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Two new acoels (Acoelomorpha) of the genus *Haplogonaria* from the northwest Atlantic

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Abstract

Two previously unknown species of *Haplogonaria* (Acoela), *H. schillingi* **sp. nov.** and *H. baki* **sp. nov.**, are described from the coastline of Maine, USA. The two species are morphologically similar to each other but *H. schillingi* can be distinguished from *H. baki* by its red pigmentation, its possession of a large genital atrium that branches posteriorly to the seminal vesicle and anteriorly to the vagina, a seminal vesicle that is more ellipsoid-shaped than spherical, and a well-defined wall in the seminal bursa.

We provide a description of the new species using live observation, light microscopy of serial sagittal sections, and confocal microscopy imaging of F-actin. We compare the morphology of the new species with other members of the genus and discuss the phylogenetic position of *H. schillingi* in light of conflicting morphological and molecular data.

Key words: Meiofauna, turbellarians, Acoela, Xenacoelomorpha, interstitial

Introduction

The Acoela is a diverse group of flatworms that live in intertidal, subtidal and pelagic habitats. The majority of known acoel species are interstitial, and the many sheltered coves and bays along the coastline of the northeast United States create ideal habitats. Hooge & Tyler (2003a) described 12 species from Maine, and provided new distributional records for another six species, bringing the total number of acoels known from Maine to 28 (Tyler *et al*. 2006-2015). We herein describe two additional species from Maine, closely related species belonging to the genus *Haplogonaria*.

Materials and methods

Sampling. Sediment samples were transported to the University of Maine for extraction and observation of the animals. Specimens were extracted from sediment using magnesium-chloride anesthetization (Sterrer 1971). Live animals were viewed by light microscopy in squeeze preparations and photographed.

Histological study. Specimens were fixed in warm Stefanini's fixative (Stefanini *et al*. 1967), washed in phosphate buffer (Millonig's, 0.1 M), fixed in phosphate-buffered 1% (v/v) osmium tetroxide, dehydrated in acetone, and embedded in EMBed/Araldite epoxy resin. Dehydration was quickened by microwave radiation (Samsung oven, two 7-sec irradiations at 650 W separated by 20-sec interim, with specimen-vial on ice and with water ballast of two filled 300-ml beakers [Giberson & Demaree 1995]). Serial thick sections of 2.0 µm were made according to Smith & Tyler (1984) using a Diatome diamond knife mounted in a Butler trough (Butler 1979) and stained with toluidine blue or with hematoxylin and eosin after deresination.

Phalloidin staining. Body musculature was revealed through F-actin staining of whole mounts with fluorescently labeled phalloidin (Alexa 488; Molecular Probes, Eugene, OR) according to Hooge (2001) and viewed with Leica TCS SP2 confocal laser scanning microscope using a glow LUT (flourescence intensity represented through yellow-orange look-up table).

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Type material. Holotype and paratype material has been deposited in the American Museum of Natural History (AMNH), New York, New York, USA.

Results

Family Proporidae Graff, 1882

Genus *Haplogonaria* Dörjes, 1968

Haplogonaria schillingi sp. nov.

(Figs. 1–3)

Type material. Holotype. AMNH_IZC 249974, one set of 2-µm-thick serial sagittal sections of epoxy-embedded specimen stained with toluidine blue. Paratype. AMNH_IZC 249975, epoxy-embedded whole mount.

Type locality. Reid State Park, Maine, USA (43°47'N, 69°44'W); medium-grained subtidal sediment, June 2003.

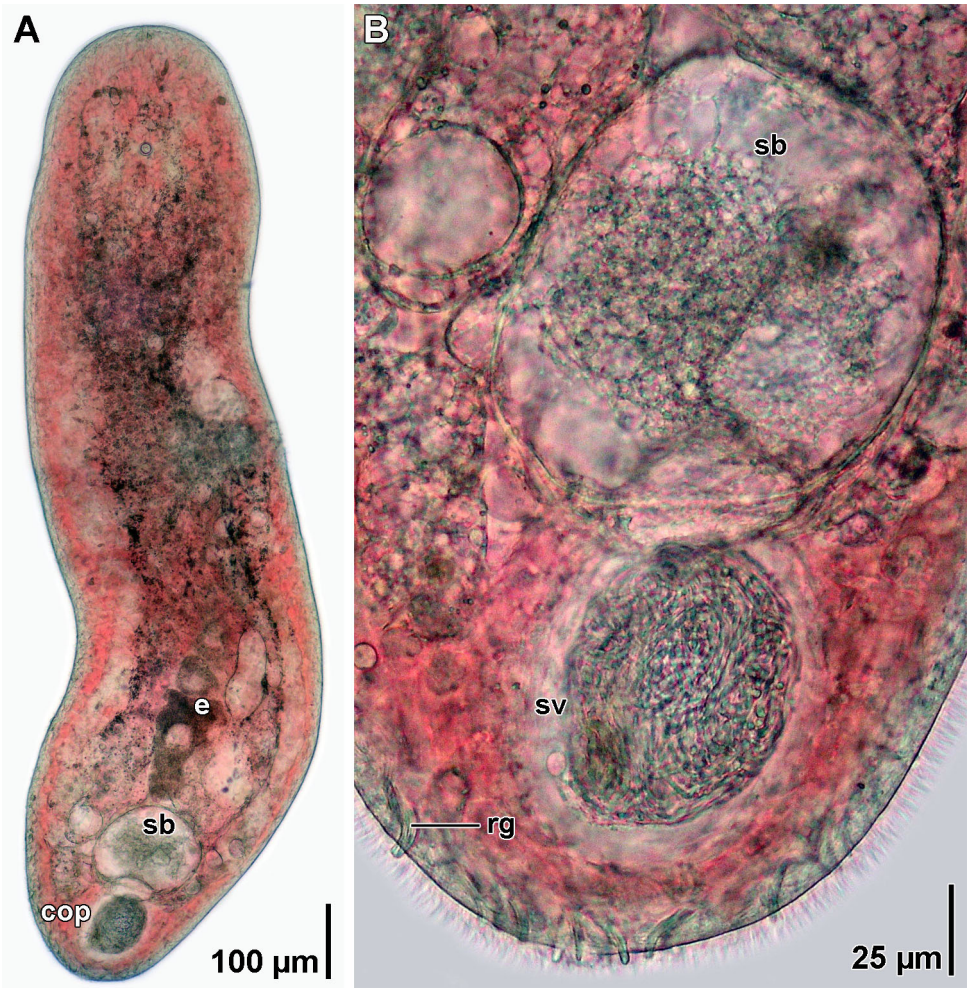

FIGURE 1. *Haplogonaria schillingi* **sp. nov.**; photomicrographs of living specimen. A. Dorsal view of lightly squeezed specimen. B. Dorsal view of posterior end of specimen. *cop* male copulatory organ, *e* egg, *rg* rhabdoid gland, *sb* seminal bursa, *sv* seminal vesicle.

Other material examined. Living specimens in squeeze preparations from Reid State Park in June 2003 and Crow Neck, Cobscook Bay, Maine, USA (44°52'34.6"N, 67°07'35.9W), from medium-grained subtidal sediment at reversing falls in June 2004; 2-µm-thick serial frontal sections of epoxy-embedded specimen stained with

toluidine blue; four specimens collected in 1995 from Reid State Park, serially sectioned at 1.5 µm, one frontally and three sagitally, and stained with hematoxylin and eosin; whole mount for fluorescence imaging of musculature.

Etymology. Species name in honor of Steve Schilling in recognition of his significant contribution to the Turbellarian Taxonomic Database (Tyler *et al*. 2006-2015).

Synonyms. *Haplogonaria* "schillingi": Jondelius *et al*. 2011; *Haplogonaria schillingi*: Jondelius *et al*. 2011; Nilsson, Wallberg & Jondelius 2011; Haplogonaria schillingi: Kånneby & Jondelius 2013.

Description. Living specimens ~1.3 mm long and 350 µm wide (Fig. 1A). Body somewhat flattened. Anterior and posterior ends rounded. Bright red pigmentation in parenchyma. Epidermis completely ciliated. Many large, scattered rhabdoid glands present (Figs. 1B, 2B). Frontal organ moderately developed and visible in sections as an ampule-like collection between frontal pore and statocyst and with its gland cell bodies positioned approximately one-third of the body-length behind frontal pore. Mouth opening on ventral surface, middle of body. Digestive central syncytium extends from posterior end of frontal glands to posterior tip of body (Fig. 2A).

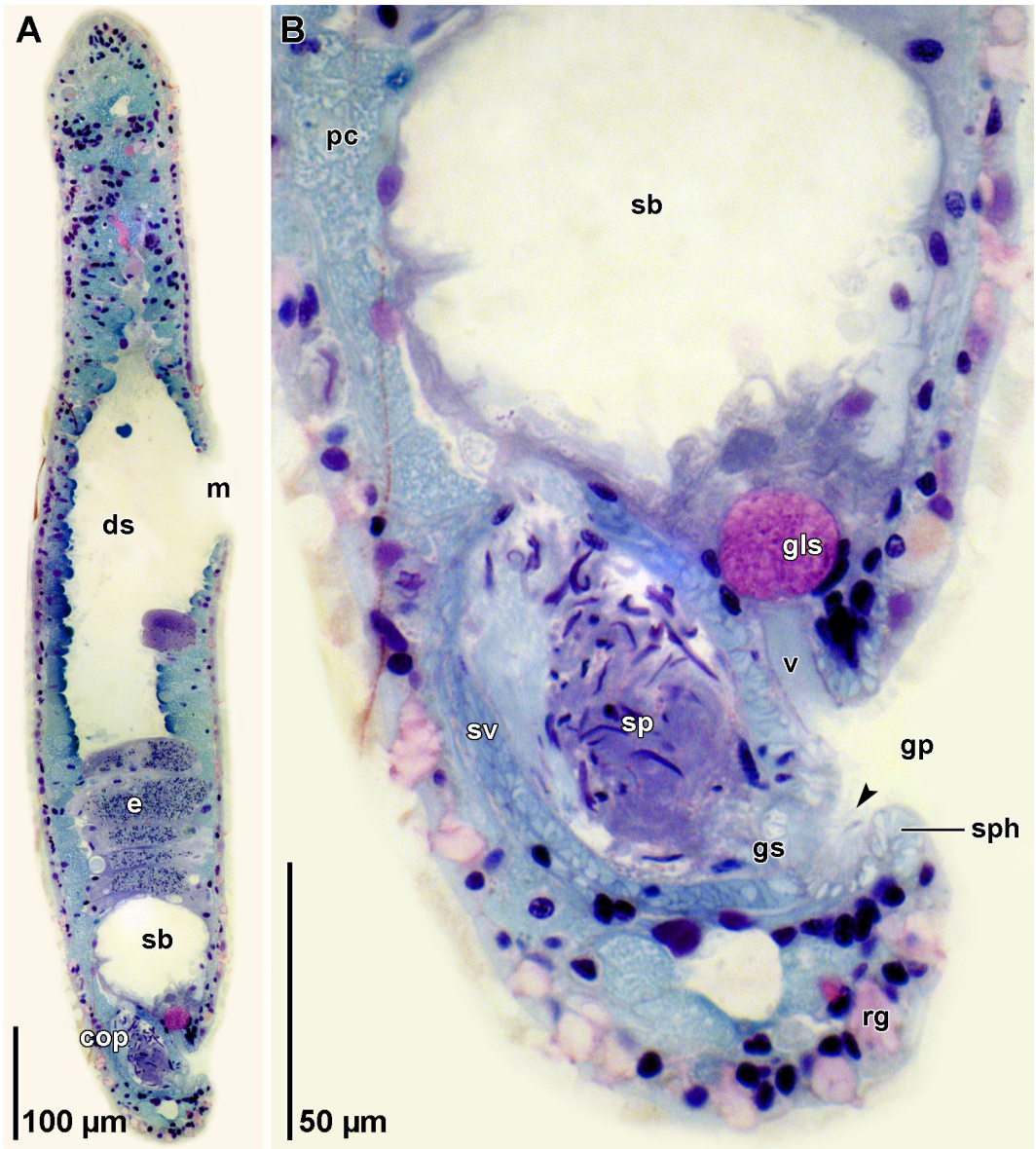

FIGURE 2. *Haplogonaria schillingi* **sp. nov.**; Sagittal histological section of holotype stained with toluidine blue. A. Whole animal. B. Copulatory organs. *cop* male copulatory organ, *ds* digestive syncytium, *e* egg, *gls* sphere of glandular secretions, *gp* gonopore, *gs* glandular secretions, *m* mouth, *pc* parenchymal cell, *rg* rhabdoid gland, *sb* seminal bursa, *sv* seminal vesicle, sp sperm, sph sphincter, *sv* seminal vesicle, *v* vagina. Arrowhead points to opening of seminal vesicle.

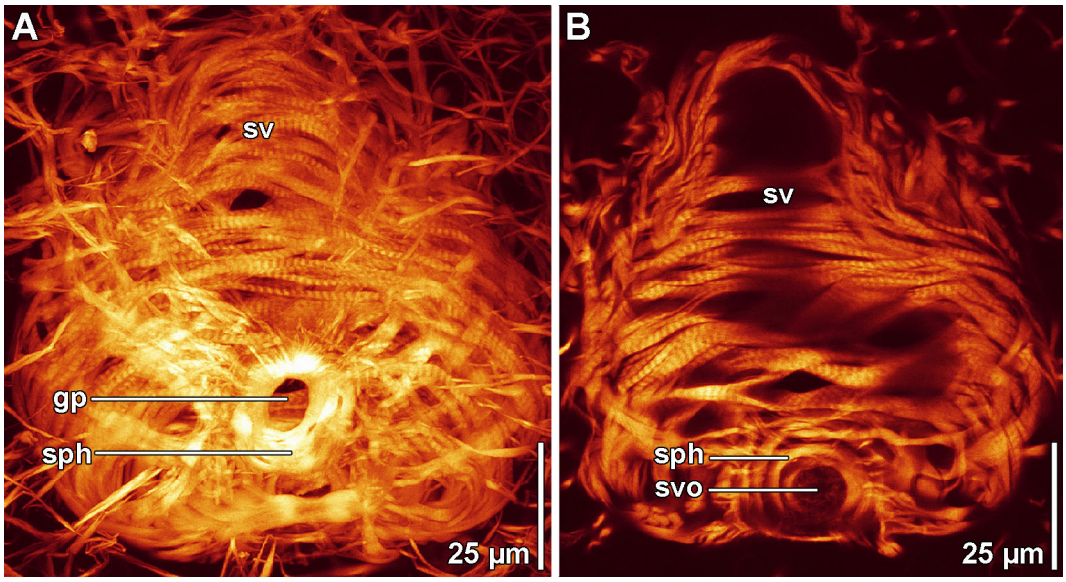

FIGURE 3. *Haplogonaria schillingi* **sp. nov.**; musculature of gonopore and male copulatory organ in whole-mount stained with Alexa-488-labeled phalloidin and viewed with confocal microscopy. A. Projection of gonopore sphincters and underlying seminal vesicle. B. Seminal vesicle. *gp* gonopore, *sph* sphincter, *sv* seminal vesicle, *svo* seminal vesicle opening.

Musculature with circular muscles that encircle the body along entire length of animal; straight longitudinal muscles present between frontal organ and anterior edge of mouth; longitudinal-cross-over muscles (fibers with a longitudinal orientation anteriorly, but bend medially to cross diagonally) present in both dorsal and ventral body wall; longitudinal muscles in anterior half of body that wrap around posterior rim of mouth (U-shaped muscles) present in ventral body wall; anterior end with ventral diagonal muscles positioned between outer circular and inner longitudinal muscles.

Ovary unpaired, ventral; extends from level of mouth posteriorly to seminal bursa (Figs. 1A, 2A). Testes paired, lateral to ovaries; extend length of body from frontal glands to male copulatory organ.

Common gonopore with thick sphincter muscles (Figs. 2B, 3A); opens to ciliated atrium with muscular walls. Vagina well-defined, tubular, and ciliated; opening from anterior side of the atrium and leading to large seminal bursa (Fig. 2B). Seminal bursa with wall that is thickened on its anteroventral side (bursal cap); sperm present in only three examined specimens (Figs. 1B, 2B); most bursae appeared empty in live animals or with a finely flocculant content in sectioned specimens. Proximal opening of vagina capped with sphere of glandular secretions that stain pink with toluidine blue (Fig. 2B) and that are cyanophilic with hematoxylin and eosin. Seminal vesicle composed of thick pseudostriated muscle fibers surrounding bundle of sperm and with strong sphincters around its opening on posterior wall of atrium (Figs. 1B, 2B, 3B). Penis absent; some glandular secretions present at seminal vesicle opening. (Fig. 2B).

Remarks. Several specimens of *H. schillingi* collected at the same time as the type material were preserved in 95% ethanol and used for DNA extraction and molecular analysis. The sequence data was included in a phylogenetic analysis of the Acoela, using sequences of the nuclear ribosomal SSU (18S), LSU (28S), and a portion of the mitochondrial cytochrome oxidase subunit I (COI) (GenBank accession numbers: FR837859, FR 837700, and FR837782) (Jondelius *et al*. 2011). Although it was not yet formally described, the species was preliminarily identified in Jondelius *et al.* (2011) as belonging to *Haplogonaria*, and was listed in their Table 1 as *Haplogonaria* "schillingi"—with the specific epithet in quotation marks to denote that it was not a valid species. However, in Figures 2, 3, 4, and 8, and Table 8, the epithet is shown without quotation marks. The analysis of Jondelius *et al*. (2011) produced gene trees in which *H. schillingi* grouped most closely with another species from the same habitat, *Pseudaphanostoma smithrii* Hooge & Tyler, 2003a in the family Isodiametridae, and away from members of Proporidae. As a result, the authors state that *H. schillingi* is to be formally transferred to the genus *Pseudaphanostoma* in the Isodiametridae.

The sequence data of *H. schillingi* was later used in analyses of gene sequences in Nilsson *et al*. (2011) and Kånneby & Jondelius (2013), but was still listed as a species of *Haplogonaria*, rather than *Pseudaphanostoma*. In these studies, the *H. schillingi* gene sequences again grouped with species of Isodiametridae rather than Proporidae.

The morphology of *H. schillingi* is incongruous with the diagnostic characters of the Isodiametridae, which includes a male copulatory organ with a muscular, isodiametric, tubular penis, with penis musculature composed of inner circular and outer non-anastomosing longitudinal fibers (Hooge & Tyler 2005). In the absence of morphological data supporting placement in the Isodiametridae, we herein persist in placing our new species in the genus *Haplogonaria* (Proporidae) and assume the molecular-sequence data may have resulted from an error in handling specimens. We hope future studies will help explain the lack of concordance between the morphological and molecular data.

Haplogonaria is likely a polyphyletic taxon, consisting of 15 species (Tyler *et al*. 2006-2015) that all possess a seminal bursa that lacks a bursal nozzle and have more or less spherical seminal vesicles (Table 1). Hooge & Eppinger (2005) recognized that some species of *Haplogonaria* have non-muscular, or weakly muscular seminal vesicles, while other species have thick musculature (Table 1). We agree with Hooge & Rocha (2006) that these differences likely indicate separate phylogenetic lineages within the genus.

H. schillingi is most morphologically similar to *Haplogonaria baki* **sp. nov.**; a comparison of these species is provided below. Among previously described species, *H. schillingi* is similar to *H. phyllospadicis* Hooge & Tyler, 2003b, and *H. sophiae* Hooge & Rocha, 2006, both of which have seminal vesicles with thick musculature, although neither are as muscular as that of *H. schillingi*. *H. schillingi* differs from these two species in several ways, including the presence of red pigmentation in the parenchyma, muscular sphincters associated with the gonopore, and a seminal vesicle that is more ellipsoid than spherical in shape (Table 1).

***Haplogonaria baki* sp. nov.**

(Figs. 4–5)

Type material. Holotype. AMNH_IZC 249976, one set of 2-µm-thick serial sagittal sections of epoxy-embedded specimen stained with toluidine blue. Paratype. AMNH_IZC 249977, epoxy-embedded whole mount.

Type locality. Bakeman Beach, Maine, USA (44°18’39”N, 68°48’11”W); medium to coarse-grained subtidal sediment.

Other material examined. Living specimens in squeeze preparations from Bakeman Beach in July 2003, Wadsworth Cove, Maine (44°24’N, 68°49’W) in April 1999, and Deer Island Point, Deer Island, New Brunswick, Canada (44°55’31.2”N, 66°59’02.3W) in June 2004; 2-µm-thick serial frontal sections of epoxy-embedded specimen stained with toluidine blue; whole mount for fluorescence imaging of musculature.

Etymology. Species name refers to the type locality.

Description. Living specimens ~600 µm long and 130 µm wide (Fig. 4A). Body somewhat flattened. Anterior and posterior ends rounded; posterior more narrow. Body without color by transmitted light. Epidermis completely ciliated. Many large, scattered rhabdoid glands present (Fig. 5B). Frontal organ poorly developed; cell bodies of frontal glands positioned approximately one-fourth body-length behind frontal pore. Mouth opening on ventral surface, anterior of body. Digestive central syncytium extends from posterior end of frontal glands to seminal vesicle (Fig. 5A).

Musculature with circular muscles that encircle the body along entire length of animal; straight longitudinal muscles present between frontal organ and anterior edge of mouth; longitudinal-cross-over muscles (fibers with a longitudinal orientation anteriorly, but bend medially to cross diagonally) present in both dorsal and ventral body wall; longitudinal muscles in anterior half of body that wrap around posterior rim of mouth (U-shaped muscles) present in ventral body wall; anterior end with ventral diagonal muscles positioned between outer circular and inner longitudinal muscles.

Ovary unpaired, ventral; extends from middle of body to seminal bursa (Figs. 4A, 5A). Testes paired, lateral to ovaries; extend length of body from frontal glands to male copulatory organ.

Common gonopore with thick sphincter muscles (Figs. 4B, 5B); opens anteriorly to tubular, ciliated vagina leading to syncytial seminal bursa, the wall of which is thickened on the anteroventral side (bursal cap). Sperm in seminal bursa scattered within syncytial tissue (Fig. 5B). Gonopore opens dorsally to spherical sperm-filled seminal vesicle composed of thick pseudostriated muscle fibers (Figs. 4C, 5B). Penis absent; some glandular secretions present at seminal vesicle opening (Fig. 5B).

Table 1. Comparison of sampling localities, body size and morphological characteristics among species of the genus *Haplogonaria*. 1 body length [µm]; 2 color; 3 rhabdoids: + present, - absent; 4 ovary: u unpaired, p paired; 5 testis: u unpaired, p paired; 6 gonopore: c common, s separate, m male only; 7 vagina: + present, - absent; 8 bursa: + present, - absent; 9 cap of cells at anterior end of bursa: + present, - absent; 10 position of vagina: - absent, a anterior to male antrum, p posterior to male antrum and extends dorsally over seminal vesicle; 11 sphincter location: - absent, g gonopore, m male antrum, v vagina; 12 seminal vesicle shape: s spherical, e ellipsoid; 13 seminal vesicle musculature: - absent, + thin, ++ thick; 14 position of the distal end of seminal vesicle relative to gonopore: m medial, a anterior, p posterior.

Species	Authority	Localities	1	2	3	4	5	6	7	8	9	10	11	12	13	14
amarilla	Hooge & Eppinger, 2005	Gulf of California	650	yellow	+	u	p	c	+	+	-	p	-	s	+	a
arenaria	(Ax, 1959)	Sea of Marmara, Black Sea	500	yellow	+	u	p	c	-	+	-	-	-	s	-	m
arenicolae	Faubel & Warwick, 2005	Isle of Scilly, UK	1200	yellow / gray	-	u	p	s	?	?	?	a	?	e	?	a
baki	This work	Maine	600	colorless	+	u	p	c	+	+	+	a	g	s	++	a
elegans	Faubel, 1976	North Sea	600–800	red / brown	-	u	p	c	+	+	-	a	m	e	-	a
glandulifera	Dörjes, 1968	North Sea	1200	colorless	+	u	p	c	+	+	-	a	-	s	+	a
idia	(Marcus, 1954)	Chile	400	colorless	+	p	p	c	-	+	-	-	-	s	-	m
macrobursalia	Dörjes, 1968	North Sea	800–1300	colorless	+	u	p	c	+	+	+	a	-	e	-	m
minima	(Westblad, 1946)	Adriatic, Gullmarfjord	400–900	yellow	+	p	p	c	+	+	-	a	-	s	+	m
pellita	(Marcus, 1951)	Brazil	200–300	colorless	+	p	p	c	+	+	-	a	-	s	?	a
phyllospadicis	Hooge & Tyler, 2003	California	450	colorless	+	u	p	s	+	+	-	a	v	s	++	p
psammalia	Faubel, 1974	North Sea	700–900	colorless	+	u	p	c	+	+	+	a	m	s	+	p
schillingi	This work	Maine	1300	red	+	u	p	c	+	+	+	a	g	e	++	a
simplex	Dörjes, 1968	North Sea, Celtic Sea	400–800	colorless	+	u	p	c	+	+	+	a	-	s	+	m
sinubursalia	Dörjes, 1968	North Sea	800–1000	brown	+	u	p	c	+	+	+	p	g	s	-	a
sophiae	Hooge & Rocha, 2006	Brazil	450–650	colorless	+	p	p	m	-	+	-	-	-	s	++	a
stradbrokensis	Hooge, 2003	Coral Sea	440	yellow	+	u	p	c	+	+	-	a	-	e	-	a

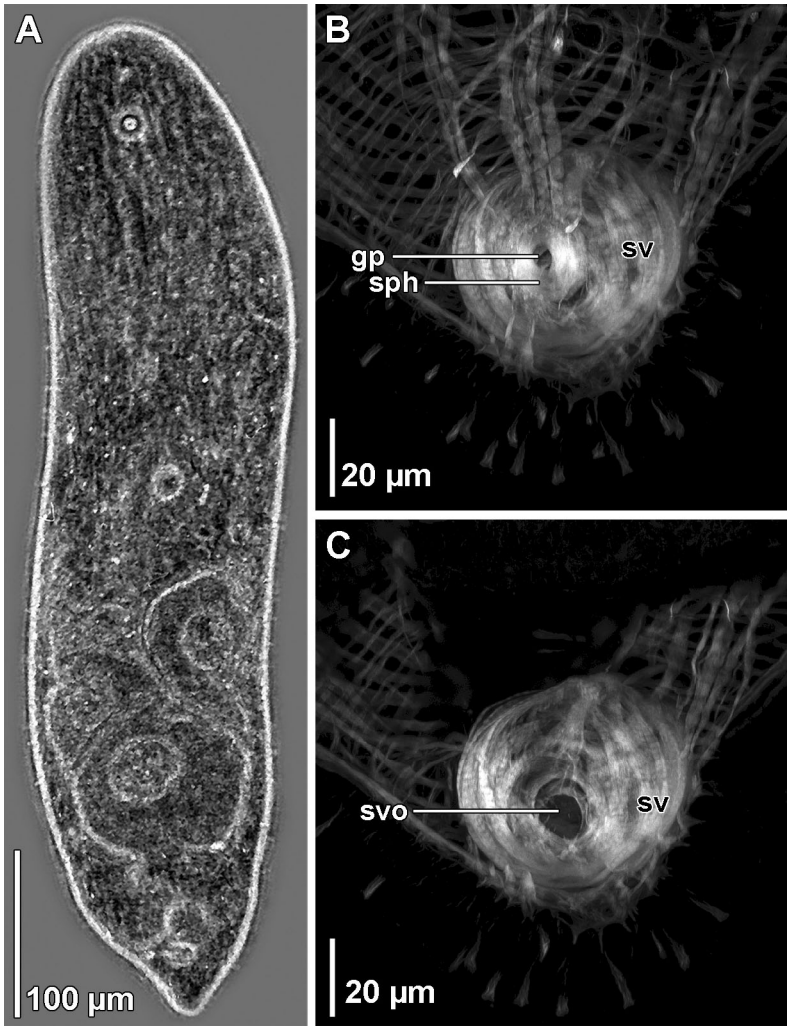

FIGURE 4. *Haplogonaria baki* **sp. nov.**; photomicrographs. A. Dorsal view of lightly squeezed living specimen. B–C. Projection of male copulatory organ in whole-mount stained with Alexa-488-labeled phalloidin and viewed with confocal microscopy. *gp* gonopore, *sph* sphincter, *sv* seminal vesicle, *svo* seminal vesicle opening.

Remarks. *Haplogonaria baki* is most similar to *H. schillingi*, which has remarkably similar internal morphology (Table 1). Nevertheless, the two are easily distinguished by *H. baki*'s smaller size and lack of red parenchymal pigmentation. The copulatory organs of these two species are similar. However, in *H. baki* the seminal vesicle opening is positioned immediately dorsal to the gonopore, whereas in *H. schillingi* it is positioned more posteriorly, allowing for the presence of a large atrium that branches posteriorly to the seminal vesicle and anteriorly to the vagina. The seminal vesicle of *H. baki* is more spherical than the ellipsoid-shaped seminal vesicle of *H. schillingi*. The bursae of these species also differ. *H. baki* appears to have a syncytial bursa in which clusters of sperm are scattered, while in *H. schillingi* the bursa is a large empty cavity with a well-defined wall, only rarely having sperm in small clusters along the ventral side. The presence of a cyanophilic/metachromatic ball of secretions between the vagina and the bursa is another useful character for distinguishing *H. schillingi* from *H. baki*.

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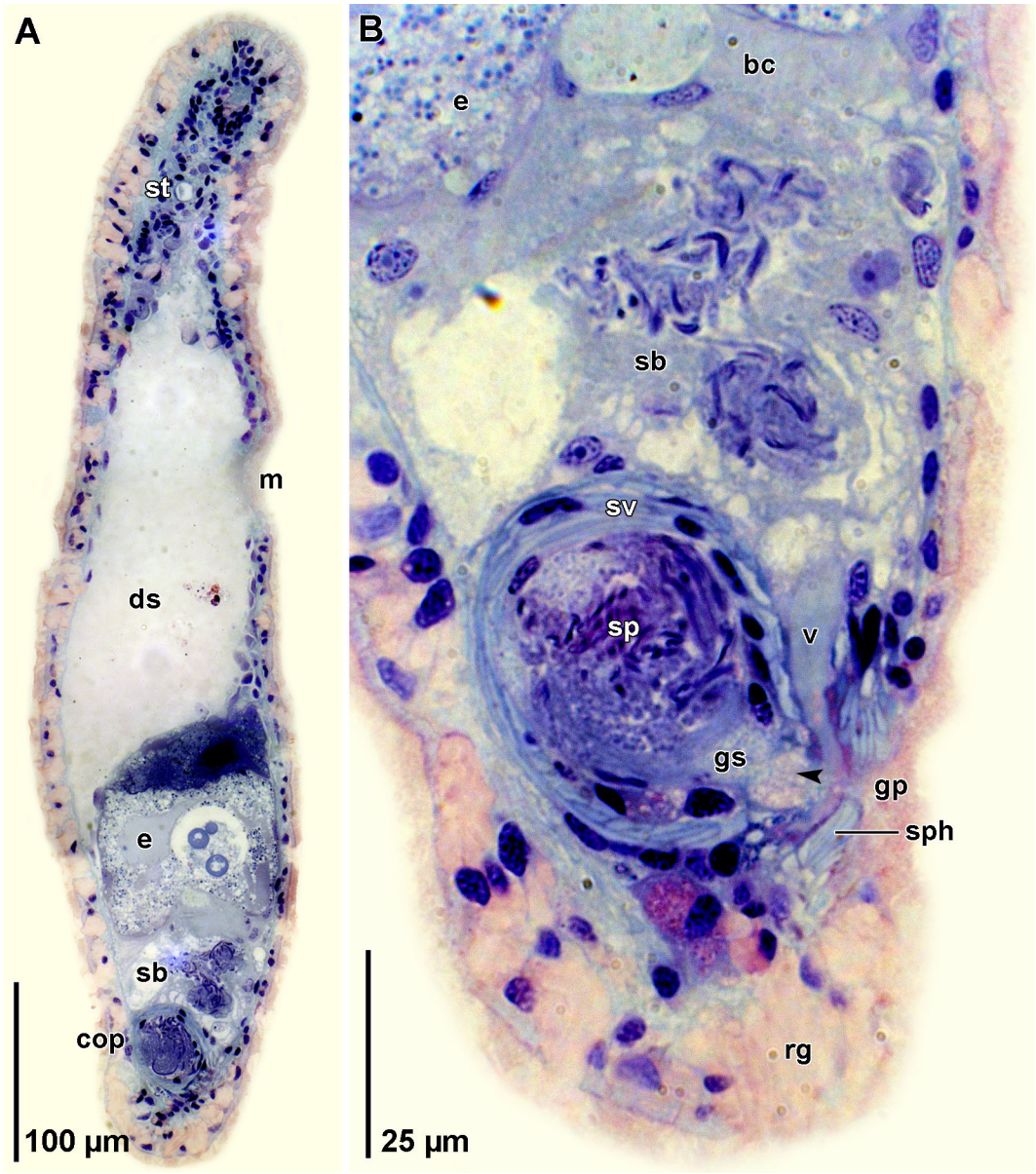

FIGURE 5. *Haplogonaria baki* **sp. nov.**; Sagittal histological section of holotype stained with toluidine blue. A. Whole animal. B. Copulatory organs. *bc*, bursal cap, *cop* male copulatory organ, *ds* digestive syncytium, *e* egg, *gp* gonopore, *gs* glandular secretions, *m* mouth, *rg* rhabdoid gland, *sb* seminal bursa, *st* statocyst, *sv* seminal vesicle, *sp* sperm, sph sphincter, *sv* seminal vesicle, *v* vagina. Arrowhead points to seminal vesicle opening.

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