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***Cicerina debrae* n. sp. (Platyhelminthes: Kalyptorhynchia, Cicerinidae) from the Southern Atlantic Coast, USA**

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Cicerina debrae is described as a new species of kalyptorhynch flatworm belonging to the Cicerinidae. This species was found in surface sediment from the lower half of the beach at two sites in North Carolina and is identical to museum material previously collected from North Carolina and from the Atlantic coast of Florida. *C. debrae* differs from its congeners in the shape of the ductus spermatici and the copulatory cirrus.

Specimens were found in surface sediment collected from the lower half of a high-energy beach near Emerald Isle (EI), North Carolina (within 10 m of N34°38'41"; W77°5'22.8"—Type Locality) and from a beach near Salter Path (SP), North Carolina (within 10m of N34°41'18.28"; W76°51'54.25") in October of 2011, in January, February, March, and October of 2012, and in May of 2013. Specimens were extracted from the sand samples, photographed and drawn, and processed for confocal laser-scanning microscopy (CLSM) and resin-embedment/serial-sectioning (Whitson *et al.* 2011; Smith & Tyler 1984). Wholemount resin slides were prepared of all meiofauna from ten lower-mid-tide level core samples (100cc each) taken in March 2012 at Emerald Isle (EI) (see "Locales" below), using a modification of the method originally described by Rieger & Ruppert (1978). Changes from their protocol comprised initial fixation and storage (refrigerated) in a variant of Trump's fixative (4% Paraformaldehyde, 1% Glutaraldehyde, 200mM Sucrose, 0.1mM CaCl₂ in 0.1M Sorensen's Phosphate buffer, pH 7.2), and secondary fixation in 1% Osmium tetroxide. DNA was separately extracted from two specimens as described in Whitson *et al.* (2011) and a portion of the 18S rDNA gene was recovered by PCR and sequenced as described in Maghsoud *et al.* (2014).

Material examined comprised: four specimens prepared for CLSM, three specimens prepared for TEM and serially-sectioned for light microscopy, one specimen observed as a whole-mount in a resin-slide preparation, and one specimen thin-sectioned for TEM examination. Additional material examined comprised seven slides of preserved, whole-mounted specimens inventoried at the Swedish Museum of Natural History, Stockholm (SMNHS), originally collected from Bogue Banks, NC, near Morehead city January 1971, and from the Atlantic coast of Florida near Marineland in January of 1971 (SMNHS).

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Authors' Contributions: KT carried out the initial confocal studies and drafted the manuscript. CS conducted a serial-section study of the copulatory bulb by TEM and also contributed LM and confocal observations to the present paper. JSIII contributed DIC micrographs, prepared the serial sections, made CLSM observations, obtained the DNA sequences, drew the figure, and made final revisions to the manuscript.

#134201-02, #134204-06 and #134197-98, respectively; leg. Tor Karling) and drawings, notes, and one whole-mount of “*Cicerina orthocirri*” collected at the site of the old Iron Steamer Pier on Bogue Banks, NC, probably in winter of 1969-1970, by Reinhard Rieger. In the description below, positions along the longitudinal body axis are expressed as percentage of the body length and given as “U-values,” where U0 is the anterior tip of the body and U100 is the posterior end (Rieger & Sterrer 1968).

The specific epithet is in honor of the first author's mother. This species is listed in the unpublished notes by Rieger (see below) as both “CinciAugi” and “*Cicerina orthocirri*”. It is identical with *Cicerina* “*debrae*” in Maghsoud *et al.* (2014). Type material was deposited at the Smithsonian National Museum of Natural History (NMNH) and the SMNHS as follows: Holotype—a sagittally sectioned specimen from EI (NMNH XXXXXXXX); Paratypes: One whole-mount (NMNH XXXXXXXX) and two serially-sectioned specimens from EI (NMNH XXXXXXXX, SMNHS XXXXXXXX), and two whole-mounted specimens from SMNHS (XXXXXX, XXXXXX).

In transmitted light, the free-swimming animal was faintly golden-brown and the body was slightly flattened, most obviously at the posterior end. Proboscis, eyes, pharynx, and posterior adhesive papillae were easily seen. Specimens were threadlike, measuring approximately 1 mm long 75 μ m wide when fully extended. This species was present year-round, but generally more abundant in our samples during fall, winter, and early spring.

Cicerina debrae possessed intraepithelial nuclei throughout. CLSM labeling with anti-acetylated tubulin revealed that epidermal ciliation was restricted to the dorsal and ventral surfaces of the body except at the anterior end; the sides of the body exhibited only scattered (sensory?) cilia from U20 posteriorly, and the dorsal ciliation did not extend as far posteriorly as the ventral ciliation (U76 vs. U83). The 98 μ m-long proboscis possessed a small terminal cone and four prominent secretory ampules; the proboscis sheath was not ciliated. Prominent adhesive papillae occurred at the posterior end and in four somewhat disordered belts—between the proboscis and eyes at U15, posterior to the pharynx at U42, and anterior and posterior to the copulatory organs at U66 and U85. A pair of large eyes with prominent retinal bulbs occurred at U16 (Fig A).

The pharynx was vertically directed and located shortly behind the brain. The pharynx contained mostly intrapharyngeal glands of two types—with either eosinophilic or basophilic secretions. The mouth occurred near the anterior extent of the pharynx, at U27. Identifiable contents of the digestive tract in various specimens were nematodes, and in one case, including pair of male copulatory spicules and accompanying gubernacula. On three occasions, we saw an individual capture and swallow a nematode in the extraction dish; that this species preys (perhaps not exclusively) on nematodes is also supported by molecular data (Maghsoud *et al.* 2014).

Although the testes are paired, and appear to occur in four lobes (Fig A), the position of the testes is clearly variable. In living specimens, paired testes were observed mostly posterior to the pharynx; however, a central mass of testicular tissue was found anterior to the pharynx in sections, and paired testes anterior to the pharynx were figured in an unpublished

drawing of this species by R. M. Rieger. Paired vasa deferentia extended posteriorly from the testes to paired seminal vesicles located at U63. A short duct exited each seminal vesicle and extended posteriorly to enter the copulatory bulb at its anterior face, located at U67 (Fig A). The 66 μm -long, ovoid copulatory bulb was bipartite (Fig B), with a proximal glandular portion that also contained an internal ductus ejaculatorius (Fig C). The distal portion of the copulatory bulb contained a short (28 μm) cirrus bearing a proximal wreath of small granular glands (Figs D,E), and a single large matrix cell (Fig B). The glands associated with the male organ appeared to be exclusively intra-capsular. The copulatory bulb opened into a common genital atrium that arose from the common gonopore at U75.

An internal vagina extended anteriorly from the genital atrium ventrally and slightly to the left of the male copulatory organ. Initially spacious, the vagina narrowed to a sphincter just anterior to the copulatory bulb (Fig C), and opened into a mass of bursal tissue that extended in the midline from just anterior to the copulatory bulb to just posterior to the germaria. A pair of C-shaped ductus spermatici (37 μm long) connected the bursa to near the paired germaria at U61 (Fig B); the ductus spermatici did not stain with Alexa488/phalloidin, but stained intensely in serial sections with iron hematoxylin. Vitellaria extended from just ventral to the testes lateral to the gut as far posterior as the germaria. The common duct originated in the vicinity of the germaria and extended postero-dorsally, passing dorsal to the copulatory bulb to enter the genital atrium. A group of glands occurred posterior to the genital atrium. Their connections to the atrium could not be traced; however, granules like those in the glands occurred in the epithelium of the genital atrium where the common duct entered.

A 1696-base sequence from the 18S rDNA gene was obtained from a specimen collected in the lower half of the beach at EI in January 2012 (GenBank accession number KJ206554). A blastn search returned the sequence from *Linguabana tulai* Rundell & Leander 2014 (JN205121) at e^0 and 96% identity. Blasting the sequence for *C. debrae* against the unpublished sequence for *Cicerina tetradactyla* (Tessens *et al.* submitted) resulted in e^0 and 97% identity.

The most recent key to the Family Cicerinidae (Brunet 1973) places *Cicerina debrae* in the genus *Cicerina*. Compared to the 7 presently described congeners, *C. debrae* shares with *C. eucentrota* the character of a vagina interna (Brunet, 1973); the remaining species have an external vagina, opening anterior to the common gonopore and leading into the bursa. The position of the testes is variable in this genus, being pre-pharyngeal in *C. bicirrata*, *C. triangularis* (Karling 1989), and *C. elegans* (Evdonin 1971) and post-pharyngeal in *C. brevicirrus*, *C. remanei*, *C. tetradactyla* (Karling 1952), and *C. eucentrota* (Ax 1959). *C. debrae* is, however, clearly distinguished from the other 7 congeners by its very short cirrus and by the elongate and curved ductus spermatici.

The traditional phylogenetic placement of the family Cicerinidae within the Eukalyptorhynchia, already cast in doubt (see Van Steenkiste *et al.* 2008), is also not supported by molecular data which show the majority of cicerinid species forming a clade at the base of the Schizorhynchia (Tessens *et al.* submitted). Our own results based on 18S sequence from this new species agree with theirs (Smith *et al.* 2013). Accordingly, a

comprehensive revision of the Cicerinidae, including both molecular and morphological data, is certainly to be desired.

Our results once again demonstrate the utility of confocal microscopy in routine species descriptions: beyond the ease with which CLSM allows three-dimensional reconstruction of major organ systems, it seems likely that the unusual pattern of epidermal ciliation could easily have been missed in live observations. We also emphasize the utility of the resin-slide procedure (Rieger & Ruppert, 1978). Our modifications of the original procedure (primary fixation in modified Trump's and decreased osmium tetroxide concentration in the second fixative) appear to be useful improvements, as the former allows short- to long-term sample storage in primary fixative, and the combination of these two modifications prevents specimens from being so overstained by osmium tetroxide that internal structures cannot be clearly seen. Finally, we make a plea to current workers on flatworm systematics for more wide-spread use of resin-embedment serial sectioning and a corresponding move away from whole-mounts as the sole type material for new species. As originally pointed out (Smith & Tyler 1984), the anatomical detail obtained by this technique is far superior to that obtained in ordinary paraffin sections.

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Figure 1. *Cicerina debrae* n. sp

Anterior end is to the left in Fig C and to the top in the remaining figures. **A.** General anatomy; arrows, adhesive belts; br, brain; cb, copulatory bulb; dsp, ductus spermaticus; ge, germarium; go, common gonopore; ph, pharynx; sv, seminal vesicle; t, testes; v, vitellaria.

B. Bright-field micrograph of a specimen from a resin-slide preparation, showing genital region with paired germaria, ducti spermatici with flanges where they enter the bursal tissue (bu), proximal prostatic region of copulatory bulb (pr), and distal portion of copulatory bulb with cirrus (ci) and matrix-cell nucleus (arrow). **C.** CLSM stack (Alexa488/phalloidin—

green; Hoechst 33342—blue) of genital region, lateral view showing seminal vesicle, proximal region of copulatory bulb with invaginated internal ductus ejaculatorius (de), distal (di) region of copulatory bulb with separate muscle layer, and vagina interna (vi) ending in a sphincter (sph) at the bursal tissue. **D, E.** Brightfield micrographs of same specimen as B, more-dorsal and more-ventral (respectively) focal planes of the cirrus, showing arrangement of spines, and hemi-circular wreath of gland cells (gl) located at the base of the cirrus.