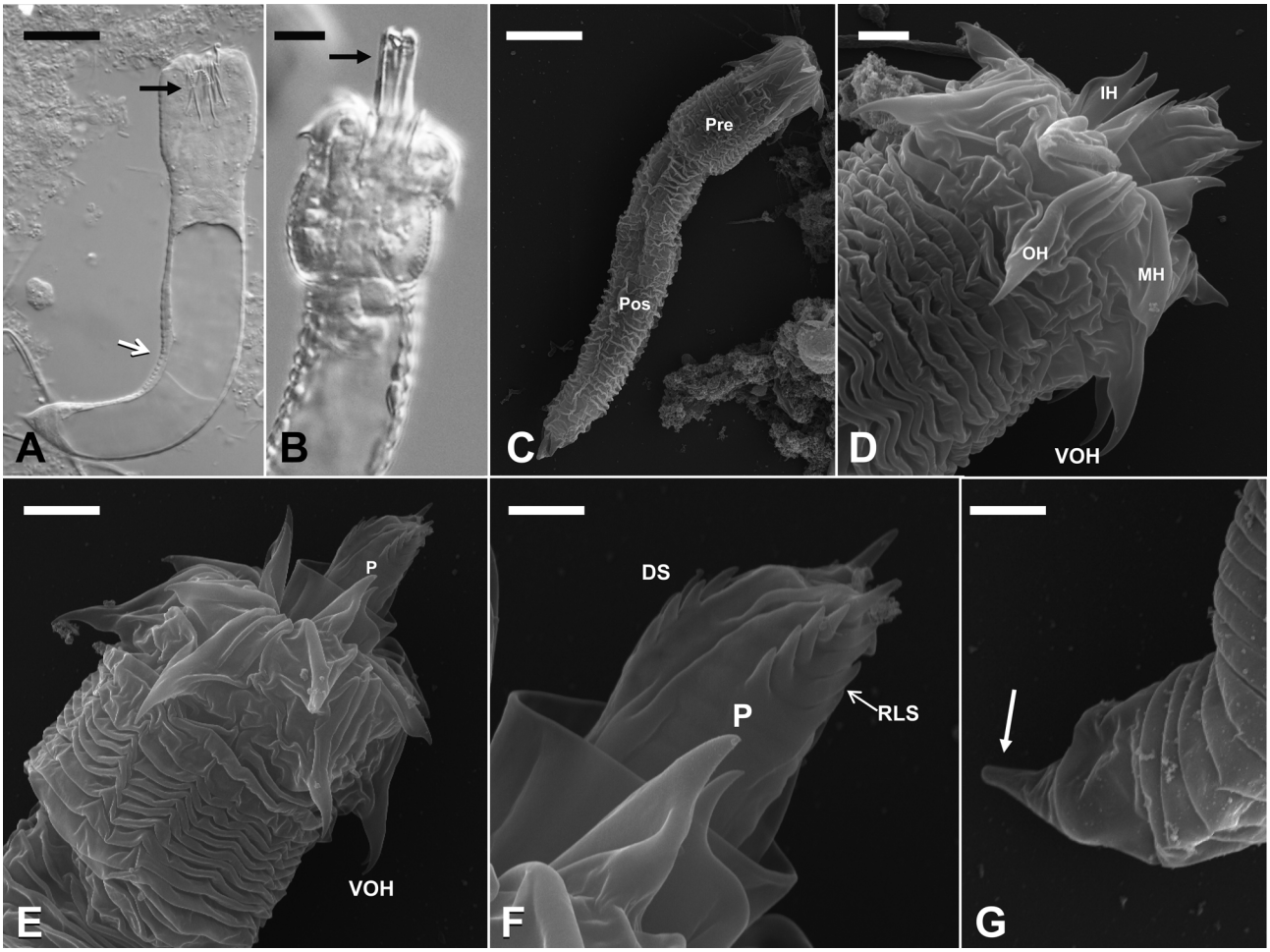

FIGURE 2. Larval characteristics of the genus *Gordius*. (**A**) DIC photomicrograph of a live *G.* cf. *robustus* #2 larva. Note the stylets (black arrow) and subdivided pseudo intestine (white arrow). Scale bar = 18.8 µm. (**B**) DIC photomicrograph of anterior end of *G.* cf. *robustus* #2 larva. Note the everted proboscis (arrow). Scale bar = 8.0 µm. (**C**) SEM photomicrograph of an entire *G.* cf. *robustus* #1 larva. Note the distinct preseptum (Pre) and very long postseptum (Pos). Scale bar = 11.0 µm. (**D**) SEM photomicrograph of the preseptum of a *G.* cf. *robustus* #1 larva. Note the inner row of hooks (IH), middle row of hooks (MH), and outer row of hooks (OH) containing two ventral outer hooks (VOH). Scale bar = 2.0 µm. (**E**) SEM photomicrograph of the preseptum of *G.* cf. *robustus* # 1 larva. Note the orientation of the proboscis (P) to the ventral hooks (VOH). Scale bar =3.0 µm. (**F**) SEM photomicrograph of the proboscis (P) of a *G.* cf. *robustus* #1 larva. Note the arrangement of the nine right lateral spines (RLS), and seven dorsal spines (DS). Scale bar = 1.0 µm. (**G**) SEM photomicrograph of the posterior end of a *G.* cf. *robustus* #2 larva. Note single posterior spine (arrow). Scale bar = 3.0 µm.

As in *Gordius* and *Paragordius* species, larvae in the genus *Chordodes* were superficially annulated, cylindrical in shape, the body was divided by a septum into two regions and the eversible proboscis was supported by three internal stylets (Figs 4A, 4B, 4G). However, larvae of all *Chordodes* species were much shorter in length and the pseudointestine was v-shaped with one smaller and one larger branch positioned anteriorly; a preintestinal gland was located above the anterior end of the larger branch (Fig. 4A). As in *Paragordius* species, larvae contained two pairs of terminal spines located ventrally on the postseptum and the pseudointestine exterior opening was centrally located above the anterior terminal spines (Figs 4B, 4C, FG). The preseptum contained seven outer, six middle and six inner cuticular hooks (Figs 4B, 4D, 4F). However, the proboscis in all *Chordodes* species was

laterally compressed and the distal end of the dorsal, ventral and left lateral sides contained spines. Two pairs of spines were arranged in tandem and one single spine was located above on the dorsal and ventral side of the proboscis; whereas the left lateral side of the proboscis contained four spines (two pairs arranged in tandem; Figs 4D–4F). Three to four weeks after hatching free-living larvae of *C. janovyi* and *C. kenyaensis* secreted thread like projections by empting their pseudointestine and stopped moving (Fig. 4G).

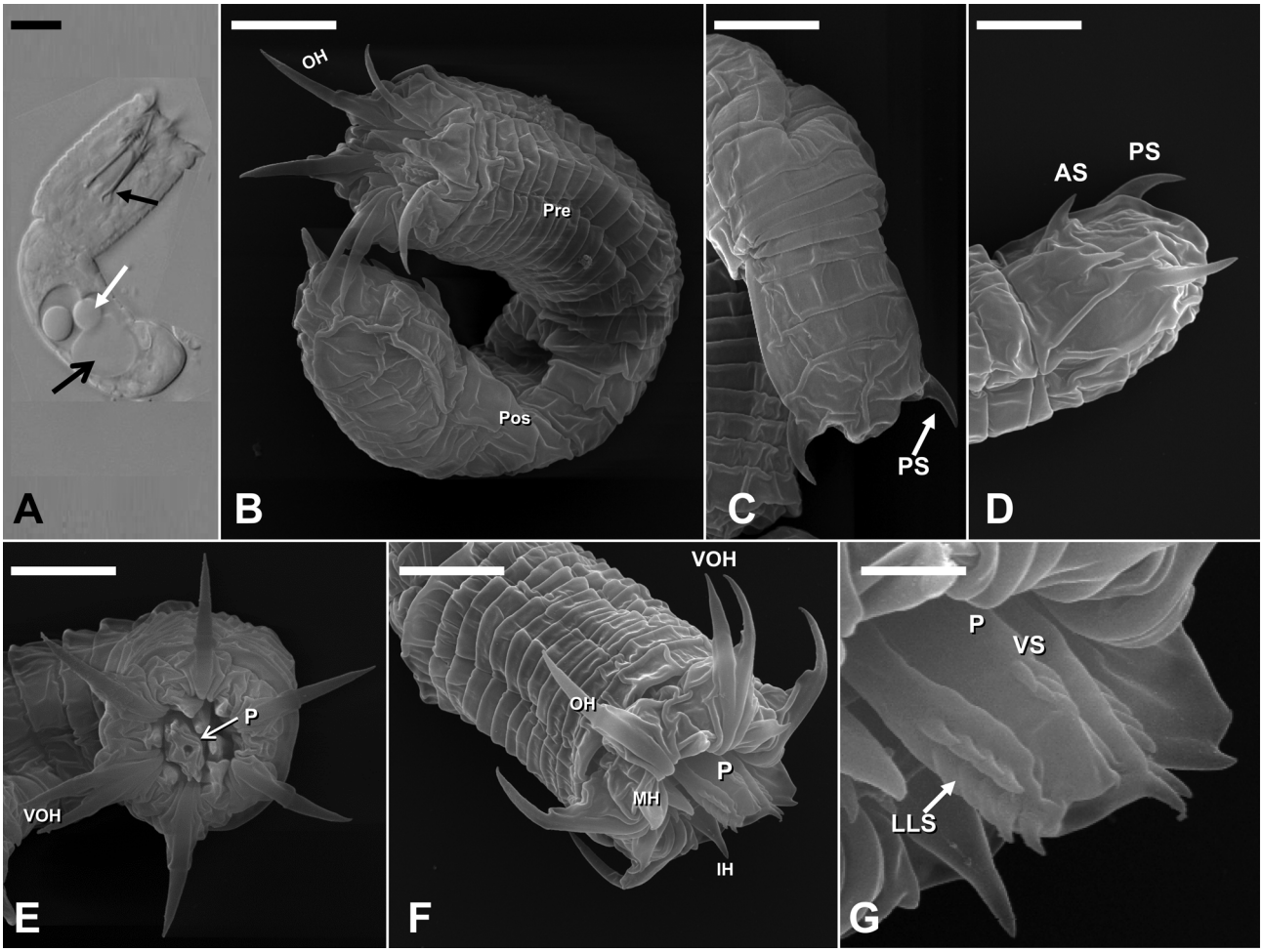

FIGURE 3. Larval characteristics of the genus *Paragordius*. (**A**) DIC photomicrograph of a live *P. obamai* larva. Note the stylets (thin black arrow), pseudointestine with two anterior granules (white arrow) and posterior mass (thick black arrow). Scale bar = 7.0 µm. (**B**) SEM photomicrograph of a *P. varius* larva. Note the distinct preseptum (Pre), postseptum (Pos) and relatively long outer hooks (OH). Scale bar = 5.5 µm. (**C**) SEM photomicrograph of the posterior end (dorsal side) of a *P. varius* larva. Note two posterior spines (PS). Scale bar = 6.0 µm. (**D**) SEM photomicrograph of the posterior end (ventral side) of a *P. varius* larva. Note the two anterior spines (AS) and two posterior spines (PS). Scale bar = 5.0 µm. (**E**) SEM photomicrograph of the anterior end (*en face* view) of a *P. varius* larva. Note the orientation of the proboscis (P) to the ventral outer hooks (VOH). Scale bar = 4.5 µm. (**F**) SEM photomicrograph of the anterior end (ventral side) of a *P. varius* larva. Note partially everted proboscis (P), inner row of hooks (IH), middle row of hooks (MH), and outer row of hooks (OH) containing two ventral outer hooks (VOH). Scale bar = 4.6 µm. (**G**) Close up SEM photomicrograph of the proboscis (P) of a *P. varius* larva. Note the arrangement of the five ventral spines (VS) and nine spines on the left lateral side (LLS). Scale bar = 1.5 µm.

Larvae of *N. occidentalis* were morphologically most similar to larvae in the genus *Chordodes* (Figs 5A, 5B, 5F, 5G, 5H). However, two preintestinal glands were visible above each arm of the pseudointestine when pressure was applied to larvae (Fig. 5C). Three weeks after hatching free-living larvae were observed empting the content of their pseudointestine (Figs 5D, 5E).

Cyst morphology: Morphological characteristics and measurements for cysts are presented in Table 2. Cysts of *G. difficilis*, *G.* cf. *robustus* #1 and *G.* cf. *robustus* #2 contained a clear cyst wall. During cyst formation the content of the larval pseudointestine was emptied and larvae folded their postseptum twice around the preseptum (Figs 6A, 6B, 6C, 6D). The posterior end of the postseptum always reached the posterior end of the preseptum (Fig.

6C) and protruding spines were never visible on the anterior end of fully formed cysts (Figs 6B, 6C, 6D). During cyst formation, larvae of the two *Paragordius* species emptied the content of their pseudointestine and folded their postseptum twice around the preseptum (Figs 6E, 6F, 6G, 6H). However, in larvae of *P. obamai* and *P. varius* the posterior end of the postseptum never reached the posterior end of the preseptum (Fig. 6G). Fully formed cysts contained a clear cyst wall and clearly visible large spines were always present on the anterior end of larvae (Figs 6E, 6F, 6G, 6H). Cysts of the three species of *Chordodes* and *N. occidentalis* also possessed a clear cyst wall; larvae emptied the content of their pseudointestine during cyst formation (Figs 6I, 6J, 6K, 6L). However, in contrast to species of *Gordius* and *Paragordius*, larvae of species of *Chordodes* and *N. occidentalis* folded their postseptum only once and folded larvae contained much smaller and less visible spines on the anterior end (Figs 6I, 6J, 6K, 6L).

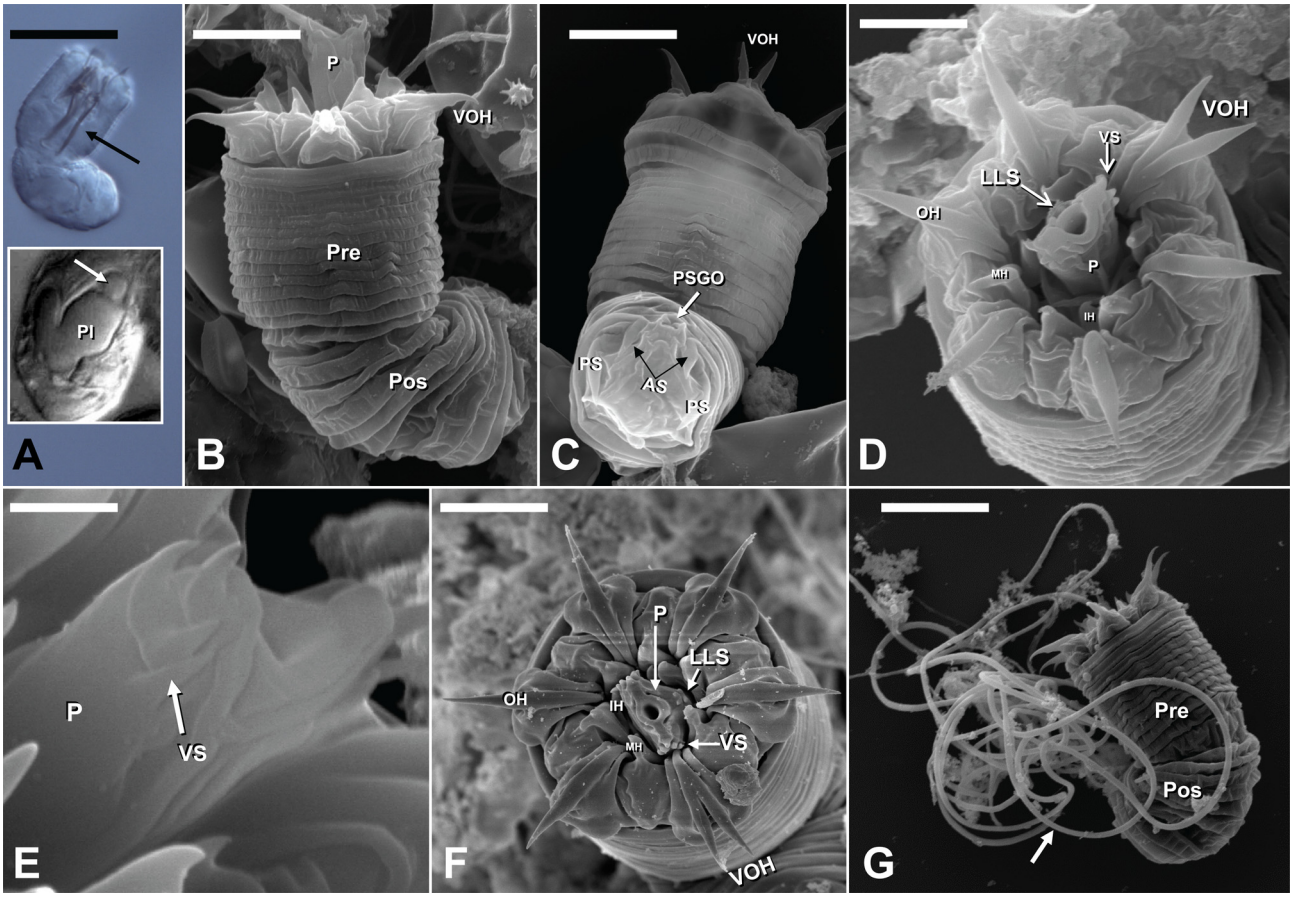

FIGURE 4. Larval characteristics of the genus *Chordodes*. (**A**) DIC photomicrograph of a live larva of *C. morgani*, and postseptum of *C. kenyaensis* (insert). Note the stylets (black arrow). Insert: v shaped pseudointestine (PI) of *C. kenyaensis*. Note the preintestinal gland (white arrow) located above the anterior end of the larger branch of the pseudointestine. Scale bars = 15 µm for A and 10 µm for insert. (**B**) SEM photomicrograph of an entire *C. morgani* larva. Note the distinct preseptum (Pre), postseptum (Pos), proboscis (P) and ventral outer hooks (VOH). Scale bar = 8.0 µm. (**C**) SEM photomicrograph of the ventral side of a *C. morgani* larva. Note the two anterior spines (AS), two posterior spines (PS), pseudointestine gland opening (PSGO) and ventral outer hooks (VOH). Scale bar = 8.0 µm. (**D**) SEM photomicrograph of the anterior end (*en face* view) of a *C. morgani* larva. Note the orientation of the proboscis (P) with left lateral spines (LLS) and ventral spines (VS). Additionally note the inner hooks (IH) on the inner row, middle row of hooks (MH), and outer row of hooks (OH) containing two ventral outer hooks (VOH). Scale bar = 3.0 µm. (**E**) SEM photomicrograph of the ventral side of the proboscis (P) of a *C. morgani* larva. Note the two rows of ventral spines arranged in tandem and one single ventral spine on top (VS). Scale bar = 1.0 µm. (**F**) SEM photomicrograph of the anterior end (*en face* view) of a *C. janovyi* larva. Note the orientation of the proboscis (P) with left lateral spines (LLS) and ventral spines (VS). Additionally, note the arrangement of the inner row of hooks (IH), middle row of hooks (MH), and outer row of hooks (OH) containing two ventral outer hooks (VOH). Scale bar = 3.0 µm. (**G**) SEM photomicrograph of an entire *C. kenyaensis* larva in the process of empting the contents of its pseudointestine (white arrow). Note the relative size of the preseptum (Pre) and postseptum (Pos). Scale bar = 12.0 µm.

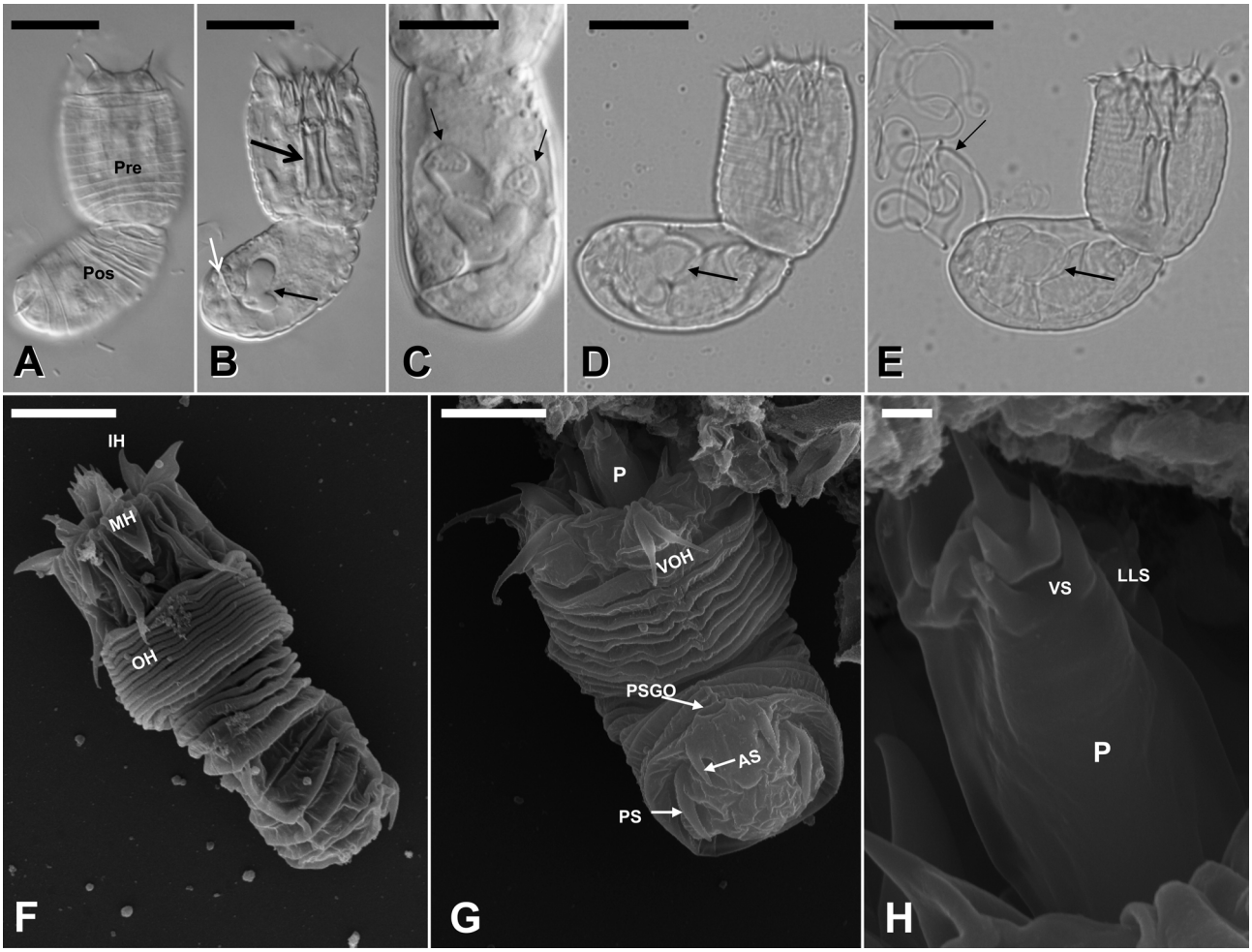

FIGURE 5. Larval characteristics of *Neochordodes occidentalis*. (**A**) DIC photomicrograph of a live larva. Note the distinct preseptum (Pre) and postseptum (Pos). Scale bar = 13.0 µm. (**B**) DIC photomicrograph of a live larva. Note the three stylets (large black arrow), the v shaped pseudointestine (small black arrow) and the pseudointestine gland opening (white arrow). Scale bar = 13.0 µm. (**C**) DIC photomicrograph of the postseptum (lateral view). Note the complex nature of the v shaped pseudointestine containing two preintestinal glands (black arrows) above the two arms of the v-shaped pseudointestine. Scale bar = 13.0 µm. (**D** and **E**) Light photomicrograph of an entire larva in the process of empting the contents of its pseudointestine (black arrow). Scale bars = 13.5 µm. (**F**) SEM photomicrograph of an entire larva (dorsal view). Note the inner hooks (IH), median hooks (MH) and outer hooks (OH) on the preseptum. Scale bar = 7.0 µm. (**G**) SEM photomicrograph of an entire larva (ventral view). Note the orientation of the proboscis (P) to the ventral outer hooks (VOH) and two anterior spines (AS), two posterior spines (PS) and pseudointestine gland opening (PSGO). Scale bar = 4.5 µm. (**H**) SEM photomicrograph of the ventral side of the proboscis (P). Note the arrangement of the five ventral spines (VS) and the presence of left lateral spines (LLS). Scale bar = 1.0 µm.

Morphometric comparisons among cysts: Comparisons among all cyst measurements within each data set (*Gordius*; *Paragordius*; *Chordodes* and *Neochordodes*) indicated that highly significant differences existed among species (Tables 3). However, when each character was examined individually, significant differences were not always observed for specific cyst morphological characters among all species within each data set (Table 3). Significant differences existed only for cyst wall length and width among cysts of all three species of *Gordius* (Table 3). Principal component one accounted for 66.30% of the variation, and the first two components accounted for 91.78% of the variation among species of *Gordius* (Table 4). DFA considered all cyst characters as significant in the discrimination process (see Table 5). A plot of the canonical function showed a clear graphic separation between the three species of *Gordius* (Fig. 7A). In contrast, ANOVA only identified cyst larval length and total cyst length as significantly different among species of *Paragordius* (Table 3). PCA analysis indicated that principal component one accounted for 51.27% of the variation, and the first two components accounted for 72.66% of the variation among species (Table 4). DFA identified only two of six cyst characters as significant in the

discrimination process (Table 5). A plot of the canonical function showed that there was significant overlap in morphology in cysts of *P. obamai* and *P. varius* (Fig. 7B). Finally, the 1-way ANOVA indicated that there was significant difference among five of the six cyst characteristics among the three species of *Chordodes* and *N. occidentalis* (Table 3). However, Scheffe's post hoc test indicated that not all species were significantly different among each other (Table 3). Principal component one accounted for 57.95% of the variation, and the first two components accounted for 75.63% of the variation (Table 4). DFA identified five of six cyst characteristics as discriminating (see Table 5). However, a plot of the canonical function showed that all species overlapped and could not be differentiated based on cyst morphometrics (Fig. 7C).

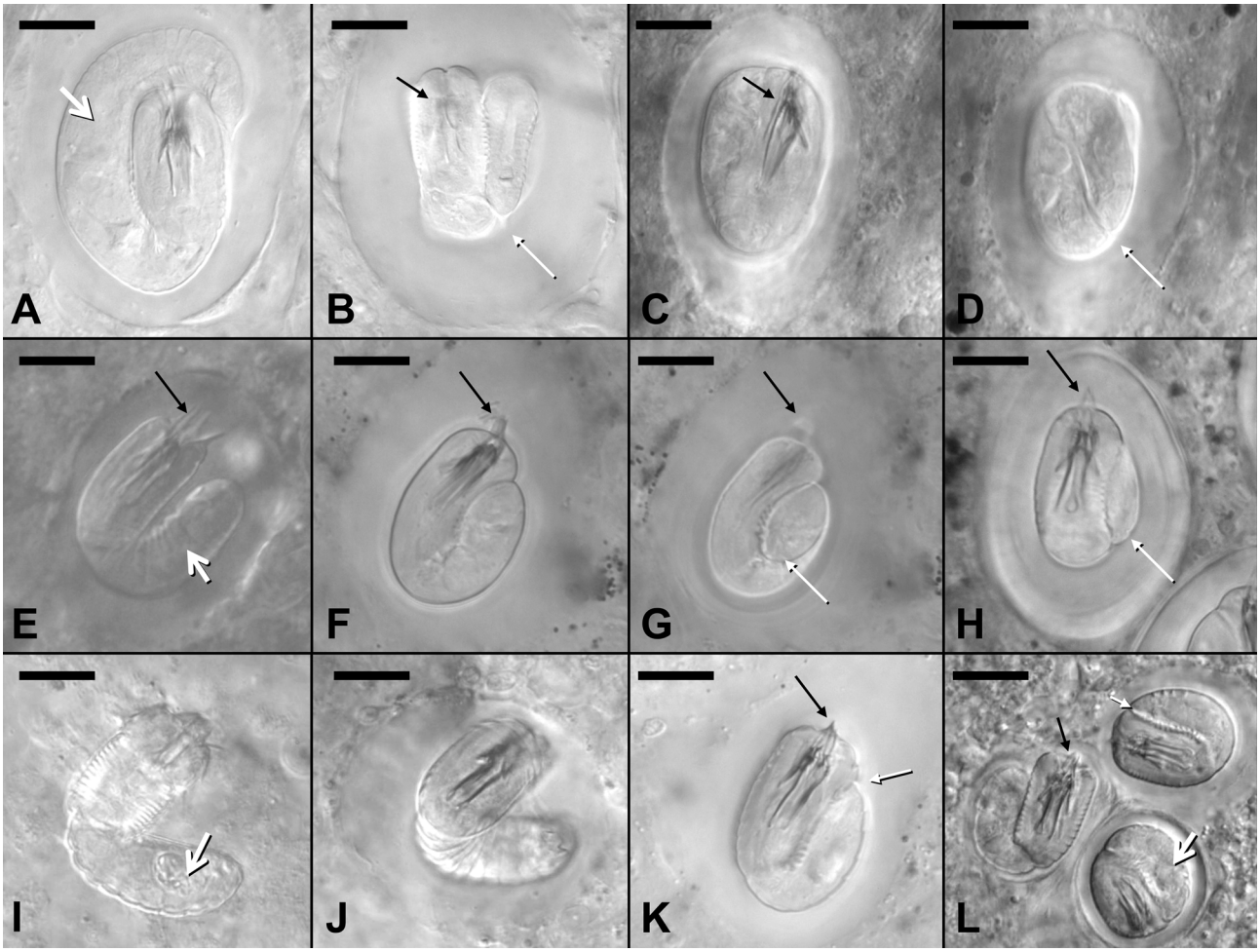

FIGURE 6. Larval folding pattern during cyst formation in four genera of gordiids. (**A–D**) DIC photomicrograph of cyst characteristics of the genus *Gordius*. Lateral (**A**) and dorso-ventral (**B**) view of larvae of *G.* cf. *robustus* #2 in the process of forming a cyst. Note the reduced content of the pseudointestine (white arrow in **A**); clear cyst wall, no protruding spines (black arrow) and the double folded postseptum with its posterior end reaching the posterior end of the preseptum (white arrow in **B**). Scale bars = 15.0 µm. (**C–D**) Lateral view of cysts of *G. difficilis*. Note the clear cyst wall, no protruding spines (black arrow) and the posterior end of the postseptum reaches the posterior end of the preseptum (small white arrow). Scale bars = 15.0 µm. (**E–H**) DIC photomicrographs of cyst characteristics of the genus *Paragordius*. (**E–G**) Larvae of *P. varius* in the process of forming a cyst. Note the clear cyst wall, empty pseudointestine (large white arrow), distinct spines on the preseptum (black arrows) and the position of the posterior end of the postseptum (small white arrow). Scale bars = 13.0 µm. (**H**) DIC photomicrograph of a *P. obamai* cyst (dorso-ventral view). Note the clear cyst wall, distinct spines on the preseptum (black arrows), and the position of the posterior end of the postseptum (white arrow). Scale bar = 13.0 µm. (**I–K**) DIC photomicrographs of *C. kenyaensis* larvae in the process of cyst formation. Note the clear cyst wall, reduced content of the pseudointestine (large white arrow), the postseptum folded once (small white arrow) and the presence of relatively small protruding spines on the preseptum (black arrow). Scale bars = 10.0 µm. (**L**) DIC photomicrograph of *N. occidentalis* larvae in the process of cyst formation. Note the clear cyst wall, reduced content of the pseudointestine (large white arrow), relatively small spines on the preseptum (black arrow), and the position of the posterior region of the postseptum (small white arrow). Scale bar = 19.0 µm.

TABLE 3. Significance levels of cyst character suites and each cyst character separately in distinguishing among species of *Gordius*, *Paragordius* and species of *Chordodes* and *Neochordodes occidentalis*.

	Gordius		*Paragordius*		*Chordodes*/*Neochordodes*	
Statistical Test and Character	Significance Level	Species Differences	Significance Level	Species Differences	Significance Level	Species Differences
MANOVA	$F_{(8,108)} = 30.11$, $P < 0.0005$; Wilk's ë = 0.096		$F_{(4,35)} = 4.27$, $P < 0.0005$; Wilk's ë = 0.672		$F_{(12, 193)} = 7.33$, $P < 0.0005$; Wilk's ë = 0.371	
ANOVA						
Cyst wall length	$F_{(2,57)} = 80.59$, $P < 0.0005$	All	$F_{(1,38)} = 1.49$, $P = 0.228$	None	$F_{(3,76)} = 6.76$, $P < 0.0005$	Ck (Cj,Cm)
Cyst wall width	$F_{(2,57)} = 74.28$, $P < 0.0005$	All	$F_{(1,38)} = 0.304$, $P = 0.585$	None	$F_{(3,76)} = 29.67$, $P < 0.0005$	Cj (Cm, Ck); Cm (Cj, Ck); Csp (All); No (Ck)
Cyst larva length	$F_{(2,57)} = 8.08$, $P = 0.001$	Gd (Gcfr1, Gcfr2)	$F_{(1,38)} = 6.77$, $P = 0.013$	All	$F_{(3,76)} = 3.23$, $P = 0.027$	Cm(Ck, Cj, No)
Cysts larva width	$F_{(2,57)} = 43.16$, $P < 0.0005$	Gd (Gcfr1, Gcfr2)	$F_{(1,38)} = 0.003$, $P = 0.958$	None	$F_{(3,76)} = 1.60$, $P = 0.196$	None
Total cyst length	$F_{(2,57)} = 71.83$, $P < 0.0005$	Gcfr1 (Gcfr2, Gd)	$F_{(1,38)} = 6.08$, $P = 0.01$	All	$F_{(3,76)} = 8.65$, $P < 0.0005$	Cj (Cm, Ck); Cm (Cj, Ck); Ck (Cj, Cm)
Total cyst width	$F_{(2,57)} = 94.26$, $P < 0.0005$	Gcfr1 (Gcfr2, Gd)	$F_{(1,38)} = 0.35$, $P = 0.556$	None	$F_{(3,76)} = 25.40$, $P < 0.0005$	Cj (Cm, Ck); Cm (Cj, Ck); Csp (All); No (Ck)

Cj = *C. janovyi*; Ck = *C. kenyaensis*; Cm = *C. morgani*; Gd = *G. difficilis*; Gcfr1 = *G.* cf. *robustus* #1; Gcfr2 = *G.* cf. *robustus* #2, No = *N. occidentalis*

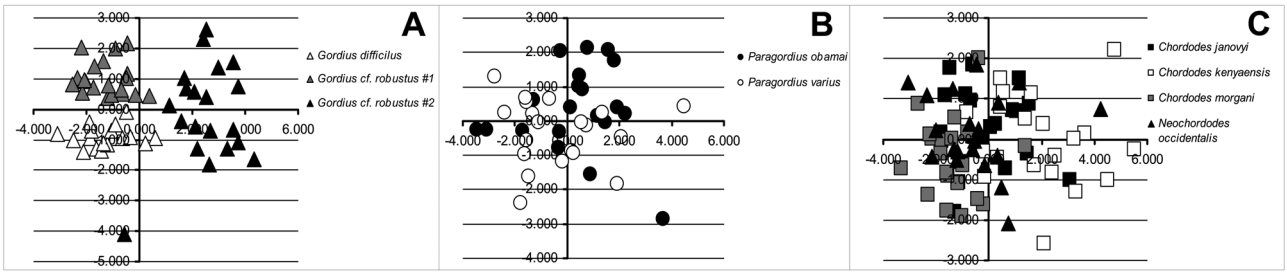

FIGURE 7. Canonical plots of gordiid cysts. (**A**) Cysts of three species of *Gordius*. (**B**) Cysts of two species of *Paragordius*. (**C**) Cysts of three species of *Chordodes* and *N. occidentalis*.

TABLE 4. Partial tabulation of principal component analyses of correlation matrix for six attributes for three types of gordiid cysts (*Gordius*; *Paragordius*; *Chordodes*/*Neochordodes*) for nine species of gordiids (only the first 2 principal components (PC) are shown).

	Gordius		*Paragoridius*		*Chordodes/Neochordodes*	
Character	PC1	PC2	PC1	PC2	PC1	PC2
Cyst length	0.914	-0.342	0.868	-0.177	0.915	-0.146
Cyst width	0.915	-0.327	0.876	0.066	0.932	-0.063
Larval length	0.300	0.887	-0.190	0.828	0.152	0.880
Larval width	0.566	0.719	-0.171	0.680	-0.271	-0.459
Total length	0.970	-0.012	0.862	0.149	0.923	0.119
Total width	0.977	-0.050	0.865	0.278	0.907	-0.193
% Variance	66.296	25.487	51.271	21.389	57.950	17.682
Cumulative	66.296	91.783	51.271	72.660	57.950	75.632

TABLE 5. Stepwise discriminant analyses of morphological characters for three types of gordiid cysts (*Gordius*; *Paragordius*; *Chordodes*/*Neochordodes*) for nine species of gordiids.

	Gordius			*Paragordius*			*Chordodes/Neochordodes*		
Character	Wilks' ë	$F_{(2,57)}$ =	P	Wilks' ë	$F_{(1,38)}$ =	P	Wilks' ë	$F_{(3,76)}$ =	P
Larval length	0.779	8.086	< 0.0005	1	0.003	0.958	0.941	1.602	0.196
Larval width	0.398	43.169	< 0.0005	0.992	0.304	0.585	0.887	3.237	0.027
Cyst width	0.277	74.289	< 0.0005	0.962	1.499	0.228	0.745	8.653	< 0.0005
Cyst length	0.261	80.59	< 0.0005	0.862	6.082	0.018	0.499	25.408	< 0.0005
Total cyst length	0.284	71.83	< 0.0005	0.991	0.352	0.556	0.789	6.765	< 0.0005
Total cyst width	0.232	94.268	< 0.0005	0.849	0.352	0.013	0.461	29.672	< 0.0005

Discussion

The major contribution of our study is that it is the first comparative evaluation of morphological characteristics of non-adult stages of gordiids from multiple genera and continents. Our study clearly indicates that three distinct types of oviposition behaviors, as well as three distinct morphological types of egg string, larva, and cysts were present among the four genera of gordiids examined in this study. All three species of *Gordius* deposited short pieces of egg strings in the water column; but the two species of *Paragordius* deposited a single long egg string in the water column. Although little information is available on oviposition behavior of other species of *Gordius* and *Paragordius*, data presented in Schmidt-Rhaesa (1997) for the European *Gordius aquaticus* Linné, 1758 and our unpublished observations on undescribed species of North American *Gordius* and *Paragordius* (B. Hanelt & M. Bolek, 2013) indicate that other species in these genera deposit short and long egg strings in the water column

respectively, suggesting that these are conserved characteristics in these genera. In contrast, all species of *Chordodes* for which oviposition behavior is known and our observations on *N. occidentalis* indicate that these species attached their egg strings in a zig-zag pattern on objects and never deposited them free in the water column. Observations on egg string morphology and oviposition behavior in the Japanese species *Chordodes japonensis* Inoue, 1952, the North American *C. morgani*, and the recently discovered Taiwanese species *C. formosanus*, indicate that these species glue their egg strings to objects such as sticks or rocks in a zig-zag pattern but never deposit eggs strings free in the water column (Inoue 1958; Hanelt & Janovy 2002; Chiu *et al.* 2011).

As with oviposition behavior and egg string morphology, we observed three distinct larval types. Larvae of *Gordius* species were much longer than those of the other genera of gordiids examined in this study. They had an elongated oval pseudointestine subdivided into unequal portions, a postseptum 2.6–3.0 times the length of the preseptum and a single terminal spine on the postseptum (Table 2). Studies on four other species of *Gordius* including *G. aquaticus*, *Gordius villoti* Rosa, 1882, *Gordius dimorphus* Poinar, 1991 and an unidentified species of *Gordius* all indicate that larvae of these species have a single terminal spine on the postseptum and the postseptum is approximately three times as long as the preseptum (Adrianov *et al.* 1998; Valvassori *et al.* 1988; Schmidt-Rhaesa 1997; Marchiori *et al.* 2009). Additionally, data on *G. aquaticus* in Schmidt-Rhaesa (1997) and *G. difficilis* in Bolek & Coggins (2002) indicate that larvae of these species have an oval subdivided pseudointestine. In contrast, our observations on larvae of *Paragordius* species indicate that they are shorter, with a postseptum to preseptum ration of 1.3: 1.0, an elongate pseudointestine with a pair of anterior granules and a large posterior mass, two pairs of spines on the postseptum and very long hooks on the outer ring of the preseptum. Finally, morphological characteristics of larvae of the African and North American species of *Chordodes* are most similar to larvae of *N. occidentalis*. Both genera contained a v-shaped pseudointestine, a postseptum to preseptum ration of 1.1: 1.0, and four terminal spines on the postseptum, and agree with larval descriptions for two other species of *Chordodes* from Taiwan and Japan (Inoue 1958; Chiu *et al.* 2011) and *N. occidentalis* (Poinar & Doelman 1974).

One remarkable observation in our study suggests an evolutionary switch in the proboscis morphology and proboscis spine orientation among the three types of gordiid larvae. Our SEM observations indicate that the proboscis is dorsoventrally compressed in two genera (*Gordius* and *Paragordius*) and laterally compressed in the genus *Chordodes* and *N. occidentalis*. The genus *Gordius* contains spines on both lateral sides and the dorsal side of the proboscis, whereas the genus *Paragordius* has spines on both lateral sides and the ventral side of the proboscis. In contrast, larvae of all species of *Chordodes* and *N. occidentalis* contain spines on the dorsal, ventral and left lateral sides of the proboscis. SEM studies on larvae of four other species of gordiids exist in the literature and support our observations. As in our study, Chiu *et al.* (2011) showed that the proboscis in the Taiwanese *C. formosanus* was laterally compressed and contained spines on the dorsal, ventral and left lateral sides. In contrast, work by Adrianov *et al.* (1998), Valvassori *et al.* (1988) and Marchiori *et al.* (2009) indicates that the proboscis in larvae of an unidentified *Gordius* sp., *G. villoti* and *G. dimorphus* were dorsoventrally compressed with spines present on both lateral sides. Unfortunately, only one of those studies shows clear images of the dorsal and ventral side of the proboscis (Adrianov *et al.* 1998) and the proboscis contains spines on the left, right and dorsal sides. Because the eversible proboscis is a muscular structure covered by a thin cuticle and used for host penetration, it is unclear why or how this structure would rotate over evolutionary time among different genera of gordiids. One possible explanation for this interesting observation may be related to the three stylets forming the skeletal support structure of the proboscis. Transmission electron microscopy work on larvae of *P. varius* by Jochmann & Schmidt-Rhaesa (2007) indicates that the three stylets contain apical teeth on their distal ends. More importantly, Jochmann & Schmidt-Rhaesa (2007) showed that the three stylets were arranged on the ventral and left and right lateral sides of the proboscis. Our SEM work on the proboscis of *P. varius* and *P. obamai* indicate that in these species, spines are located on the distal end of the left, right and ventral side of the proboscis. In contrast, an *en face* drawing of a *Gordius* larva by Mühldorf (1914) indicates that the three stylets are arranged on the left, right and ventral side of the proboscis. Finally, an *en face* drawing of a larva of *N. occidentalis* by Poinar & Doelman (1974) and larval ultra-structural work on that species in Poinar (2010) indicates that the three stylets are arranged on the dorsal, ventral and left lateral sides of the proboscis. Because tissue distortion can occur during fixation of specimens for SEM, we corroborated our SEM observations by examining the orientation of the stylets in living larvae of these species using DIC microscopy. Taken together, these observations indicate that the different orientations of the proboscis we observed in the three types of gordiid larvae may be determined by the orientation of the three internal stylets. Clearly, other ultra-structural studies will have to be conducted on other genera and species of gordiids to confirm this hypothesis.

Our work indicates that morphological differences in the three types of cyst folding patterns are correlated to the three types of larval morphologies. *Paragordius* was the only genus with distinctly longer hooks on the outer ring of the larval preseptum, and these were clearly visible as large spines in the cyst stages of the two species of *Paragordius*. Additionally, larvae of *Gordius* and *Paragordius* species had a postseptum which was significantly longer than the preseptum (Table 2). As a result, larvae within cysts of both of these genera always folded twice. However, the postseptum in *Gordius* species was 2.6–3.0 times as long as the preseptum and consequently the posterior end of the postseptum always reached the posterior end of the preseptum. In contrast, the postseptum of *Paragordius* species was only 1.3 times as long as the preseptum, and as a result the posterior end of the postseptum never reached the posterior end of the preseptum. Finally, larvae of *Chordodes* species and *N. occidentalis* had a postseptum to preseptum ratio which was almost equal in length (1.1: 1.0) and consequently larvae of species in these genera only folded once.

The only other available information for a laboratory reared cyst of another gordiid species is a line drawing of a cyst of the European *G. aquaticus* (reviewed in Schmidt-Rhaesa, 1997). That description indicates that larvae of *G. aquaticus* have a postseptum to preseptum ratio of 3.0: 1.0 and, during cyst formation, larvae fold twice with the posterior end of the postseptum reaching the posterior end of the preseptum. Taken together these data suggest that larval length, postseptum to preseptum ratio, and the relative size of hooks on the outer ring of the preseptum may be useful in predicting cyst morphology among other gordiid genera and/or species. Unfortunately, few detailed larval descriptions exist for other gordiid genera. However, drawings of gordiid larvae and some measurements are available for two species in the genus *Gordionus* Müller, 1927 and a single species in the genus *Parachordodes* Camerano, 1897 (reviewed in Schmidt-Rhaesa 1997). Morphological data indicate that both of these genera have a postseptum to preseptum ratio of 1.1: 1.0 and relatively small hooks on the outer ring of the preseptum, suggesting that their cysts should be morphologically similar to cysts of *Chordodes* species and *N. occidentalis*. Recently, Poinar *et al.* (2004) described cysts putatively identified as *Parachordodes tegonotus* Poinar, Rykken & LaBonte, 2004 from naturally infected aquatic insects collected from the type locality for this species. Photomicrographs of those cysts indicate that larvae within them were folded only once and contained small and indistinct spines on their anterior ends.

While our work indicates that species identification of gordiids based on cyst stages is not always possible, it suggests that cyst morphology as well as other non-adult characteristics is conserved among some genera of gordiids (Table 2). These observations are particularly interesting when evaluated from a phylogenetic perspective. The only available phylogenetic hypothesis on the relationships of multiple genera and species of gordiids was presented by Bleidorn *et al.* (2002). Their study indicates that all species within the basal genus *Gordius* and all species within the sister genus *Paragordius* are monophyletic; whereas the more derived genus *Chordodes* is polyphyletic, and *N. occidentalis* is nested within species of *Chordodes*. Our observations on the three distinct types of cysts along with the predicted cyst morphology in the genus *Gordionus* clearly support their findings and suggest that only three distinct types of cysts occur in the Gordiida.

Our abilities to identify and predict gordiid genera and/or clades based on cyst morphology have two important implications for biodiversity studies on these worms. First, collecting and identifying cysts in aquatic snails over large geographical areas will be a useful tool for studies on the distribution and biodiversity of gordiids over large geographical regions because free-living adult gordiids are difficult to find (De Villalobos & Voglino 2000; Hanelt *et al.* 2001; Szmygiel 2012). Second, recent advances in culturing gordiids in the laboratory from field collected cysts have provided a framework for discovering new species of gordiids and completing their life cycles and these techniques will undoubtedly allow others to discover new species of gordiids from around the world (Hanelt & Janovy 2004; Hanelt *et al.* 2012; Bolek *et al.* 2013a; 2013b). We hope our study stimulates others to conduct comprehensive morphological descriptions of non-adult life stages of other genera and species of gordiids using DIC and SEM, which will aid in alleviating the current problems in our understanding of nematomorph distribution and biodiversity.

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