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FISH PARASITOLOGY - ORIGINAL PAPER

# First report of the metacestode *Caulobothrium* sp. in the Peruvian scallop *Argopecten purpuratus* from Sechura Bay, Piura, Peru

**Teresa Castro**[1] · **Dante R. Mateo**[2] · **Spencer J. Greenwood**[3] · **Enrique C. Mateo**[1]

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## Abstract

In recent parasitological surveys performed on the Peruvian scallop, *Argopecten purpuratus*, from bottom cultures of Sechura Bay, Piura, Peru, free and encysted metacestodes were frequently found in their gonads. The objective of this study was to identify this metacestode, determine their prevalence and intensity and briefly assess the histopathological impact in the affected tissues. A parasitological study of 890 scallops over a 3-year period was performed in order to determine the parasite prevalence and intensity. Microscopical observation of details of the scolex and histopathological study of the affected host tissues were performed as well as molecular characterization of the parasite based on 18S and 28S rDNA sequences. The prevalence of the metacestode was 82.2% in August of 2013, 90.4% in November of 2014, and 83.1% and 85.6% in April and September of 2015, respectively. The highest average intensity (218.4) was found in spring of 2014. The histopathological study showed that plerocercoids reduced the gonadal space where the ovules develop. The molecular characterization and phylogenetic analysis revealed that the metacestodes belong to the genus *Caulobothrium* having high sequence similarity to *Caulobothrium opisthorchis*. This study constitutes the first report of *Caulobothrium* metacestodes in the scallop *A. purpuratus*.

**Keywords** *Argopecten purpuratus* · Metacestode · *Caulobothrium* · Microscopy · Histopathology · rDNA · Peru

## Introduction

The Peruvian scallop, *Argopecten purpuratus* (Lamarck, 1819), inhabits the coastal areas of Peru and is the only bivalve cultivated at a commercial level in the country (Cavero-Cerrato and Rodríguez-Pinto 2008). Aquaculture is mostly performed as wild ranching and, to a lesser degree, in hanging systems (Mendo et al. 2008) it occurs mainly in the Samanco and Sechura Bays, in Ancash and Piura regions, respectively (PRODUCE 2015).

Despite the importance of this resource to Peruvian aquaculture, there is a lack of studies in health and diseases of local marine bivalves. The only previous report was on the presence of sporocysts containing cystophorus cercariae of hemiurid digeneans in the gonads and adductor muscle of *A. purpuratus* from Laguna Grande, Independencia Bay, Pisco region (Mateo et al. 1985). In addition to the significant mortality and castration associated with these trematodes, around 20% of the scallops harvested from that location were unmarketable due to esthetic reasons derived from the presence of the parasites.

Given the scarce information available, a parasitological study of the scallops grown out in bottom cultures in the Sechura Bay from 2013 to 2015 was performed. The study is aimed at taxonomically identifying the parasites affecting the gonads and assessing their prevalence, intensity, and, briefly, their histopathological impact. A molecular characterization and phylogenetic analysis was conducted because tetraphyllidean larvae dramatically differ in their morphology when they reach the adult stage (Jensen and Bullard 2010) making identification especially difficult (Caira et al. 2014, 2017; Caira and Jensen 2014).

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✉ Dante R. Mateo
dmateo@upei.ca

[1] Laboratorio de Patobiología Acuática, Dirección General de Investigaciones en Acuicultura, Instituto del Mar del Perú (IMARPE), Esquina Gamarra y General Valle s/n. Casilla Postal 22, Callao, Peru

[2] Diagnostic Services, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave., Charlottetown, PE C1A 4P3, Canada

[3] Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave., Charlottetown, PE C1A 4P3, Canada

## Material and methods

### Area of study and sampling

A total of 890 Peruvian scallops, *A. purpuratus* (average weight and size of 116 g and 81 mm respectively), were sampled at four times in a period over three years: 280 in August 2013 (water temperature 16.8 °C), 150 in November 2014 (water temperature 18.5 °C), 160 in April 2015 (water temperature 20.4 °C), and 300 in September 2015 (water temperature 19.9 °C) from bottom cultures at different point locations of the Sechura Bay, Piura region, Peru. The scallops were maintained in aerated tanks with filtered seawater until further study.

### Microscopic study

Scallop gonads were thoroughly dissected and inspected using stereoscopic (Leica M80) and compound microscopy (Nikon Eclipse 90i). Cestodes encountered in individual scallops were counted and overall prevalence and intensity by sampling period was calculated. A saline solution (equal parts 0.85% saline solution and seawater) was used to keep parasites alive during manipulation. Fresh parasites were measured and photographed while others were processed for further study.

Twenty metacestodes were measured. All measurements are given in micrometers (μm), as the range followed by the mean with standard error. The morphology was further analyzed following classic keys for identification (Wardle and McLeod 1952; Yamaguti 1959; Schmidt 1970; Cake 1976; Euzet 1994). Scanning electron microscope (SEM) photomicrographs of individual metacestodes were obtained at the Laboratorio de Equipamiento Especializado, Universidad Nacional Mayor de San Marcos, Lima, Peru. Samples for histopathology were fixed in Davidson solution in a proportion 1:20 (sample:fixative) (Shaw and Battle 1957). Dehydration, paraffin infiltration, sectioning, and hematoxylin and eosin (H&E) staining followed known methods (Luna 1968; Yasutake and Wales 1983). The larval terminology used is according to Chervy (2002).

### Molecular identification and phylogeny

Total genomic DNA was extracted from ethanol-preserved specimens using the DNeasy Blood & Tissue kit (Qiagen) according to manufacturer's instructions except that the AE elution buffer was incubated at 70 °C.

Amplification of the partial 18S and 28S ribosomal DNA (rDNA) regions used the previously published primers (Caira et al. 2014; Table 1) generating a number of overlapping fragments. For 18S rDNA, fragments were obtained from the products of primers WormA and WormB, 18S and WormB, and 18S-8 and 1200R. For 28S rDNA, products were obtained from primers LSU5 and 1500R, LSU5 and LSUD6–3, LSU5 and L1642, ZX-1 and 1200R, ZX-1 and 1500R, and 1600F and OR-2. Each PCR reaction was performed in a 50-μL reaction volume, comprising 2 μL (~ 8–20 ng) of DNA, 5 μL 10× PCR buffer, 1 μL of dNTP at 10 mM, 5 μL each of forward and reverse primers (10 μM), 0.5 μL (2.5 units) of HotStarTaq, 10 μL of Q-Solution (all PCR components from Qiagen, Toronto, ON, Canada) and the remaining volume made of nuclease-free water. Negative control samples contained 2 μl of nuclease-free water in place of DNA. PCR followed a touchdown protocol with an initial denaturation/activation step at 95 °C for 2 min, followed by three cycles of 94 °C for 45 s, 60° for 45 s, 72 °C for 2 min, 10 cycles of 94 °C for 45 s, 60 °C for 45 s (decreasing by 1 °C each cycle), and 35 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. PCR amplicons were visualized after 1% agarose gel electrophoresis with SYBR Safe DNA gel stain (Thermo Fisher Scientific, Ottawa, ON, Canada) under an ultraviolet light source.

**Table 1** PCR primers used to amplify ribosomal 18S and 28S (18S/28S rDNA) of the metacestode found in the scallop *A. purpuratus*

| Target | Primer | Sequence 5′-3′ |
|---|---|---|
| 18S rDNA | WormA (F) | GCGAATGGCTCATTAAATCAG |
| | WormB (R) | CTTGTTACGACTTTTACTTCC |
| | 18S-8 (F) | GCAGCCGCGGTAATTCCAGC |
| | 1200R | GGGCATCACAGACTTG |
| 28S rDNA | LSU5 (F) | TAGGTCGACCCGCTGAAYTTAAGCA |
| | 1500R | GCTATCCTGAGGGAAACTTCG |
| | ZX-1 (F) | ACCCGCTGAATTTAAGCATAT |
| | 1200R | GCATAGTTCACCATCTTTCGG |
| | LSUD6–3 (R) | GGAACCCTTCTCCACTTCAGTC |
| | L1642 (R) | CCAGCGCCATCCATTTTCA |
| | 1600F | GCAGGACGGTGGCCATGGAAG |
| | OR-2 (R) | TTCTGACTTAGAGGCGTTCAG |

*F* forward, *R* reverse

PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen), and bidirectional sequencing (ACGT Corp.) of 18S and 28S rDNA was performed. Contiguous sequences of 18S and 28S rDNA were assembled combining the various primer pair products. The consensus sequence was aligned using CLC Main Workbench v7 and deposited in GenBank (Accession nos. MH129528 and MH124632, respectively).

For comparative purposes and phylogeny reconstruction, the newly generated 18S and 28S rDNA sequences were aligned with sequences of related genera, under default values for gap opening extension penalties.

Phylogenies were constructed by the maximum likelihood (ML) method using MEGA version 6.0 (Kumar et al. 2016). Statistical support values (%) for the ML tree topology were obtained and indicated next to the nodes on the trees.

The ML trees' evolutionary distance was computed using the maximum composite likelihood method based on the

Tamura-Nei (TN93) model (Tamura and Nei 1993) for 18S rDNA and based on the general time reversible (GTR + G) model (Nei and Kumar 2000) for 28S rDNA. The distances are in the units of the number of base substitutions per site. A total of 14 and 13 nucleotide sequences were used in the tree with 1440 and 1209 positions in the final dataset for 18S and 28S rDNA, respectively.

Sequence similarity was determined using CLC Main Workbench v7 by pairwise comparison of 18S and 28S rDNA regions, with the 8 sequences of *Caulobothrium* Baer, 1948 isolates found in GenBank.

## Results

### Morphological study

According to the morphological characteristics of the scolex and the bothridia (Fig. 1a, b), the parasites found in the gonads and in the intestine were identified as metacestodes of the order "Tetraphyllidea" van Beneden, 1850 relics (Caira et al. 2017). Some of these structures were also observed in SEM (Fig. 2a–d).

Description of the plerocercoid (based on 20 specimens): body pyriform, slightly pointed at posterior end, 827.7–1741.6 (1061.2 ± 249.2) long by 261.4–1587.9 (488.2 ± 287.6) wide. Scolex with four bothridia and apical sucker. Bothridia elongate and foliate, 421.8–963.2 (575.4 ± 169.0) long by 81.6–224.8 (141.5 ± 36.5) wide, divided longitudinally by single median septum and horizontally by 22 septa forming two parallel rows of 23 loculi, total of 46 loculi per bothridium. Bothridial pedicels are short. Apical sucker terminal, 159.2–902.4 (270.7 ± 169.2) in diameter. Parenchyma full of calcareous corpuscles.

### Histopathology

Grossly visible whitish cysts present in the female gonads were found in most scallops. In the gonads, plerocercoids were found in formation and encysted (Fig. 1a–c). The average size of the procercoids was 200 μm.

Histopathological observation revealed that the cysts contained one or more metacestodes (Fig. 3a) that were exerting mechanical pressure upon and displacement of the adjacent tissues (Fig. 3b) and stimulating an inflammatory tissue reaction surrounding each cyst (Fig. 3c). In the intestinal mucosa, epithelial detachment and severe atrophy were observed to be associated with the presence of the parasites.

### Molecular identification and phylogeny

BLAST analysis showed that compared with sequence data in GenBank, the new partial 18S (1480 bp) and 28S (2819 bp) rDNA sequences shared the highest identity with members of the genus *Caulobothrium* (93.58–99.85% and 80.81–100%, respectively) and, among them, *Caulobothrium opisthorchis* Riser 1955 was the closest sequence corresponding to both rDNA datasets (18S, FJ177066; 28S, FJ177106). This was further supported by the phylogenetic trees for the 18S and 28S rDNA regions (Fig. 4a, b).

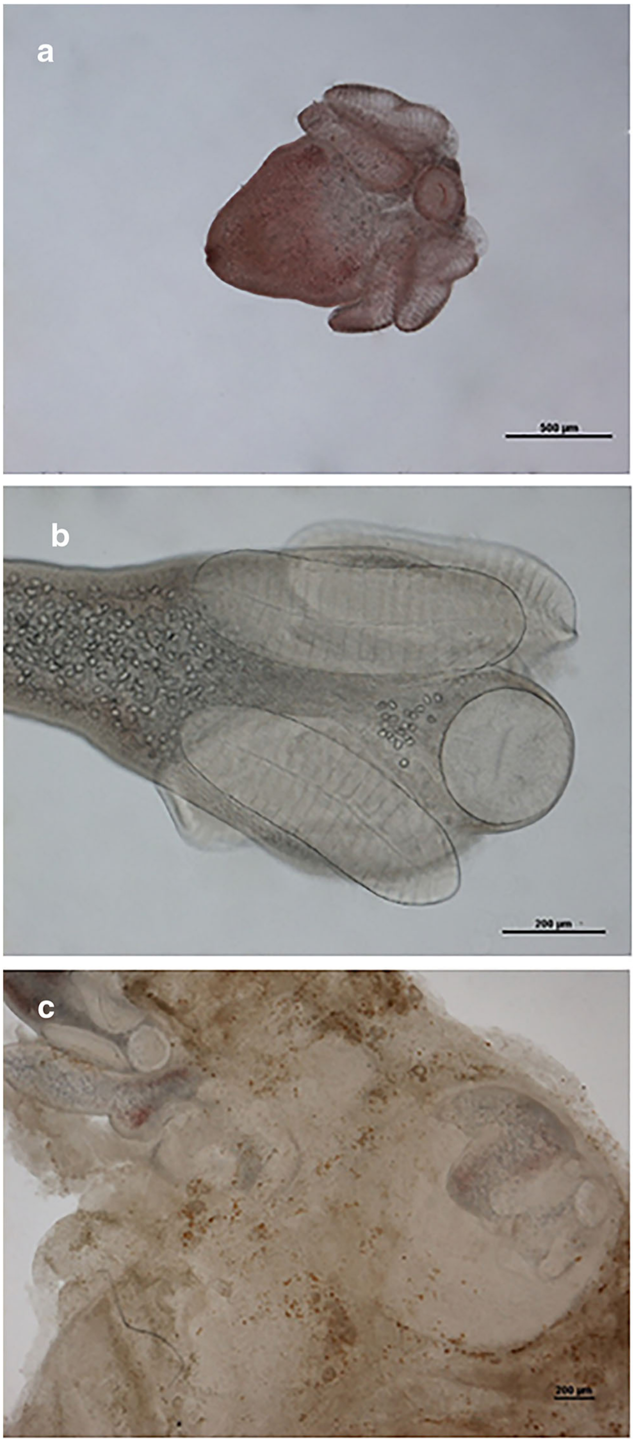

**Fig. 1** Photomicrographs of plerocercoid larvae found in *A. purpuratus*. **a** Stained with neutral red (× 4). **b** Plerocercoid (× 10). **c** Plerocercoid within cystic capsule (× 4)

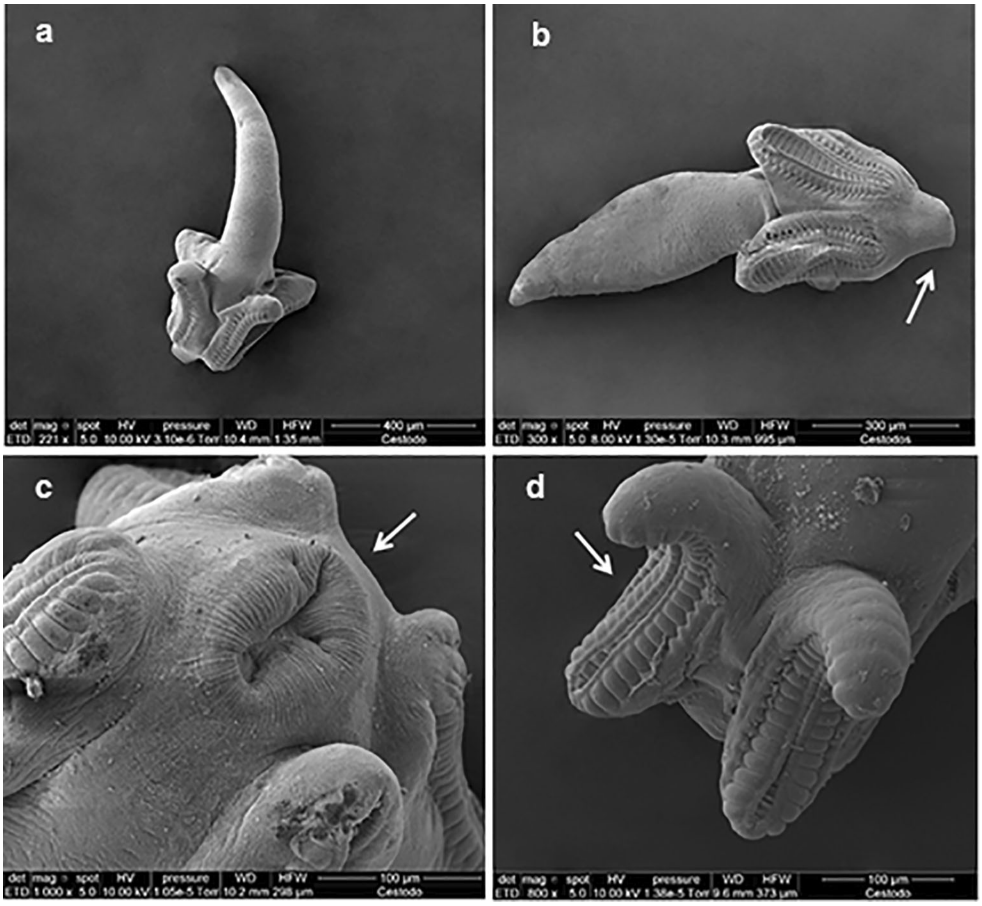

**Fig. 2** Scanning electron micrographs of parts and structures of metacestodes found in *A. purpuratus*. **a** Plerocercoid. **b** and **c** Apical sucker (arrow). **d** Bothridia (arrow)

## Prevalence and intensity

The average prevalence of the parasites found in scallop gonads was 82.2% in August 2013, 90.4% in November 2014, 83.1% in April 2015, and 85.6% in September 2015. The overall average prevalence during the whole sampling period was 85.3%.

The average intensity (number of parasites per infected individual) of infection was recorded only for scallops obtained in the various point locations in August 2013 and in a single point location in November 2014. The intensities in scallops obtained in these two samplings were 20.7 and 218.4, respectively.

# Discussion

In this study, metacestodes of the genus *Caulobothrium* were found in the Peruvian scallop, *A. purpuratus*, from the Sechura Bay, the main area for scallop aquaculture in Peru. The high and persistent parasitic prevalence (85.3%) found in the scallops sampled in a three-year period suggests a permanent significant presence of the definitive host in the Sechura Bay.

Although the intensity was measured only in 2013 and 2014, it was found to be highly variable, 20.7 and 218.4, respectively. It should be highlighted that the water temperature in November 2014 was over 2 °C higher than that in August 2013 in the Sechura Bay. This could explain the much higher intensity in the latter sampling date; seasonal variation is considered one of the stress factors that affects the physiology and immunity of mollusks, making them more susceptible to parasites and microbial pathogens (Chu and La Peyre 1993; Paillard et al. 2004; Perigault et al. 2011). It is also possible that higher temperatures in the area might have influenced an increased abundance of elasmobranchs, the definitive hosts of *Caulobothrium* species. Regardless, the high-intensity parasitic infection may have an impact on scallop health (Lauckner 1983). Intense parasitic infection by cestode larvae is known to cause physiological stress, thereby affecting growth and reproduction in bivalves (Cake 1977; Mouritsen and Poulin 2002). In the present study, displacement of gonadal tissue caused by encysted larvae could result in increased mechanical pressure that, in conjunction with the persistent and prominent host inflammatory tissue response surrounding the metacestodes, may be detrimental for reproduction.

Classic taxonomy identification of cestodes is based on the characteristics of the adult scolex, the estrobila, the proglottids and the disposition of the genital organs. Given the undeveloped nature of the plerocercoid larvae found in the scallop *A. purpuratus*, it was necessary to resort to molecular analysis to define its true taxonomic position.

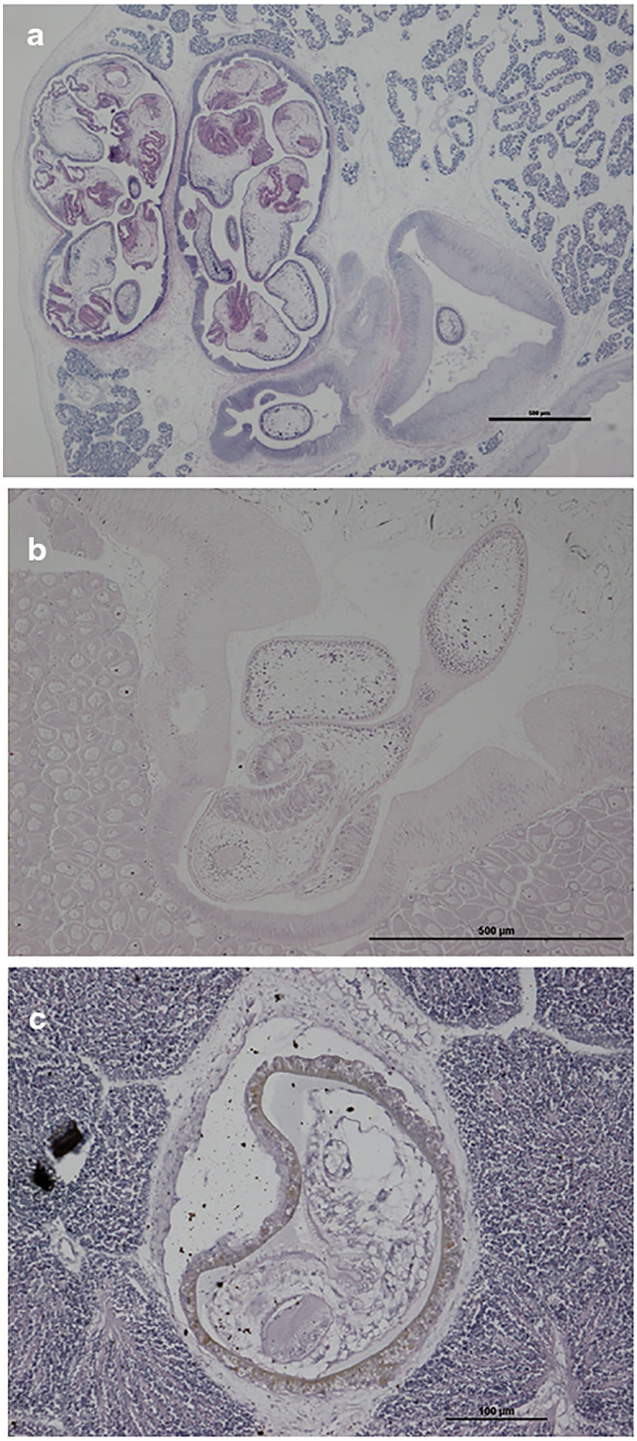

**Fig. 3** Histopathological views of metacestodes in scallop *A. purpuratus*. **a** Encysted plerocercoid larvae within gonadal tissue (H&E, × 4). **b** Metacestode within the intestinal tract exerting mechanical pressure on the gonadal tissues (H&E, × 10). **c** Inflammatory tissue reaction surrounding a metacestode (H&E, × 20)

The plerocercoid larvae, found in *A. purpuratus*, possess a scolex with an apical sucker and four bothridia with short pedicels. The body, the scolex proper, and the bothridia extend and contract when the larva is still very active. The bothridia of these larvae are flat and have one longitudinal septum in the middle of each bothridium and 22 transverse septa, making two parallel columns of 23 facial loculi. The tips of the bothridia end in a pair of loculi; therefore, each bothridium have a total of 46 loculi. Using the key of Euzet (1994), this larva, characterized by the presence of loculi in their bothridium, was tentatively placed in the Tetraphyllidean subfamily Rhinebothriinae Euzet, 1953. With the molecular analysis of the ribosomal DNA subunits, it was determined that this larva belongs to the *Caulobothrium* genus. Of the seven species of *Caulobothrium* mentioned as valid in Caira et al. (2017), the morphological characteristics of the scolex of the plerocercoid larvae found in *A. purpuratus* have greater concordance with the scolex of *C. opisthorchis*, except for the presence of an apical sucker in the plerocercoid of the former larvae and in the number of transverse septa present in the bothridia, 18 for *C. opisthorchis* and 22 for the plerocercoid larva in *A. purpuratus*. The closeness of the plerocercoid larvae found in *A. purpuratus* to *C. opisthorchis* was also supported by the phylogenetic analysis of rDNA sequences.

The genus *Caulobothrium* was erected by Baer in 1948 in the family Phyllobothriidae Braun, 1900 in the order Tetraphyllidea (Wardle and McLeod 1952). In 1953, *Caulobothrium*, together with *Rhinebothrium* Linton, 1890 and *Rhabdotobothrium* Euzet, 1953 was included in a nonrostellate subfamily Rhinebothriinae Euzet, 1953 of the Phyllobothriidae (Campbell 1975). The Tetraphyllidea, however, has always been considered a confused and poorly understood order (Olson et al. 1999) and shown to be paraphyletic (Caira et al. 1999). In the last three decades, this large and unstable polyphyletic order has been split, as a result of an exhaustive revision, into the following monophyletic orders: Litobothriidea Dailey, 1969 (Olson and Caira 2001), Cathetocephalidea Schmidt and Beveridge, 1990 (Caira et al. 2005), Rhinebothriidea Healy, Caira, Jensen, Webster & Littlewood, 2009 (Healy et al. 2009), Onchoproteocephalidea Caira, Jensen, Waesbach, Olson & Littlewood, 2014, and Phyllobothriidea Caira, Jensen, Waesbach, Olson & Littlewood, 2014 (Caira et al. 2014). All molecular analyses performed on the species of *Caulobothrium* supported the suggestion of Brooks and Barriga (1995) and the work of Olson et al. (1999) that *Caulobothrium* does not belong to the Rhinebothriinae which includes the subfamily Rhinebothriinae.

In Caira et al. (2017), ten independent groups, six families and four clades, which contain a total of 27 genera, remain as "tetraphyllidean" relics and the genus *Caulobothrium* is placed in Clade 4 with *Pithophorus* Southwell, 1925 and new genus 9; and in the genus *Caulobothrium* Baer, 1948, the following seven species are considered valid by Caira et al. (2017): *Caulobothrium longicolle* (Linton, 1890) Baer 1948 (type), *Caulobothrium myliobatidis* Carvajal 1977, *C. opisthorchis* Riser 1955, *Caulobothrium ostrowskiae* Brooks, Mayes & Thorson 1981, *Caulobothrium tetrascaphium* Riser 1955, *Caulobothrium tobijei* (Yamaguti, 1934) Baer 1948, and *Caulobothrium uruguayense* Brooks,

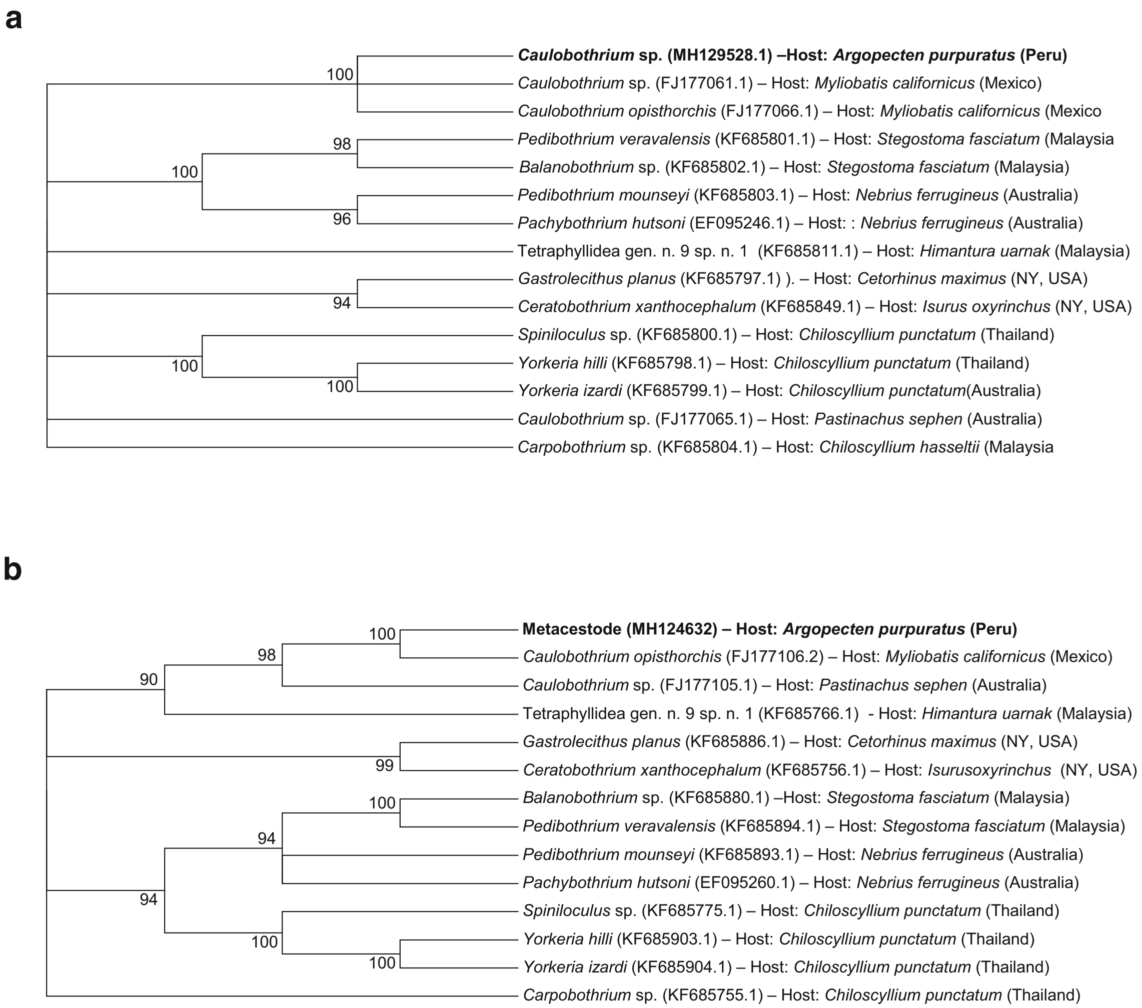

**Fig. 4** Maximum likelihood consensus tree based on 18S (**a**) and 28S (**b**) rDNA datasets. Values at nodes represent the bootstrap percentages. Sequences are indicated by the organism name followed by the GenBank accession number, the host species, and the location (in parenthesis) where they were found. The newly described metacestode sequence is in bold; the other sequences correspond to related genera

Mayes & Thorson 1981. In Euzet's (1994) diagnosis of the genus *Caulobothrium* Baer, 1948 it is mentioned: "scolex with four sessile bothridia: divided into loculi by several transverse septa and one longitudinal septum and no myzorhynchus". Of the valid species mentioned by Caira et al. 2017, only the following species fulfill these diagnostic characteristics: *C. ostrowskiae*, *C. opisthorchis*, *C. myliobatidis*, and *C. longicolle*. *Caulobothrium tetrascaphium* do not meet the diagnosis criteria for *Caulobothrium* in the possession of strongly developed bothridial pedicels and *C. tobijei* along with *C. uruguayense* for not possessing a longitudinal septum in the bothridia.

The morphology of the scolex of *Caulobothrium* became more complicated when Healy et al. (2009) highlighted the presence of bothridial stalks in *Caulobothrium* species and Caira et al. (2017) mentioned a new species of *Caulobothrium* with marginal loculi and bothridia with apical sucker, which are morphological characteristics not described for any other species of *Caulobothrium*. Thus, *Caulobothrium* clearly must be considered one of the morphologically enigmatic tetraphyllidean genera that is in need of careful revision.

Few reports exist on the presence of cestode larvae in bivalves of the eastern Pacific Ocean with reviews by Ching (1991) and more recently, Getchell et al. (2016), including references of cestode larvae in bivalves from this area. In California, USA, Sparks and Chew (1966), Katkansky and Warner (1969), and Warner and Katkansky (1969) reported the presence of *Echeneibothrium* sp. larvae in the bivalves *Protothaca staminea* and *Protothaca laciniata*, and MacGinitie and MacGinitie (1949) (cited by Warner and Katkansky 1969) reported the larva of *Anthobothrium* sp. in the bivalves *Tresus nuttallii*, *Macoma nasuta*, and *P. staminea*. In Chile, Carvajal (1977) described the larva of *Caulobothrium myliobatidis* from the Chilean clam *Mesodesma donacium*, and Oliva et al. (1986) reported unidentified larvae belonging to the families Phyllobothriidae and Oncobothriidae in *A. purpuratus*. The larva assigned to the Phyllobothriidae was initially suspected to be either a *Rhinebothrium* or *Caulobothrium*, but later, Oliva and Sanchez

(2005) dismissed the latter by stating that only the presence of *Acanthobothrium* sp. and *Rhinebothrium* sp. larvae, an unidentified hirudinid and the commensal *Polydora* sp., have been reported in the same scallop species. In relation to these findings, we consider it important to point out that the metacestodes described by Katkansky and Warner (1969) as *Echeneibothrium* sp. in the clam *P. laciniata* from Morro Bay, California, are morphologically very similar to the *Caulobothrium* metacestodes described here in *A. purpuratus*; we speculate that they may also be metacestodes of the genus *Caulobothrium*.

Adult *Caulobothrium* species have been reported from elasmobranchs (rays and skates) of the Atlantic, Indian, and Pacific Oceans. In the area of the eastern Pacific Ocean, they have been reported from the USA, Chile, and Peru. In California, USA, Riser (1955) reported *C. opisthorchis* and *C. tetrascaphium* from the stingray *Myliobatis californicus*; in Chile, Carvajal (1977) reported *C. myliobatidis* in the stingray *Myliobatis chilensis*; and in Peru, López (1987) reported a *Caulobothrium* sp. in the ray *Rhinoptera steindachneri* and Tantaleán (1991) reported *C. myliobatidis* in *M. chilensis*.

The presence of procercoid and plerocercoid larvae observed in the gonads of *A. purpuratus* suggests that this bivalve would act as the first intermediate host, probably by ingesting embryonated eggs and/or free coracidia. This finding is in agreement with those of Cake (1976, 1977), Lauckner (1983), Gosling (2003), and Figueras and Novoa (2011) who indicate that bivalves act as the first intermediate host of tetraphyllidean cestodes; and also with the opinion of Ben-Horin et al. (2015) who indicate that, in most cases, the transmission of bivalve parasites occurs by exposure to water-borne parasite stages through suspension feeding processes. An alternative case is that copepods might be playing the role of first intermediate hosts.

The parasites found in *A. purpuratus* from the Sechura Bay may cause serious consequences in local bivalve aquaculture. A potential reduction of spats in natural banks adjacent to the culture areas, due to infected scallops with impaired gonads, and commercialization problems related to the unaesthetic appearance of infected individuals, a problem already experienced (Mateo et al. 1985), may occur in the future. In order to elucidate the whole life cycle of this parasite, more morphological and genetic studies are necessary to discover the adult stage and its host, possibly a ray species of the genera *Rhinoptera* or the species *Myliobatis peruvians*, the most common ray in the northern coast of Peru (Córdova-Zavaleta et al. 2016). This would further our understanding of the ecological development of this parasitic infection.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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