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## Two ‘new’ renicolid trematodes (Trematoda: Digenea: Renicolidae) from the California horn snail, *Cerithidea californica* (Haldeman, 1840) (Gastropoda: Potamididae)

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### Abstract

This manuscript describes the daughter parthenitae (sporocysts) and cercariae of two species of renicolid xiphidiocercaria that infect the California horn snail, *Cerithidea californica*, which serves as first intermediate host for a diverse and ecologically important guild of digenean trematode parasitic castrators. The two species described here have previously been considered to be a single morphospecies in ecological and evolutionary research. We provide provisional species names to respect that digenean alpha taxonomy is currently focused on sexual (adult) stages, while simultaneously respecting the spirit and utility of formal nomenclature in providing unambiguously unique, species-level names that also clarify to the extent possible species' taxonomic affiliations. The first species, *Renicola* sp. “polychaetophila” is most readily distinguishable from previously described renicolid xiphidiocercariae by a combination of (1) having a penetration gland duct arrangement of  $2[(1+3+1)+1]$ , (2) having one pair of penetration glands positioned anteriorly to the main gland cluster, (3) lacking tegmental spines, and (4) infecting *Cerithidea californica*. The second species, *Renicola* sp. “martini”, is most readily distinguishable from other renicolid xiphidiocercariae that also have tegmental spines by a combination of (1) having a simple, bullet-shaped oral stylet sclerotized for 50–80% of its length, (2) having a cystogenous-gland field with an anterior-most extent about half way between the oral and ventral suckers, and (3) in infecting *Cerithidea californica*. Phylogenetic analyses using DNA (COI and ITS1) sequence data support that these two trematodes represent distinct species of *Renicola*. We also (1) provide an emended diagnosis for renicolid cercariae, (2) highlight a few morphological characters that may be useful for future taxonomic work involving renicolid xiphidiocercariae, and (3) suggest that future descriptive work involving trematode parthenitae include more information pertaining to the group of parthenitae as a whole.

**Key words:** parthenitae, colony, first intermediate host, cercariae, parasites, parasitic castrators, *Renicola*, estuary

### Introduction

Dr. Walter Martin appears to have never seen the two renicolid xiphidiocercaria species described in this manuscript. This is noteworthy because, for over two decades, Martin studied the digenean trematodes that infect the California horn snail, *Cerithidea californica* (Haldeman, 1940) (e.g., see Martin 1950;1955;1972). This snail serves as first intermediate host for a diverse and ecologically important guild of trematode parasitic castrators (e.g., see Kuris *et al.* 2008; Martin 1972) and resides in coastal estuaries from central California (USA) to Peru (Keen 1971; Miura *et al.* 2010). Martin's studies took place in several localities in southern California, and he created an identification key for these trematodes (Martin 1972). The key included species that he had described, and those that he had encountered but not thoroughly described. However, the key did not include any renicolid xiphidiocercariae. This is odd, as researchers, both before and after that time, have regularly encountered renicolid xiphidiocercariae from the California horn snail. For instance, in an unpublished thesis, Hunter (1942), also working in southern California, included what appear to be two renicolid xiphidiocercariae. More recently, ecological and evolutionary research involving this trematode guild has included what was considered to be a

single, undescribed renicolid xiphidiocercaria (for example, “REN2” in Sousa (1993), and “Large Xiphidiocercaria” in Hechinger *et al.* (2007)). A few years ago, while carefully examining the morphology of this “Large Xiphidiocercaria,” it became clear that it represented not one, but two species. Hence, despite examining thousands of infections, Martin’s studies missed two species of renicolid xiphidiocercaria, while more recent researchers, including the authors of this manuscript, have likely encountered both species, but mistakenly pooled them into one.

This manuscript presents descriptions of the daughter parthenitae and cercariae of these two renicolid species, including analysis of DNA sequence data. The two species are readily distinguishable genetically and morphologically. We also highlight a few morphological traits that may inform future taxonomic work focused on this group of cercariae. Further, we include more information than is typical concerning the entire group of sporocysts that comprise an infection. This is particularly warranted given the recent recognition that parthenitae in first intermediate hosts can be understood as comprising a colony or society—given that they represent groups of individuals that cooperatively live together to reproduce and operate the castrated host phenotype (see Hechinger *et al.* 2011b).

## Material and methods

Material for these descriptions came from Carpinteria Salt Marsh, California. Horn snails were hand-collected (usually by technicians or students) from intertidal pans, intertidal “back flats”, and tidal creeks. Fully developed cercariae were shed from infected snails by placing snails in individual seawater containers placed under a warm light. Often, snails were individually marked on the shell and maintained at the laboratory or in field enclosures (sometimes for over a year) to permit additional shedding of cercariae before being dissected to examine parthenitae.

Morphological and behavioral descriptions are based on both live and fixed cercariae shed from snails, and on sporocysts gathered from dissected snails, using stereomicroscopes and standard bright-field and phase-contrast microscopy up to 1000x magnification. We sought to obtain measurements from ten individual cercariae or sporocysts from each of 4–5 infections for each species (a target *n* of 40–50). Most morphometrics were taken using a compound microscope ocular micrometer on cercariae and sporocysts that were fixed in hot 10% formalin, stained with acetocarmine (cercariae only), and temporarily mounted in glycerin. Cercaria bodies and tails typically fixed with dorso-ventrally flexion, so body-length measurements were taken in several straight sections along the lateral view. In addition to standard length and width measurements, we also provide dorso-ventral heights for several attributes to better indicate body shape. Measurements for oral stylets and sporocyst cercaria counts were taken from additional fresh material, as these traits were not readily observable in the fixed, non-flattened material. Material has been deposited at the United States National Parasitology Collection.

We note that the cercaria flame cells for these two species are very difficult to discern, largely due to the obscuring cystogenous glands (as is typical for renicolid xiphidiocercariae (e.g., see Cable 1956)). Further, we obtained no increase in visibility after immersing cercariae in various solutions (seawater at various dilutions down to fresh water, or urine at several concentrations). Hence, the entire set of flame cells was rarely observed in a single specimen.

Prevalence information is not available for the material focused on in the descriptions, but we provide information from a subsequent dissecting of 5,392 snails dissected from the same locality.

For DNA sequence analyses, we used three infections of one species and eight infections of the other. Fresh sporocyst or cercaria tissue was fixed and stored in 70% EtOH. DNA was extracted using a CTAB method following Miura *et al.* (2005). Polymerase chain reaction (PCR) was used to amplify the mitochondrial cytochrome c oxidase subunit 1 gene (COI) and the nuclear internal transcribed spacer 1 gene (ITS1). For primers, we used JB3 (Bowles & McManus 1993) and COI-R trema (Miura *et al.* 2005) for COI, and ITS1tre-R (5'-AATTCACACAGTTGGCTGCRC-3', developed in this study) and BD (Bowles & McManus 1993) for ITS1. For COI, PCRs were run for 35 cycles under the following conditions: denaturing at 94 °C for 60 s, annealing at 45 °C for 60 s, and extension at 72 °C for 90 s. The 35 cycles were preceded by an initial denaturing at 94 °C for 60 s followed by a final extension at 72 °C for 7 minutes. For ITS1, the annealing temperature was 65 °C and other PCR conditions were the same as for COI. PCR products were separated by electrophoresis on a 1% low melting temperature agarose gel. The DNA fragments were excised from the gel and DNA was extracted from the agarose

using  $\beta$ -agarase. We sequenced both strands of the DNA fragments using an automated sequencer (ABI 3130xl). COI and ITS1 sequences have been deposited at GenBank.

The sequence alignment was obtained separately for each gene using ClustalW (Thompson *et al.* 1994), implemented in Geneious v. 6.1.6 (created by Biomatters, <http://www.geneious.com>). To examine phylogenetic relationships using the COI gene, we included sequences of an additional renicolid xiphidiocercaria species from New Zealand, *Renicola* sp. (sensu Martorelli *et al.* (2008)), available on GenBank (Leung *et al.* 2009a), and sequences for two non-styleted renicolid species that also infect *Cerithidea californica*, *Renicola buchmanii* (Martin & Gregory, 1951) and *Renicola cerithidicola* Martin, 1971 (Miura *et al.* unpublished). We used sequences from two microphallid species that were also available on GenBank (Keeney *et al.* 2009; Leung *et al.* 2009a), to comprise the outgroup, based on the higher taxonomy of the Digenea (Olson *et al.* 2003). All insertions and deletions (indels) were removed from the alignment. Phylogenetic trees for each gene were constructed using Maximum likelihood algorithms. We used Bayesian Information Criterion (BIC) to select the best evolutionary model using MEGA5 (Tamura *et al.* 2011); the HKY + G + I model was selected for COI and K2P model was selected for ITS1. The Maximum likelihood analyses were conducted by MEGA5 using an automatically generated initial tree and NNI heuristic search. Node robustness was assessed using bootstrapping with 1000 replicates.

There are several ways workers have named trematode parthenitae and cercariae (larvae). Although the International Code of Zoological Nomenclature (ICZN, 4<sup>th</sup> Edition) permits naming species based on any life stage (e.g., ICZN Art. 23.3.2 and 72.5), the formal nomenclature of digenean alpha taxonomy is typically based on sexual (adult) stages (e.g., see Bray *et al.* 2008; Gibson *et al.* 2002; Jones *et al.* 2005). Known adult stages are not available for the larval stages described herein. In such cases, one tradition has been to provide formal names using the collective-group name of *Cercaria* as genus (e.g., *Cercaria opaca* Holliman, 1961 and *Cercaria roscovita* Stunkard, 1932). Such species names have formal nomenclatural validity, and should be associated with types. However, there appears to be reluctance among many workers to formally name digeneans based on larval stages, presumably because of potential downstream issues concerning types. Hence, another naming tradition has been to provide provisional species names (which are not regulated by the ICZN). A common way to construct provisional names is to use *Cercaria* followed by a pseudo-specific epithet and a number (e.g., *Cercaria caribbea* XXXIII of Cable (1956) and *Cercaria queenslandae* III of Cannon (1976)). A problem with using *Cercaria*, formally or provisionally, is that it conveys no taxonomic information between the levels of order to genus, yet knowledge about trematodes has progressed such that this information is often available. This is a further problem in formal names, as collective-group names, as indicated in the ICZN glossary, are to be used when one cannot place specimens into a nominal genus. To avoid problems associated with using *Cercaria* as a genus, some workers have provided provisional names that indicate the lowest known taxonomic unit for the described stages (e.g., *Galactosomum* sp. and *Renicola* sp. of Martorelli *et al.* (2008), and *Philophthalmid* sp. I and *Renicola* sp. I of Hechinger (2007)). This type of naming scheme solves the problems of using *Cercaria*, but has another problem in that the names, by themselves, are not unambiguously unique (e.g., there may be several, different *Renicola* sp. I). Being unambiguously unique is a major goal of formal nomenclature (see the Preamble, Introduction, and General Recommendation 1 of the ICZN), and there have been recent calls to adopt this attribute in provisional nomenclature (e.g., see Schindel and Miller 2010).

Therefore, although perhaps not the final or only solution to the issues concerning naming digenean larval stages, the schema used in this manuscript is to provide provisional names comprised of the genus (which is known) followed by “sp.” and a potential specific epithet, which is in quotes and upright font to clarify that the name does not represent a formal binomial. This naming procedure has the advantage of respecting the current practice of digenean alpha taxonomy being based on adult stages, while simultaneously respecting the spirit and utility of formal nomenclature in providing unambiguously unique, species-level names that also clarify to the extent possible the species’ taxonomic affiliations.

## Results

### Diagnosis for renicolid cercariae (emended from Cable (1963))

Distomate cercariae developing in simple sporocysts that reside in the visceral mass or the mantle of “prosobranch”

gastropods (including at least caenogastropodans and patellogastropodans). Tail variable, with or without fins, leptocercous when stylet present, leptocercous, magnacercous, or zygotercous when stylet absent. Tegument of body with or without fine spines, ventral sucker with or without spines, eyespots absent, prepharynx very short or absent, pharynx and ventral sucker sometimes reduced. Cystogenous glands abundant, conspicuous, largely concealing numerous penetration gland bodies that open at pores anterior or posterior to mouth. Excretory system mesostomate; bladder Y-shaped, epithelial, from simple with long stem and short arms partly embracing ventral sucker, to complex V- or Y-shaped, with numerous diverticula of stem and long arms that extend to sides of oral sucker. Main excretory tubules join bladder near base of arms, receive anterior and posterior collecting tubules without extending into forebody. Each collecting tubule serves 3 groups of 5 or 6 flame cells each in species with tail fins, and 3 flame cells each in species lacking fins, so that the excretory formula is  $2[(n+n+n)+(n+n+n)]=2 \times 6n$  flame cells. Species with stylets encyst in mollusks or polychaetes; species without stylets encyst in fishes. All species sexually mature in kidneys and ureters of aquatic or marine birds.

## Descriptions

### *Renicola* sp. “polychaetophila”

(Figs. 1–3)

**Diagnosis:** Distome, leptocercous xiphidiocercariae with penetration gland ducts opening in a  $2[(1+3+1)+1]$  arrangement and no tegmental spines. Cercariae produced by daughter sporocysts comprising a colony that resides in the gonadal and digestive gland regions of the first intermediate host.

**Host:** *Cerithidea californica* (Haldeman, 1840)

**Location in host (daughter sporocysts):** gonadal and digestive gland regions

**Prevalence:** 0.7% in 5392 snails dissected in 2012

**Locality:** Carpinteria Salt Marsh, Santa Barbara County, California, USA (34.4°N, 119.54°W)

**Habitat:** Estuaries (intertidal flats, pans, channels)

**Dates of collection:** May–October 2009

**Deposited Material:** Cercariae and sporocysts from 5 infections (USNPC nos. 107294–107306).

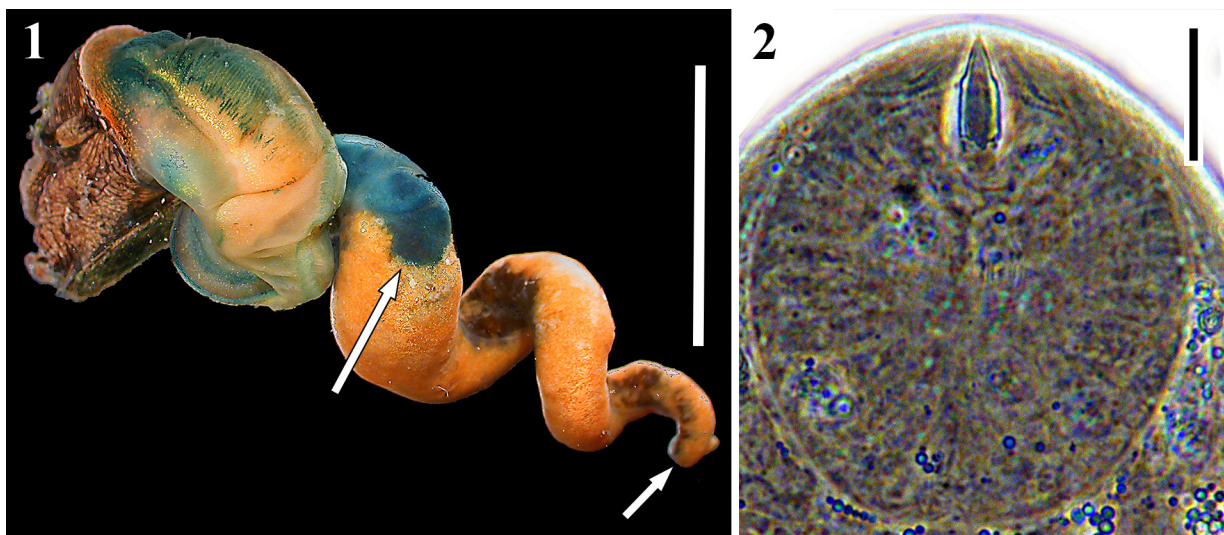
**GenBank accession numbers:** CO1 KF512550–KF512552; ITS1 KF512569–KF512571.

**Description:** Daughter sporocysts. Body immobile, shape variable, elongate to pyriform to spheroidal, sometimes pinched at one end to form a nipple or neck that appears to mark the birth pore. Tegument translucent, typically covered with thicker paletot that gives sporocysts lemon-cream to orangish appearance and causes them to stick together, and lined on interior by tissue that likely represents germinal cells. Ripe sporocysts with cercariae in various stages of development; germinal balls to developed cercariae distributed in sporocyst with no clear spatial polarity. Developed cercariae impart a whitish coloration to sporocysts under reflected light that mottles with the orange and clear tegument color. Distribution of sporocysts includes gonadal space with some infiltration of digestive gland space. Sporocysts exhibit seasonal variation, wherein sporocysts in developed infections physically regress in winter, becoming thinner-bodied, somewhat flaccid, more orangish, with zero to few developed cercariae; sporocysts ripen up again during spring, becoming more plump, filled with more developed and developing cercariae, and collectively form a more compact mass. Morphometrics and meristics are presented in Table 1 and are based on 40 individuals from four infections, while cercaria counts are of the well-developed cercariae from 31 sporocysts from four infections.

Cercariae. Distome, leptocercous xiphidiocercaria. Body elliptic-elongate-ovate in dorsal view, dorso-ventrally flattened, maximal width and dorso-ventral height usually just anterior to ventral sucker; with refringent, varying sized spherules scattered throughout most of interior. Cystogenous glands fill posterior  $\sim 3/4$  of body, densely packed, filled with refringent granules, reflect white light, obscure fine internal anatomy. Oral sucker well-developed, round, large, equal-subequal in size with ventral sucker; with small stylet; stylet embedded horizontally at anterior oral sucker, dorsal to mouth, small, usually appears less than  $1/4$  length of oral sucker, sclerotized for 70–90% of its length, bullet-shaped, tapering anteriorly for about  $1/2$  sclerotized length, difficult to carefully observe unless cercaria is flattened. Mouth subterminal; prepharynx absent; pharynx small, ovoid-spheroid, abutting posterior margin of oral sucker; esophagus thin and thin-walled, branching into two ceca approximately midway

between oral and ventral suckers near anterior margin of cystogenous gland field; ceca thin and thin-walled, posterior-most extent obscured by cystogenous glands. Ventral sucker positioned at mid-body, well developed, round, protrudes from ventral surface, lacking spines. Tegument thin, apparently with no spines on body or tail (there are potentially extremely minute spines embedded within the tegument); with few, thin sensillae > 10 long observed laterally and dorsally, not counted. Penetration gland bodies extremely difficult to observe given obscuring cystogenous glands, but are primarily clustered ~2/5 into body from anterior edge, anterior extent of cluster close to anterior extent of cystogenous gland field; appear to be five pairs (matching duct number) of unknown number of types. Penetration gland ducts pass anteriorly from glands in bundles of five that pass around lateral margins of oral sucker; each bundle diverges into a 1+3+1 arrangement at lateral and anterolateral edge of oral sucker; one pair exits body slightly dorsal, one pair slightly ventral, and three middle pairs empty just lateroventral to oral stylet tip (the 5 ducts rarely distinguishable from each other before diverge into groups). Additional pair of probable penetration glands present ~1/5 into body from anterior edge, anterior to and more lateral than main penetration gland cluster; each with a single, usually distinct “cross duct” extending medially and ventrally from gland body that opens on ventral body surface. Three pairs of lateral gland ducts of unknown type opening on lateral body margins just posterior to ventral sucker (third frequently not visible); gland bodies not reliably observed. Two pairs of posterior gland ducts of unknown type openings at posterior body margin, one more dorsal to the other (one frequently not readily visible); gland bodies not reliably observed. Excretory bladder prominent, Y-shaped; stem longer than arms; arms embracing posterior half or more of ventral sucker margin; excretory opening at posterior edge of bladder stem. Flame cells in a 2 [(3+3+3) + (3+3+3)] = 36 bilateral arrangement, posterior four groups usually partially obscured by cystogenous glands. Other details of collecting ducts not observed. Cerebral ganglion just posterior of, and extending laterally of, pharynx. Genital primordium a compact mass of cells, adjacent to dorso-anterior margin of ventral sucker, very evident upon acetocarmine staining. Tail attached terminally at posterior body margin in socket, simple, unadorned, slender, cylindrical in cross-section, gradually tapering, can extend and contract, approximately equal in length to body when extended, with finely annulated tegument overlying muscle layers, with scattered, refringent spherules along mid-line. Morphometrics are presented in Table 1; most are based on 40 individuals from four infections, while stylet measurements come from 14–18 individuals from three to four infections.

**Behavior:** Cercariae swim by widening and flattening the mid- and posterior-body, flexing posterior body ventrally to partly overlap ventral sucker, often also slightly flexing or protruding anterior body, and lashing the tail left to right to move in a general anterior direction. Cercariae do not clearly respond to light.



**FIGURES 1–2.** *Renicola* sp. “polychaetophila”. **1.** A fresh, deshelled first intermediate host snail (*Cerithidea californica*) infected with daughter sporocysts. The large orangish mass in the snail visceral mass is the sporocyst colony. The colony takes up the entirety of the snail gonadal space and has also infiltrated some of the digestive gland. Large arrow marks the basal-most extent of the colony, which extends to the distal tip of the visceral mass (small arrow). Scale bar = 10 mm. **2.** Phase contrast view of anterior end of a flattened, live cercaria, highlighting the sclerotized portion of the oral stylet. The apparent lateral bulges of the stylet are not consistently observed among individual cercariae. Scale bar = 10  $\mu$ m.

**TABLE 1.** Morphometrics and meristics for daughter sporocysts and cercariae of the two renicolid trematodes *Renicola* sp. “polychaetophila” and *Renicola* sp. “martini”. All values represent the range followed by mean  $\pm$  SD and sample size. Units are microns for all except counts and ratios.

stage	character	<i>Renicola</i> sp. “polychaetophila”	<i>Renicola</i> sp. “martini”
sporocyst	body length	133–739 (298 $\pm$ 124, n = 40)	108–345 (214 $\pm$ 64, n = 30) <sup>1</sup>
	body width	79–246 (145 $\pm$ 40, n = 40)	82–138 (112 $\pm$ 13, n = 30)
	cercaria count <sup>2</sup>	1–21 (9.9 $\pm$ 5.5, n = 31)	1–10 (5.6 $\pm$ 2.6, n = 30)
cercaria	body length	148–207 (179 $\pm$ 13, n = 49)	158–229 (195 $\pm$ 18, n = 40)
	body width	43–59 (50 $\pm$ 3, n = 49)	57–81 (66 $\pm$ 6, n = 40)
	body height	25–45 (35 $\pm$ 5, n = 40)	33–47 (42 $\pm$ 4, n = 39)
	tail length	59–113 (81 $\pm$ 11, n = 48)	81–165 (120 $\pm$ 24, n = 40)
	tail width	12–18 (16 $\pm$ 1, n = 44)	14–25 (19 $\pm$ 2, n = 39)
	tail height	12–17 (14 $\pm$ 1, n = 33)	14–21 (17 $\pm$ 1, n = 39)
	oral sucker length	21–30 (26 $\pm$ 2, n = 49)	28–37 (32 $\pm$ 2, n = 40)
	oral sucker width	18–26 (22 $\pm$ 2, n = 48)	23–34 (28 $\pm$ 3, n = 40)
	oral sucker height	16–25 (21 $\pm$ 2, n = 38)	21–32 (26 $\pm$ 2, n = 39)
	ventral sucker length	20–29 (25 $\pm$ 2, n = 48)	22–37 (29 $\pm$ 3, n = 40)
	ventral sucker width	21–30 (25 $\pm$ 2, n = 47)	27–38 (30 $\pm$ 3, n = 40)
	ventral sucker height	20–30 (25 $\pm$ 2, n = 36)	23–33 (27 $\pm$ 2, n = 40)
	stylet length-t <sup>3</sup>	8.9–11.5 (10.2 $\pm$ 0.7, n = 18)	10.6–12.6 (11.6 $\pm$ 0.6, n = 30)
	stylet length-s <sup>4</sup>	7.4–10 (8.3 $\pm$ 0.9, n = 14)	6.3–9.5 (8.3 $\pm$ 0.7, n = 30)
	stylet length-s / length-t	0.7–0.9 (0.8 $\pm$ 0.1, n = 14)	0.5–0.8 (0.7 $\pm$ 0.1, n = 30)
	stylet width	2.6–4.9 (3.7 $\pm$ 0.5, n = 18)	4.2–6.0 (4.9 $\pm$ 0.4, n = 30)
stylet length-t / width	2.3–3.9 (2.9 $\pm$ 0.5, n = 14)	1.9–2.8 (2.4 $\pm$ 0.2, n = 30)	
stylet length-s / width	1.8–2.9 (2.4 $\pm$ 0.3, n = 14)	1.2–2.0 (1.7 $\pm$ 0.2, n = 30)	

<sup>1</sup>Observations on additional, fresh material indicates that sporocysts can be larger than indicated by material used for morphometrics.

<sup>2</sup>Counts of well-developed cercariae.

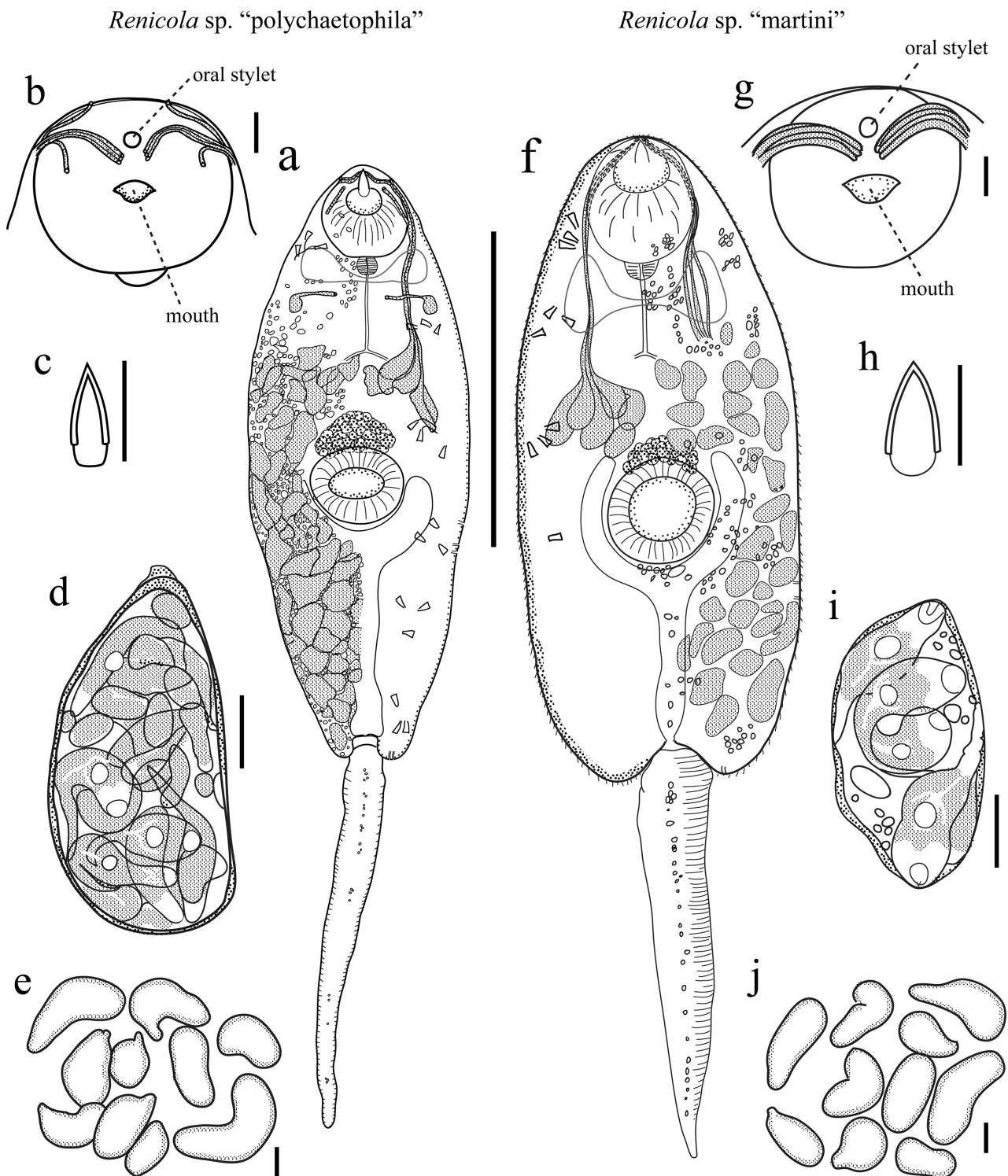
<sup>3</sup>Stylet length-t is the total length of the stylet (sclerotized and non-sclerotized portions)

<sup>4</sup>Stylet length-s is the length of the sclerotized portion of the stylet

**Other biology:** The trematode appears to usually infect the host by crossing the mid-gut, as expected given its taxonomic affiliation, and as evidenced by residual sporocyst or germinal material in the parenchymous tissue surrounding the mid-gut.

Developed infections parasitically castrate the host and usually appear to almost completely replace snail gonadal tissues. However, the seasonal (winter) regression, where cercaria production is greatly decreased and sporocysts become somewhat flaccid, is associated with infiltration of the parthenita colony by snail digestive gland tissue and sometimes a small amount of snail gonadal tissue regeneration (but there is no sign that snail reproduction takes place). These observations are consistent with the information provided in Hechinger *et al.* (2009) that the aggregate sporocyst mass takes about 20% of the infected host soft tissue mass in summer and 16% in winter. That study may have pooled this species and *Renicola* sp. “martini”, but the two species appear to be very similar concerning the way they grossly use host space.

Infections are sometimes encountered that are in the process of being invaded by other trematode species, particularly those with rediae, which can be observed ingesting the sporocysts and cercariae of *Renicola* sp. “polychaetophila”. This is consistent with the relatively low ranking of this species in the interspecific dominance hierarchy characterizing the guild of trematodes infecting the California horn snail (Kuris 1990; Sousa 1993).



**FIGURE 3.** (a–e, left) *Renicola* sp. “polychaetophila”. (f–j, right) *Renicola* sp. “martini”. (a,f) Whole cercaria, ventral view, live, slightly flattened. (b,g) Approximate *en face* view of live cercaria to clarify penetration gland duct opening arrangement. (c,h) Oral stylet. (d,i) Whole sporocyst, live, slightly flattened. (e,j) Group of sporocysts showing range of shapes and sizes. Scale bars = 100 microns, except for b, c and g, h, where scale bars = 10 microns.

Field surveys and preliminary laboratory exposure experiments indicate that the cercariae encyst as metacercariae in the hemocoel of various estuarine polychaetes (Hechinger and Smith, unpublished data; see list of probable hosts in Hechinger *et al.* (2011a)).

The final host species are not determined, but almost certainly include birds that prey on polychaetes.

**Geographic distribution:** This species may occur throughout the entire range of the California horn snail,

from North of San Francisco Bay (California, USA) to northern Peru (Keen 1971; Miura *et al.* 2010). However, we have only positively identified it from snails from southern San Francisco Bay to the Estero de Punta Banda (northern Baja California, Mexico). In addition, preliminary genetic data indicates that this species occurs in the Atlantic *Cerithidea pliculosa* (Menke) (sister species to the California horn snail), in the Gulf of Mexico and the Yucatan Peninsula (Miura *et al.* unpublished data).

**Etymology:** The potential specific epithet, “polychaetophila” (“polychaete loving”), reflects the second intermediate host use of this species.

**Remarks:** *Renicola* sp. “polychaetophila” fits well within the emended diagnosis for renicolid cercariae (above), and the molecular results (below) further indicate that it is embedded within *Renicola*.

Table 2 lists attributes of previously described renicolid xiphidiocercariae. The arrangement of the penetration gland-bodies and ducts readily distinguishes *Renicola* sp. “polychaetophila” from most other renicolid xiphidiocercariae. With one possible exception (below), it is the only species that has a 2[(1+3+1)+1] arrangement of penetration gland-duct openings, where the gland bodies for the five anterior-most duct openings are clustered together and lie within the cystogenous-gland field, while the gland bodies for the posterior-ventral “cross-duct” openings are solitary and anterior to the penetration-gland field. Of the four species that are at least roughly similar to *Renicola* sp. “polychaetophila” in having penetration gland ducts that diverge to open away from the stylet, *Cercaria caribbea* XXXIII, Cable, 1956 is most similar. However, *C. caribbea* XXXIII does not appear to have the pair of “cross-duct” penetration gland bodies isolated anteriorly from the main cluster of penetration gland bodies (which lie in the obscuring cystogenous gland field) and it also has tegmental spines whereas *Renicola* sp. “polychaetophila” does not (or at least it does not have any readily apparent tegmental spines).

Specimens of *Renicola* sp. “polychaetophila” appear to have been included in the material that Hunter used to describe “*Cercaria cerithidia* 19” in her unpublished 1942 dissertation. However, it seems her material also included *Renicola cerithidicola* Martin, 1971 (a non-styleted species with sporocysts in the mantle area), perhaps as mixed-species infections with *Renicola* sp. “polychaetophila”, as her description fuses characteristics of both those species.

### ***Renicola* sp. “martini”**

(Figs. 3–5)

**Diagnosis:** Distome, leptocercous xiphidiocercariae with all penetration-gland ducts opening in single cluster at anterior oral sucker, with fine tegmental spines, with a simple, bullet-shaped stylet that is sclerotized for 50–80% of its length, and with an obscuring cystogenous-gland field with an anterior-most extent about half way between oral and ventral suckers. Cercariae produced by daughter sporocysts that comprise a colony that resides in the gonadal and digestive gland regions of first intermediate host.

**Host:** *Cerithidea californica* (Haldeman)

**Location in host (daughter sporocysts):** gonadal and digestive gland regions

**Prevalence:** 0.3% in 5392 snails dissected in 2012

**Locality:** Carpinteria Salt Marsh, Santa Barbara County, California, USA (34.4°N, 119.54°W)

**Habitat:** Estuaries (intertidal flats, pans, channels)

**Dates of collection:** September–October 2009

**Deposited material:** Cercariae and sporocysts from 7 infections (USNPC nos. 107307–107318).

**GenBank accession numbers:** COI KF512553–KF512560; ITS1 KF512561–KF512568

**Description:** Daughter sporocysts. Body immobile, shape variable, elongate to pyriform to spheroidal, sometimes pinched at one end to form a nipple or neck that appears to mark the birth pore. Tegument translucent, typically covered with thicker paletot that gives sporocysts lemon-cream to orangish appearance and causes them to stick together, and lined on interior by tissue that likely represents germinal cells. Ripe sporocysts with cercariae in various stages of development; germinal balls to developed cercariae distributed in sporocyst with no clear spatial polarity. Developed cercariae impart a whitish coloration to sporocysts under reflected light that mottles with the orange and clear tegument color. Distribution of sporocysts includes the snail gonadal space with some infiltration of digestive gland space. Sporocysts exhibit seasonal variation, wherein sporocysts in developed infections physically regress in winter, becoming thinner-bodied, somewhat flaccid, more orangish, with zero to



few developed cercariae; sporocysts ripen up again during spring, becoming more plump, filled with more developed and developing cercariae, and together form a more compact mass. Morphometrics and meristics are presented in Table 1, and are based on 30 sporocysts from three infections while cercaria counts are of the well-developed cercariae from 30 sporocysts from four infections.



**FIGURES 4–5.** *Renicola* sp. “martini”. **5.** A fresh, deshelled first intermediate host snail (*Cerithidea californica*) infected with daughter sporocysts. The large orangish mass in the visceral mass is the colony of sporocysts. The colony takes up the entirety of the snail gonadal space and has also infiltrated some of the digestive gland. The colony extends to the distal tip of the visceral mass (small arrow). Large arrow marks the basal-most extent of the colony, which extends to the distal tip of the visceral mass (small arrow). Scale bar = 10 mm. **2.** Phase contrast view of anterior end of a flattened, live cercaria, highlighting the sclerotized portion of the oral stylet. Scale bar = 10  $\mu$ m.

**Cercariae.** Distome, leptocercous xiphidiocercaria. Body elliptic-elongate-ovate in dorsal view, dorso-ventrally flattened, maximal width and dorso-ventral height usually just anterior to ventral sucker; with refringent, varying sized spherules scattered throughout most of interior. Cystogenous glands fill posterior  $\sim 3/4$  of body, densely packed, filled with refringent granules, reflect much white light, obscure fine internal anatomy. Oral sucker well-developed, round, large, equal-subequal to ventral sucker; with small stylet; stylet embedded horizontally at anterior oral sucker, dorsal to mouth, small, usually appears less than  $1/4$  length of oral sucker, sclerotized for 50–80% of its length, bullet-shaped, tapering anteriorly from near base of sclerotized portion, difficult to carefully observe unless cercaria is flattened. Mouth subterminal; prepharynx absent; pharynx small, ovoid-spheroid, abutting posterior margin of oral sucker; esophagus thin and thin-walled, branching into two ceca approximately midway between oral and ventral suckers near anterior margin of cystogenous gland field; ceca thin and thin-walled, posterior-most extent obscured by cystogenous glands. Ventral sucker positioned at mid-body, well developed, round, protrudes from ventral surface, lacking spines. Tegument thin, with tegmental spines on ventral, dorsal, and lateral surfaces; tegmental spines fine, most readily apparent on non-flattened or slightly flattened specimens, distributed from anterior to posterior edge of body, particularly apparent laterally and dorsally, increase in size posteriorly. Penetration gland bodies extremely difficult to observe given obscuring cystogenous glands, but are primarily clustered  $\sim 2/5$  into body from anterior edge, anterior extent of cluster close to anterior extent of cystogenous gland field; potentially more than seven pairs of unknown number of types. Penetration gland ducts pass anteriorly from glands in a single bundle per side; bundles pass around lateral margins of oral sucker, contain at least three individual ducts, empty anteriorly just ventral to oral stylet tip. Two pairs of lateral gland ducts of unknown type inconsistently observed opening at lateral body margins posterior to ventral sucker; gland bodies not reliably observed. One pair of posterior gland ducts of unknown type inconsistently evident opening at posterior body margin; gland bodies not reliably observed. Excretory bladder prominent, Y-shaped; stem longer than arms; arms embracing posterior half or more of ventral sucker margin; excretory opening at posterior edge of bladder stem. Flame cell arrangement likely  $2 [(3+3+3) + (3+3+3)] = 36$  bilateral arrangement, but posterior four groups typically obscured by cystogenous glands and posterior 3 groups never entirely observed. Other details of collecting ducts not observed. Cerebral ganglion just posterior of and extending laterally to pharynx. Genital primordium a compact mass of cells, adjacent to dorso-anterior margin of ventral sucker, very evident upon

acetocarmine staining. Tail attached terminally at posterior body margin in socket, simple, unadorned, slender, cylindrical in cross-section, gradually tapering, can extend and contract, approximately equal in length to body when extended, with finely annulated tegument overlying muscle layers, with scattered, refringent spherules along mid-line. Morphometrics are presented in Table 1 and are based on 30–40 individuals from four infections.

**Behavior:** Cercariae swim by widening and flattening the mid- and posterior-body, flexing posterior body ventrally to partly overlap ventral sucker, often also slightly flexing or protruding anterior body, and lashing the tail left to right to move in a general anterior direction. Cercariae do not clearly respond to light.

**Other biology:** The trematode appears to usually infect the host by crossing the mid-gut, as expected given its taxonomic affiliation, and as evidenced by residual sporocyst or germinal material in the parenchymous tissue surrounding the mid-gut.

Developed infections parasitically castrate the host and appear usually to almost completely replace snail gonadal tissues. However, the seasonal (winter) regression, where cercaria production is greatly decreased and sporocysts become somewhat flaccid, is associated with infiltration of the parthenita colony by snail digestive gland tissue and sometimes a small amount of snail gonadal tissue regeneration (but there is no sign that snail reproduction takes place). These observations are consistent with the information provided in Hechinger *et al.* (2009) indicating that the aggregate sporocyst mass takes about 20% of the infected host soft tissue mass in summer and 16% in winter. That study may have pooled together this species and *Renicola* sp. “polychaetophila”, but the two species appear to be very similar concerning the way they grossly use host space.

Infections are sometimes encountered that are in the process of being invaded by other trematode species, particularly those with rediae, which can be observed ingesting the sporocysts and cercariae of *Renicola* sp. “martini”. This is consistent with the relatively low ranking of this species in the interspecific dominance hierarchy characterizing the guild of trematodes infecting the California horn snail (Kuris 1990; Sousa 1993).

Field surveys and preliminary laboratory exposure experiments indicate that the cercariae encyst as metacercariae in the tissues of gastropods and potentially the hemocoel of various estuarine polychaetes (Hechinger and Smith (unpublished data); see list of possible hosts in Hechinger *et al.* (2011a)). The use of gastropods is further evidenced by cercariae encysting as viable-appearing metacercariae in the mantle tissues of snails with daughter sporocyst infections, particularly when those snails have been maintained out of water for days to weeks.

The final host species are uncertain, but almost certainly include birds that prey on polychaetes.

**Geographic distribution:** This species may occur throughout the entire range of the California horn snail, from North of San Francisco Bay to northern Peru (Keen 1971; Miura *et al.* 2010). However, it has been positively identified from snails from Carpinteria Salt Marsh (the north part of southern California, USA) to the Estero de Punta Banda (northern Baja California, Mexico). In addition, preliminary genetic data indicates that this species occurs in the Atlantic *Cerithidea pliculosa* Menke (sister species to the California horn snail), in the Gulf of Mexico and the Yucatan Peninsula (Miura *et al.* unpublished data).

**Etymology:** The potential specific epithet, “martini”, represents an honorific for Dr. Walter Martin, who provided much of the taxonomic and lifecycle groundwork for the trematodes using the California horn snail as first intermediate host.

**Remarks:** *Renicola* sp. “martini” fits well within the diagnosis for renicolid cercariae (above), and the molecular results (below) further indicate that it is embedded within *Renicola*.

Table 2 lists attributes of previously described renicolid xiphidiocercariae. *Renicola* sp. “martini” is similar to most renicolid xiphidiocercariae in having a spinose tegument and having all penetration gland ducts opening very close to the stylet. It appears to be distinguishable from those similar species by having a combination of (1) possessing a simple, bullet-shaped stylet that is sclerotized for 50–80% of its length, and (2) in having the anterior-most extent of the cystogenous-gland field being about half way between the oral and ventral suckers.

*Renicola* sp. “martini” likely corresponds to what Hunter (1942) provisionally termed “Cercaria cerithidia 23” in her unpublished dissertation.

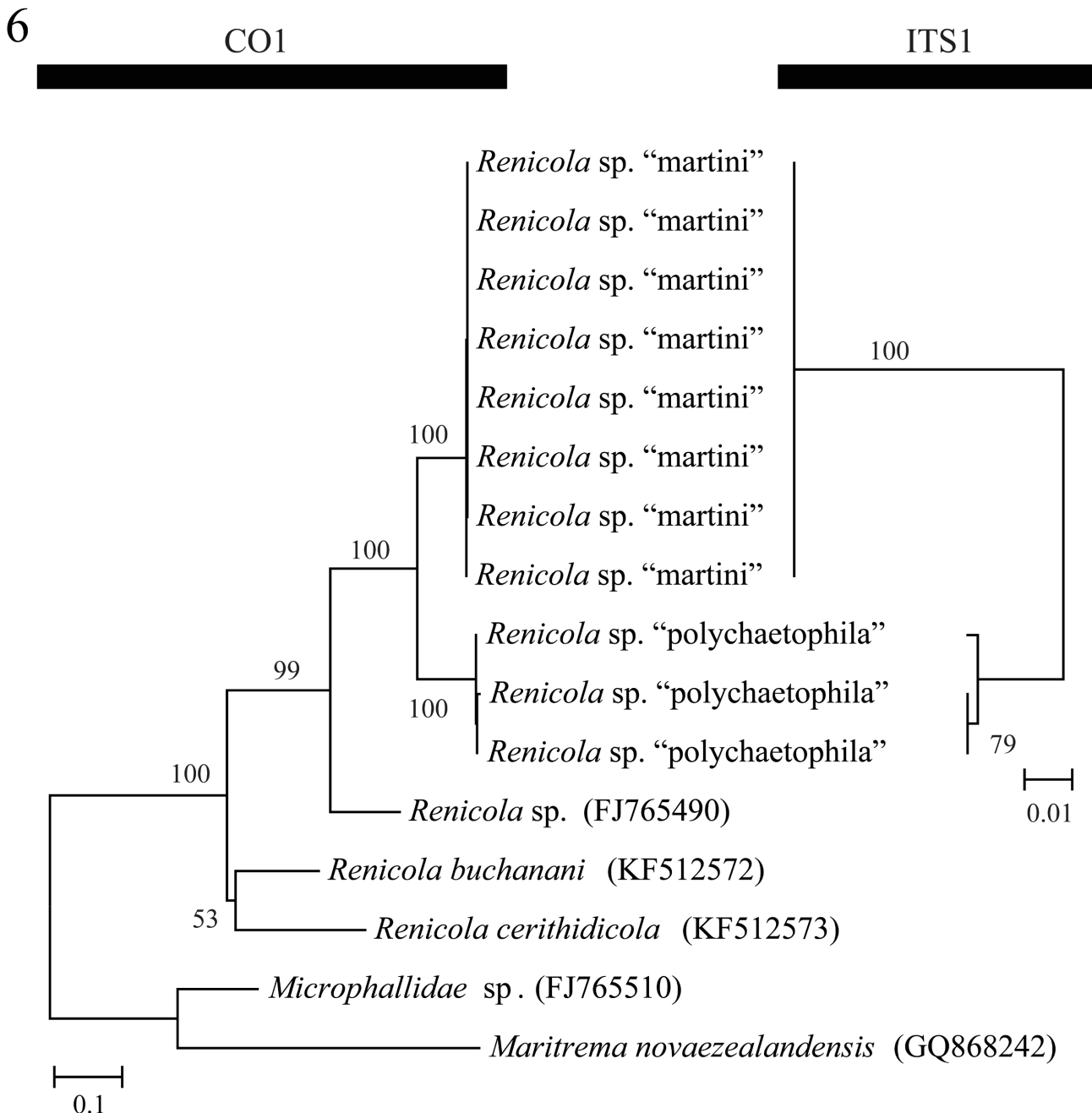
**TABLE 2.** Morphological attributes of named renicolid xiphidiocercariae. The two species described in this manuscript are bolded, and previously reported species are listed beneath each in a rough order of greatest to least similarity in terms of penetration gland duct patterns and host species used.

trematode name	host species code <sup>1</sup>	body			stylet			~ant. extent			penetration glands			"body" glands? <sup>4</sup>
		length	width	spines pres? <sup>2</sup>	length	width	length	length	lateral pres? <sup>3</sup>	length	no. in cyst	no. pairs	duct opening arrangement	
<b><i>Renicola</i> sp. "polychaetophila" of this ms</b>	ceca	148–207	43–59	n	8.9–11.5	2.6–4.9	80	n	n	~55	1 no	6	2[(1+3+1)+1]	lat & post
<i>Cercaria caribbea</i> XXXIII of Cable, 1956 <sup>5</sup>	ceco, bami	130–152	54–57	y	8–10	3	88	n	n	~70	all	6	2[1+3+1+1]	n
<i>Cercaria</i> sp. XXI of Wardle (1974) <sup>6</sup>	cepl	180–220	70–110	n	12	5.5	-	n	n	~30	all	-	2[(2+2)+1]	n
<i>Renicola</i> species A of McNeff (1978) <sup>6</sup>	cepl	151–172	46–61	y	8–12	3.0–4.5	-	n	n	~80	no	3?	-	n
<i>Renicola</i> sp. of Martorelli et al. (2008)	zesu	140–180	75–80	y	10–14	4–6	62	n	n	~55	no	6	2[1+3+1+1]	n
<i>Cercaria queenslandae</i> III of Cannon (1978)	cemo	168–200	34–45	y	13	4	70	n	n	~70	part	5	2[1+3+1]	post
<b><i>Renicola</i> sp. "martini" of this ms</b>	ceca	158–229	57–81	y	10.6–12.6	4.2–6.0	70	n	n	55	all	>7?	2[>=3]	lat & post
<i>Cercaria cerithidia</i> 23 of Hunter (1942) <sup>6</sup>	ceca	195–300	62–99	y	8	3	-	n	n	50	all	-	-	n
<i>Cercaria caribbea</i> XXXII of Cable, 1956	ceco	215–225	80–87	y	12	4	55	n	n	50	all	3	2[3]	n
<i>Cercaria</i> sp. G of Epstein (1972) <sup>6</sup>	cepl	208–273	62–99	n	12	4	-	n	n	10	all	1?	2[?]	n
<i>Cercaria opaca</i> Holliman, 1961	liir	209–240	97–107	y	9	5	80	n	n	0	all	lots	2[4]	n
<i>Cercaria</i> sp. XXII of Wardle (1974) <sup>6</sup>	liir	140–220	90–120	n	10	4	-	n	n	10	all	~8	2[4]	n
<i>Cercaria roscovita</i> Stunkard, 1932	lisa	150–300	60–120	y	16–18	2–3	-	n	n	-	all	-	2[?]	n
<i>Cercaria parvicaudata</i> Stunkard & Shaw, 1931 lili <sup>8</sup>	lili <sup>8</sup>	140–360	50–120	y	15	3.2	-	n	n	45	all	6	2[6]	n
""	lisa, liob <sup>8</sup>	-	-	-	11–13	3–4	52	y	-	-	-	3	2[3]	n
<i>Renicola thaidis</i> Stunkard, 1964	nula	250–380	60–130	y	8–10	-	-	y	y	10	all	5	2[2+3]	n
""	nula <sup>8</sup>	-	-	-	8–10	2.5–3	66	y	-	-	-	5	2[2+3]	n
<i>Cercaria</i> F of Rohde (1981) & Ching (1989)	plsu	165–220	68–90	y	8	2	-	n	n	0	all	4	2[4]	lat & post
<i>Renicola</i> sp. I of Hechinger (2007)	baat	148–233	61–74	y	8.5–8.6	2.6–2.9	-	n	n	30	all	-	-	n
<i>Cercaria renicola</i> sp. of Rybakov (1987)	baat	106–208	78–91	y	14	6.5	85	n	n	20	all	6	2[3+3]	n

Note: A dash (-) indicates not reported or extractable from figures.<sup>1</sup> Host species code key: baat-*Batillaria atramentaria*, bami-*Batillaria minima*, ceca-*Cerithidea californica*, cece-*Cerithidea costata*, cemo-*Cerithium moniliferum*, cepl-*Cerithidea pliculosa*, lili-*Littorina littorea*, liob-*Littorina obtusata*, lisa-*Littorina saxatilis*, nula-*Nucella lapillus*, plsu-*Planaxis sulcatus*, zesu-*Zeacumantus subcarinatus*.<sup>2</sup> The approximate anterior-most extent of the cyst gland field is expressed as % of distance between oral sucker posterior margin (0%) and ventral sucker anterior margin (100%).<sup>3</sup> Indicates if penetration gland bodies are completely embedded within the cyst gland field ("all"), the cluster is partially embedded and partially lying anteriorly to the field("part"), or whether one or all bodies lie anterior to the cyst gland field ("1 no" or "no", respectively).<sup>4</sup> Indicates whether the "body" glands are reported in descriptions, and if so, where on cercaria surface do the ducts empty (laterally and/or posteriorly).<sup>5</sup> Cable (1963) reported also infects *Cerithium variabile*.<sup>6</sup> These descriptions are in unpublished theses and vary in quality (Wardle 1974 is the most detailed). The information is listed here for completeness.<sup>7</sup> Information from Stunkard (1950).<sup>8</sup> Information from Galaktionov & Skirnisson (2000), who also state specimens agree in detail (or perfectly) with available descriptions.

## Molecular genetic results (for both species and other material)

The phylogenetic trees based on COI and ITS1 each show monophyly of both *Renicola* sp. “polychaetophila” and that of *Renicola* sp. “martini” with high bootstrap values (Fig. 6). The absence of any mitochondrial and nuclear alleles shared between the two species indicates that there is no gene flow between them. Sequence divergence (based on Kimura’s two parameter distance) between *Renicola* sp. “polychaetophila” and *Renicola* sp. “martini” was 12.3 % for COI and 6.6 % for ITS1, which are similar to congeneric and interspecific sequence divergences observed in other trematodes (Leung *et al.* 2009b; Locke *et al.* 2010; Miura *et al.* 2005). The COI tree further indicates that the two new species form a clade with the styleted *Renicola* sp. from New Zealand and, further, that the three styleted species are part of a broader clade with the two non-styleted *Renicola* species from the California horn snail.



**FIGURE 6.** Maximum likelihood phylogenetic trees based on 682 bp of the mitochondrial cytochrome oxidase I gene (COI, left) and 493 bp of the nuclear internal transcribed spacer 1 gene (ITS1, right). Additional species sequences from GenBank are shown with their accession nos. Numbers near nodes are the support values for the major clades. The scale bar represents the phylogenetic distances expressed as units of expected nucleotide substitutions per site.

## Discussion

The morphological and DNA sequence data clearly indicate that the two species described here are distinct species of *Renicola*.

It may appear unlikely that the species have been previously described as adults. This is because literature searching indicated that there are no *Renicola* species described from birds that frequent Pacific coast estuaries and typically prey on the likely second intermediate hosts of these two species. However, there is a chance that one or both of the species are widespread and use different snail species as first intermediate hosts throughout their range (e.g., as indicated by the preliminary molecular data mentioned in the above “geographic range” sections). If this is the case, then the adult of one or both of these species could be described elsewhere. Thus, it is possible for future research to reveal that the adult has been described, for instance, as *Renicola glandoloboides* Byrd & Heard, 1970, adults of which have been reported from birds that live in appropriate habitat in Florida and Texas (Byrd & Heard 1970; Dronen *et al.* 2002).

Table 2 highlights the probable utility of several characters for differentiating renicolid xiphidiocercariae. For instance, the arrangement of penetration gland duct openings appears to be a useful way to separate species; this is consistent with Galaktionov & Skirnisson’s (2000) documentation that the duct opening arrangement provided a clear difference between two other renicolids whose taxonomic status had previously been questioned (*Renicola thaidus* Stunkard, 1964 and *Cercaria parvicaudata* Stunkard & Shaw, 1931). In addition, the positioning of the penetration gland bodies also appears to be taxonomically informative, as does the anterior-most extent of the cystogenous gland field; this is partly parallel to how the distribution of penetration glands can distinguish two groups of Rhodometopa-type renicolid cercariae (Wright 1956). Further, it may be useful to regularly focus on neglected aspects of stylet morphology, including clearly indicating the lengths of the sclerotized and non-sclerotized portions. Continued attention to these (and doubtlessly other) morphological traits may help to improve future descriptions of renicolid xiphidiocercariae.

Also warranting study are the “body” glands of unknown function that open laterally and posteriorly. The gland bodies were extremely difficult to observe, given the obscuring nature of the cystogenous glands. However, they appear to be relatively large, and potentially shaped like elongate sacs. Similar glands have been observed in at least three other renicolid xiphidiocercariae (Cannon 1978; Ching 1989; Wardle 1974) (Table 2). Martin (1982) also noted three pairs of lateral “adhesive (?) glands” in a renicolid xiphidiocercaria from the limpet, *Collisella digitalis* (Rathke, 1833). Wardle (1974), in an unpublished thesis, depicted the glands as being a connected, convoluted series of wide ducts. These ducts not only opened laterally and posteriorly, but also connected to duct openings corresponding to that depicted in our description of *Renicola* sp. “martini” as being the openings for the sixth pair of penetration glands (the “cross-ducts”). Whether Wardle’s observations were accurate or not, he definitely recorded the occurrence of these mysterious glands. We suspect that these glands occur in many more renicolid cercariae, but that the difficulty in observing their structure has precluded workers from including them in descriptions. Although the gland bodies are very difficult to discern, we note that the duct openings on the tegument are relatively easy to see and can be more regularly included in descriptions. Whether these glands will be taxonomically informative or not, it is certainly worth better understanding their form and function.

Although sparsely populated, our phylogenetic tree reveals an intriguing pattern. First, the two xiphidiocercariae from California and the one from New Zealand form a clade, outside of which lie the two non-styleted renicolids from California. Where known, styleted species use mollusks and polychaetes, whereas non-styleted species use fishes as second intermediate hosts. Therefore, the creation of trees with more renicolid species (including those with tail fins in the “Rhodometopa-group”) will likely shed light on how host use and functional morphology relate to and potentially underpin the systematics of the Renicolidae, which is sorely in need of revision (Gibson 2008).

It is also interesting that *Renicola* sp. “polychaetophila” is not most closely related to the *Renicola* sp. from New Zealand. This was unexpected because they share a distinctive penetration gland duct arrangement characterized by a particular divergence from the oral stylet. Despite this similarity, *Renicola* sp. “polychaetophila” groups more closely with the sympatric *Renicola* sp. “martini”, which has no such duct divergence. Hence, it appears that the penetration gland duct arrangement may be an evolutionarily labile trait (but still a useful species trait, as discussed above).

Finally, relevant to any descriptions of trematode first intermediate host infections, we suggest that, when possible, researchers standardly include more detail on parthenitae, particularly information pertaining to the level

of the entire group of parthenitae in a host (that is, colony characteristics). At a minimum, this could involve brief, general notes, such as those included in this manuscript, on the distribution and density of the parthenitae within the host, including any obvious seasonal variation in colony attributes. Such observations are often readily obtained during the normal descriptive process and could easily be included in descriptions. Going further, some obvious, additional information on parthenitae could include (1) the total numbers in infected hosts, (2) the proportion of the host mass or volume taken up by the colony, and (3) evidence for different morphs and their frequencies. Such attention is warranted because, as noted in the introduction, the mass of parthenitae in their first intermediate host is comprised of individuals that cooperatively live together to reproduce and operate the castrated host phenotype. Hence, they can be understood as comprising a colony or society (Hechinger *et al.* 2011b). The degree of sociality can be developed so far as to involve a reproductive division of labor among parthenitae including the formation of a non-reproducing soldier caste (Hechinger *et al.* 2011b; Leung & Poulin 2011; Miura 2012). Whether one adopts this basic zoological/sociobiological perspective (see Oster & Wilson 1978; Wilson 1975) concerning trematode parthenitae, it is clear that it is the entire group of clonally produced parthenitae that is the functional unit of interaction with the host. Hence, information on parthenita colony attributes, including that which is often readily available to workers describing parthenitae and cercariae, will shed light on trematode biology, ecology, and evolution, and may also reveal taxonomically informative traits.

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