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Morphological description and molecular characterisation of *Opegaster mezcal* sp. n. (Trematoda: Opecoelidae) from the Mexican Tropical Pacific

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Abstract: *Opegaster* Ozaki, 1928 is one of the most species-rich genera of the trematode family Opecoelidae, commonly found in marine teleost fishes. This study describes a new species of *Opegaster* of the intertidal fish species *Labrisomus xanti* Gill (Labrisomidae) (type host) and *Tomicodon zebra* (Jordan et Gilbert) (Gobiesocidae) from the coast of Oaxaca, Mexican Pacific. *Opegaster mezcal* sp. n. is characterised by the absence of papillae on the ventral sucker and the size and shape of the testes. Sequences of the D1–D3 domains from large subunit from nuclear DNA (28S) were generated for the new species and for another opecoelid species, *Opecoelus adsphaericus* Manter et Van Cleave, 1951. The new sequences of *Opegaster mezcal* sp. n. formed a clade, which is sister to *O. adsphaericus*. However, the phylogenetic relationships among genera of Opecoelinae remain unresolved because few species have been analysed; the addition of further taxa is necessary to better understand the evolution of the subfamily.

Key words: Digenea, Plagiorchiida, taxonomy, marine fish, Oaxaca, Mexico

The Opecoelidae Ozaki, 1925 is the richest of all trematode families, comprising over 1,000 described species arranged into about 100 genera (Martin 2024). Its presence has been recorded from marine and freshwater teleost fish distributed across multiple marine ecoregions. The genus *Opegaster* was proposed by Ozaki (1928), being diagnosed by the combination of a highly reduced cirrus-sac, a common anus, simple papillae on the aperture of the ventral sucker and vitelline follicles extending well into the forebody (Cribb 2005). The last character has been used to distinguishing between the genera *Opegaster* Ozaki, 1928 and *Opecoelus* Ozaki, 1925. However, some authors have considered this character unsatisfactory and, as a consequence, there have been several proposals to consider *Opegaster* a synonym of *Opecoelus* (Aken'Ova 2007). However, recognising the two genera based on extension of the vitellarium is so entrenched in the literature that they continue to be considered valid (Cribb 2005, Bray and Justine 2013).

During zoological expeditions to the rocky shores of the Mexican Pacific from January 2019 to January 2024, addressed to survey the parasite fauna of fish inhabiting intertidal pools, specimens of a hitherto unknown species of *Opegaster* were recovered from the intestines of two fish species, *Labrisomus xanti* Gill (Labrisomidae) and *Tomicodon zebra* (Jordan et Gilbert) (Gobiesocidae). Studies of these specimens revealed unique morphological traits. Therefore, the aim of the current study is to describe a new species, combining morphological and molecular features.

MATERIALS AND METHODS

Sampling and morphological analysis

The fish were collected from the rocky intertidal zone of two localities on the Pacific coast of Mexico: Barra de Cuatunalco (15.6859N, 96.3376W) and Playa Santa Elena (15.7354N, 96.8394W), municipality of San Pedro Pochutla, Oaxaca. The hosts were collected using an anaesthetic solution composed of 10% clove oil, ethanol and seawater (Munday and Wilson 1997, Griffiths 2000); the narcotised fishes were captured using dip nets. The fish were euthanised via cerebral pithing and then dissected. The intestine was extracted and examined using a stereoscopic microscope for the detection of trematodes. Once located, they were collected and transferred to another Petri dish containing 0.75% saline solution. For morphological studies, the trematodes were fixed in boiling 70% ethanol or 4% formalin. For molecular analyses, some specimens were preserved directly in vials containing 100% ethanol.

The specimens were stained with Harris hematoxylin and subsequently mounted as permanent preparations using Canada balsam. They were observed and measured using a Zeiss Axiostar plus optical microscope (Carl Zeiss AG, Oberkochen, Germany). Illustrations were hand-drawn with the aid of a camera lucida at-

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tached to the microscope, then digitised and edited using GIMP version 2.10.36. A Leica ICC50 HD microscope (Leica, Wetzlar, Germany), and Leica Application Suite capture software version 2.1.0 were used for photographs.

Some specimens were prepared for scanning electron microscopy (SEM) study. The process involved dehydration through a graded series of ethanol and critical point dried with carbon dioxide, and mounting on aluminium stubs with carbon tape. The specimens were then coated with a thin layer of gold and examined at 10 kV using a Hitachi Stereoscan SU1510 scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

DNA extraction, amplification and sequencing

The trematodes preserved in absolute ethanol were placed individually in tubes containing 100 µl of a digestive solution with 100 mM Tris-HCl pH 7.6, 200 mM NaCl, 0.5 M EDTA pH 8.0, 10% Sarkosyl, 0.1 mg/ml proteinase K, and ultrapure water. These were incubated at 56°C overnight. Subsequently, the digestion solution was incubated at 95°C for 15 minutes to inactivate the proteinase K. Genomic DNA was extracted from the supernatant using the DNAzol reagent (Invitrogene, Carlsbad, California, USA).

The amplification of the 28S ribosomal DNA was performed using polymerase chain reaction (PCR) with the primers Forward 391 (5'-AGCGGAGGAAAAGAAACTAA-3') (Carreno and Nadler 2003) and Reverse 536 (5'-CAGCTATCCTGAGGGAAAC-3') (García-Varela and Nadler 2005). A region of the gene cytochrome oxidase subunit 1 (*cox* 1) from mitochondrial DNA was amplified using the forward primer JB3 (5′- TTT TTT GGG CAT CCT GAG GTT TAT -3') and reverse primer JB4 (5′-TAAAGAACATAATGAAATTG -3′) (Bowles and McMannus 1993). The reaction mixture contained a total 25 µl, included 2.5 µl of Platinum 10X Buffer, 1.5 µl of MgCl2, 0.5 µl of dNTPs, 1 µl of each primer, 0.125 µl of Platinum Taq polymerase (Invitrogene), 16.375 µl of distilled water, and 2 µl of genomic DNA.

PCR cycling parameters for rDNA amplifications included denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, annealing at 50 °C for 1 min for both molecular markers, and extension at 72 °C for 1 min, followed by a post-amplification incubation at 72 °C for 7 min. Sequencing reactions were performed with the primers mentioned above plus two internal primer for 28S rDNA, 503 (5'–CCTTGGTCCGTGTTTCAAGACG-3') (Stock et al. 2001), 504 (5'–CGTCTTGAAACACGGACTAAGG-3') (García-Varela and Nadler 2005), using ABI Big Dye (Applied Biosystems, Boston, Massachusetts, USA) terminator sequencing chemistry. Reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contigs were assembled and base-calling differences were resolved using Codoncode Aligner version 11.0 (Codoncode Corporation, Dedham, Massachusetts, USA).

Alignment and phylogenetic analyses

The alignment from 28S rDNA included five new sequences from *Opegaster* sp. from Oaxaca Mexico, plus a single sequence labelled as *Opegaster* sp. 1 (MK648306), two sequences identified as *Opecoelus adsphaericus* from the woolly sculpin *Clinocottus analis* (Girard) from Baja California, Mexico, and 23 sequences of the family Opecoelidae available in the GenBank database. The alignment was constructed using the software SeaView version 5.0.5 (Gouy et al. 2010) and then manually adjusted using Mesquite version 3.81 (Maddison and Maddison 2023). The final alignment was 1,359 nucleotides long and included a total of 32 taxa.

Table 1. Taxa and GenBank accession number included in the phylogenetic analysis (sequences in bold were generated in the present study).

Trematode taxa	28S rDNA	Reference
Anomalotrema koiae	KU320595	Bray et al. (2016)
Coitocaecum capense	OR129145.1	Vermaak et al. (2023)
Coitocaecum sp.	OR129276	Vermaak et al. (2023)
Discoverytrema gibsoni	MH161430	Sokolov et al. (2019)
Discoverytrema markowskii	MH161431	Sokolov et al. (2019)
Hamacreadium mutabile	KJ001209	Andres et al. (2014a)
Hamacreadium sp. 1	MN067856	Martin et al. (2019a)
Helicometra boseli	KU320600	Bray et al. (2016)
Helicometra fasciata	KU320597	Bray et al. (2016)
Helicometra manteri	KJ701238	Andres et al. (2014b)
Holsworthotrema chaoderma	MK052938.1	Martin et al. (2019b)
Holsworthotrema enboubalichthys	MK052937.1	Martin et al. (2019b)
Macvicaria bartolii	KR149464	Antar et al. (2015)
Macvicaria crassigula	KJ701237	Andres et al. (2014b)
Opecoeloides fimbriatus	KJ001211	Andres et al. (2014a)
Opecoeloides furcatus	AF151937	Tkach et al. (2000)
Opecoelus adsphaericus	**PV577184, PV577185**	**Present work**
***Opegaster mezcal* sp. n.**	**PV577186–PV577190**	**Present work**
Opegaster sp.	MK648306	Pérez-Ponce de León and Hernández Mena (2019)
Plagioporus hageli	KX553950	Fayton and Andres (2016)
Plagioporus kolipinskii	KX553952	Fayton and Andres (2016)
Podocotyle reflexa	OR439006.1	Sokolov et al. (2023)
Podocotyle atomon	OR439003.1	Sokolov et al. (2023)
Pseudopecoeloides tenuis	KU320605	Bray et al. (2016)
Pseudopecoelus vulgaris	MH161436	Sokolov et al. (2019)
Stephanostomum pristis	DQ248222	Bray et al. (2005)
Zalophotrema hepaticum	AY222255	Olson et al. (2003)

The phylogenetic analysis was performed by using the Bayesian Inference (BI) method, which was conducted using MrBayes 3.2.7 software with the computational resource Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway (Miller et al. 2010). The resulting phylogenetic tree was visualised and edited using FigTree version 1.4.2 (Rambaut and Drummond 2007). Finally, uncorrected *p* distances were estimated with the 28S rDNA and *cox*1 by using the MEGA program (Kumar et al. 2016).

RESULTS

Opegaster mezcal sp. n. Figs. 1, 2, 3A

Zoobank number for species:
urn:lsid:zoobank.org:pub:300369B4-06E5-4798-9459-DE449404EA5F

Description (based on 15 specimens; additional data from two specimens observed with SEM; all measurements are given in µm unless otherwise stated, with minimum and maximum values, followed by the mean in parentheses). Opecoelidae: Opecoelinae. Body elongated, 0.8 mm–1.27 mm (0.99 mm) long, tapering slightly towards oral sucker; maximum width 90–250 (180) at ventral sucker level. Body tegument smooth, without any spines. Anterior part of body 200–370 (280) long, representing 25.0–31.1% (28.1%) of total body length.

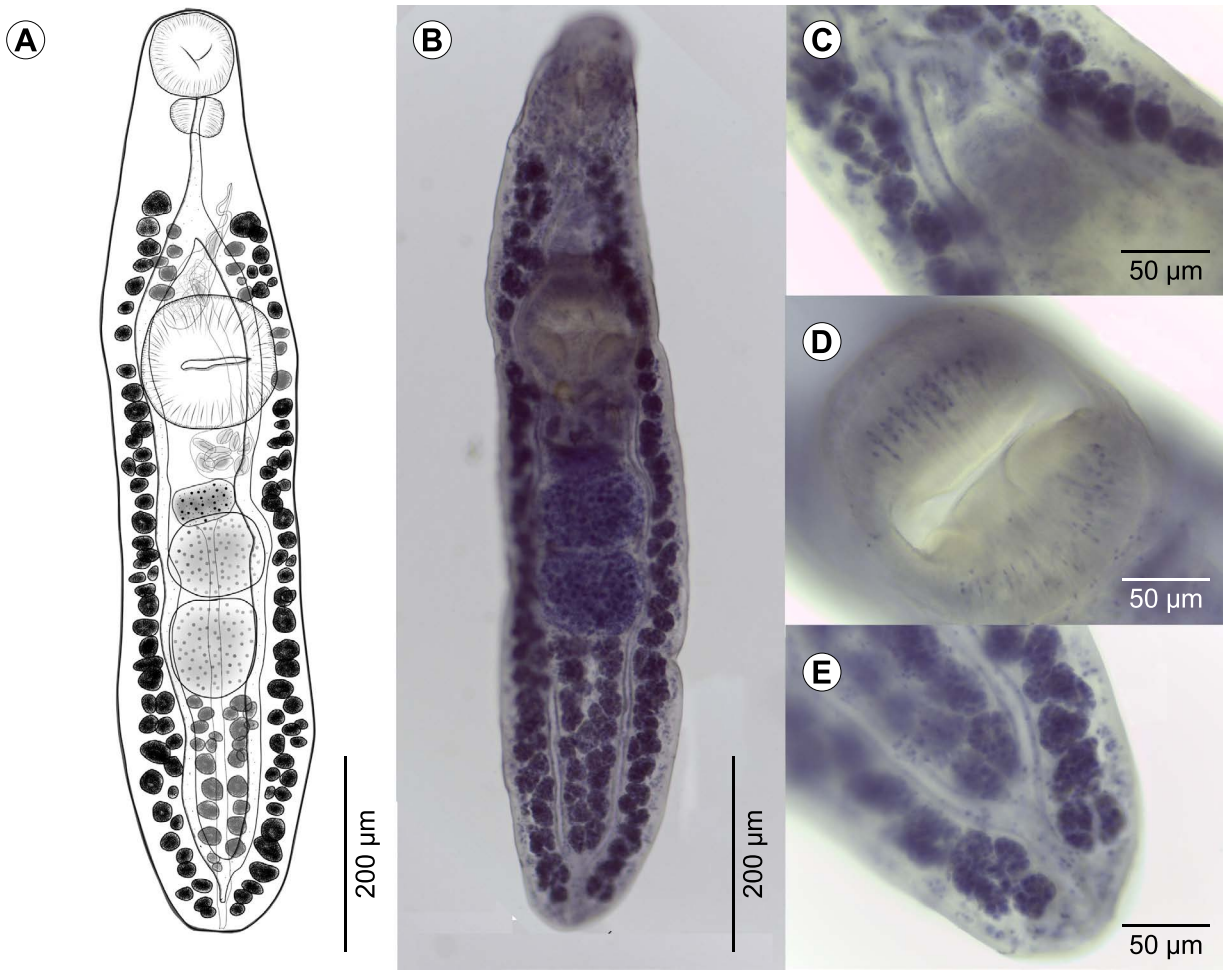

Fig. 1. *Opegaster mezcal* sp. n. from *Labrisomus xanti* Gill, Mexico. **A** – schematic representation, ventral view; **B** – photomicrograph of whole mount, ventral view; **C** – intestinal bifurcation and seminal vesicle, ventral view; **D** – ventral sucker; **E** – posterior end, ventral view.

Oral sucker subterminal, 60–90 (80) long and 60–100 (70) wide; SEM reveals clusters of papillae on sides of oral sucker near mouth (Fig. 3B). Ventral sucker 120–170 (140) long and 110–180 (140) wide, completely devoid of papillae (Fig. 3D). Length ratio of oral sucker to ventral sucker 1 : 1.5–2 (1 : 1.71), width ratio 1 : 1.4–2 (1 : 1.79). Mouth subterminal. Prepharynx short and sometimes imperceptible; pharynx muscular, subglobular, 30–70 (50) long and 40–70 (50) wide. Oesophagus long, 50–90 (70) long. Intestinal bifurcation at 190–250 (230) from anterior margin of ventral sucker reaching close to posterior end, joining at anus that appears ventrally open to exterior.

Testes of similar size, subspherical, smooth, in tandem, postovarian, located approximately in posterior half of body. Anterior testis 50–130 (97) long and 70–170 (110) wide; posterior testis 50–130 (100) and 50–150 (98). No intertesticular space. Post-testicular space 130–400 (270), representing 20.2–37% (27.3%) of total body length.

Terminal part of male genitalia comprises seminal vesicle, cirrus sac and ejaculatory duct. External seminal vesicle sac-like 70–130 (100) long and 40–70 (60) wide, dorsal to ventral sucker, extending from intestinal bifurcation to near midpoint of ventral sucker. Cirrus sac membraneous, ejaculatory duct merges with the female duct to form hermaphroditic duct. Hermaphroditic duct opens through ventral genital pore located sinistrally anterior to ventral sucker near midpoint of oesophagus, surrounded by multiple papillae that are only visible under SEM (Fig. 3C).

Ovary kidney-shaped to slightly lobulated, 20–60 (50) long and 40–90 (70) wide, pre-testicular, just anterior to anterior testis. Seminal receptacle absent. Vitelline follicles globular and distributed along body margins in bands extending towards near midpoint of oesophagus and post-testicularly restricted by margins of intestinal caeca.

Uterus pre-ovarian, situated between anterior end of ovary and genital pore. Eggs 35–45 (38) long and 18–29 (23) wide. Excretory vesicle I-shaped, extending to ovary and terminating in post-testicular region; excretory pore terminal.

Type host: *Labrisomus xanti* Gill (Labrisomidae), voucher Colección de Referencia Parásito-Huésped (CRPH), Laboratorio de Zoología Acuática, Facultad de Ciencias, Universidad Nacional Autónoma de México, Mexico (CRPH-P0023). (CRPH-P0023).

Additional host: *Tomicodon zebra* (Jordan et Gilbert) (Gobiesocidae), voucher (CRPH-P0024).

Type locality: Cuatunalco, Oaxaca, Mexico (15.6859N, 96.3376W).

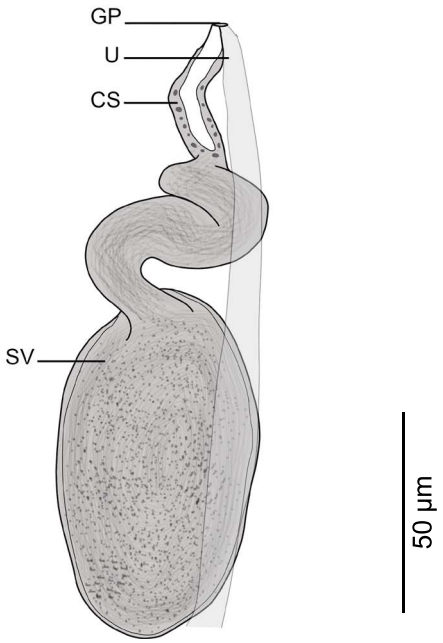

Fig. 2. *Opegaster mezcal* sp. n. from *Labrisomus xanti* Gill, Mexico. Schematic representation of terminal genitalia. CS – cirrus sac; GP – genital pore; SV – seminal vesicle; U – uterus.

Additional locality: Playa Santa Elena, Oaxaca, Mexico (15.7354N, 96.8394W).

Site in host: Intestine (anterior and middle part).

Type material: Holotype – Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México. Mexico (CHNE 12327), three paratypes (CNHE 12328, CRPH-T0041, CRPH-T0042).

Representative DNA sequences: Genbank accession numbers: PV577186–PV577190.

Prevalence: 90% (*Labrisomus xanti*, type host), 20% (*Tomicodon zebra*).

Etymology: The species name of this trematode refers to the worldwide-known traditional Mexican agave-based distilled alcoholic beverage, which carries deep historical and cultural significance in the state of Oaxaca.

Remarks: The new species is included in the genus *Opegaster* given that its general features agree with those described in the Cribb's key (2005), namely an elongated and oval body, a simple oral sucker, ventral sucker larger than the oral sucker, intestinal caeca united at the posterior end of the body to form a common anus, testes spherical to oval, smooth and arranged consecutively, seminal vesicle naked and restricted to the anterior part of the body, cirrus sac small, genital pore sinistral, ovary smooth or slightly lobulated, uterus

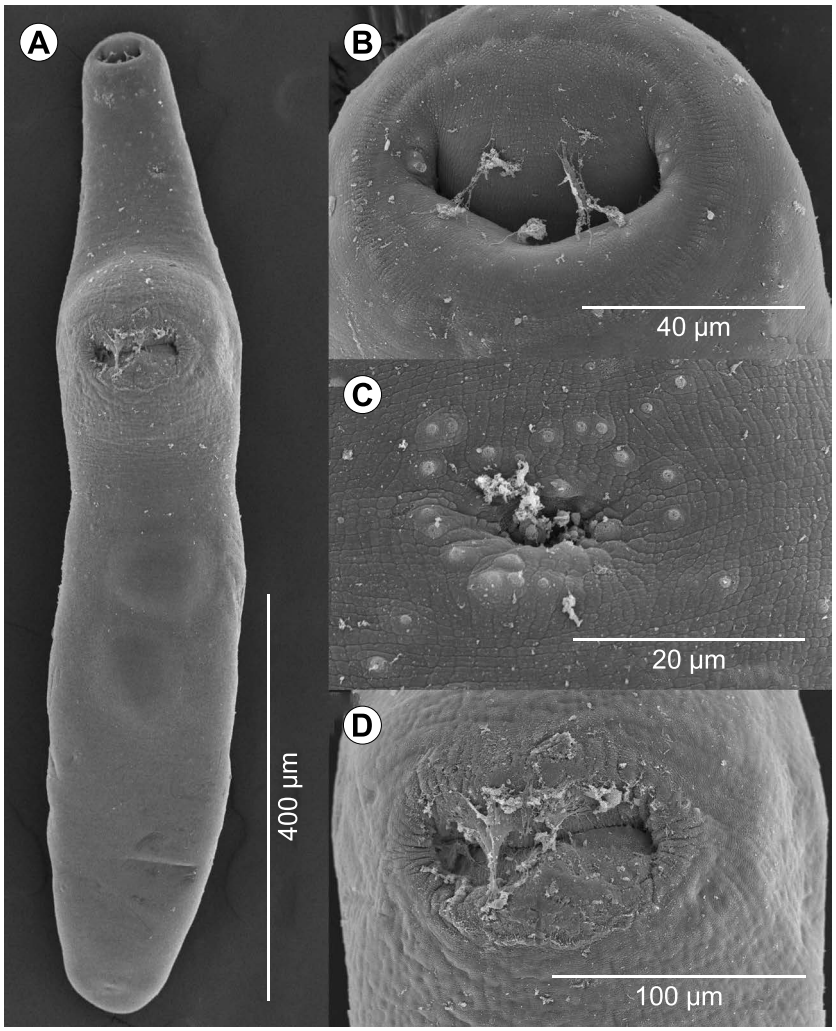

Fig. 3. *Opegaster mezcal* sp. n. from *Labrisomus xanti* Gill, Mexico; scanning electron micrographs. **A** – total view; **B** – oral sucker; **C** – genital pore; **D** – ventral sucker.

located between the ovary and the genital pore, and vitelline follicles extended towards the anterior part of the body.

The new species differs from most of the species of *Opegaster* by the absence of papillae on the ventral sucker. This is a characteristic shared by only three species within the genus: *Opegaster pritchardae* Overstreet, 1969, *O. recta* Ozaki, 1928 and *O. tamori* Yamaguti, 1938, but *Opegaster mezcal* sp. n. is distinguishable from these three species by the shape and size of the testes, which are consistently smooth and oval or spherical, while they are irregularly lobulated and transversely elongated in *O. recta* and *O. tamori*, becoming trilobed or bilobed, respectively (Ozaki 1928, Yamaguti 1938). The testes in *O. pritchardae* seem to be smooth, but have an irregular shape and different disposition in the body than in *O. mezcal* sp. n. The post-testicular space in *O. pritchardae* is quite small, representing 9–14% of the body length (Overstreet 1969), whereas this space represents about 27% of the total body length in the new species.

Other differences between *O. mezcal* sp. n. and *O. pritchardae* include the smaller dimensions of some organs in the new species, such as the oral sucker, the ventral sucker, and the ovary. Furthermore, the oesophagus in *O. pritchardae* is described as "shorter than the pharynx or more than twice its length," whereas the oesophagus is long, approximately twice the length of the pharynx in *O. mezcal* sp. n. The vitelline follicles in the new species extend halfway between the ventral sucker and pharynx, whereas the illustration of Overstreet (1969) shows that they extend beyond the genital pore, reaching the posterior margin of the pharynx in *O. pritchardae*. In addition, an evident difference between the new species and *O. tamori* is the seminal vesicle, which extends posteriorly from the ventral sucker to halfway to the ovary, whereas in *O. mezcal* sp. n. it only extends to near the midpoint of the ventral sucker.

Phylogenetic analyses

The phylogenetic tree inferred from 28S rDNA sequences found that all sequences of Opecoelinae formed a clade with a high probability value. The tree also showed that the genera *Coitocaecum* Nicoll, 1915, *Opecoeloides* Odhner, 1928, *Pseudopecoelus* von Wicklen, 1946, *Pseudopecoeloides* Yamaguti, 1940, *Anomalotrema* Zhukov, 1957 and *Discoverytrema* Gibson, 1976 formed independent subclades with high probabilities values (Fig. 4). The five new sequences for *O. mezcal* sp. n. formed a subclade together with an isolate of *Opegaster* sp 1. (MK648306) from an unidentified fish from the genus *Tomicodon* Brisout de Barneville from Barra de Cuatunalco, Oaxaca, Mexico (Fig. 4).

The genetic distances among the five isolates of *O. mezcal* sp. n. and *Opegaster* sp. 1. (MK648306) ranged from 0.1–0.3% for 28S rDNA and to *Opecoelus adsphaericus* ranged from 7–9%. Additionally, the genetic distance estimated with the *cox*1 between the two isolates of *O. adsphaericus*, generated in this study, was zero and to *O. mezcal* sp. n. ranged from 13%–14%.

DISCUSSION

This work provides molecular data from specimens belonging to the genera *Opecoelus* and *Opegaster*, which are two of the most species-rich of the family Opecoelidae, with 42 and 21 described species, respectively (Aken'Ova 2007, Bray and Justine 2013). General morphology of one of these species, especially the shape and length of the body, the size of testes, the intertesticular space, and the shape and position of ventral sucker, allow us to identify it as *Opecoelus adsphaericus*, which is well-known from California, commonly recorded from its type host, the woolly sculpin *Clinocottus analis* (Manter and Van Cleave 1951, Banerjee 1965, Aguilar-Aguilar and Martorelli 2024), whereas the other represents a new species. Neither of these genera, *Opecoelus* or *Opegaster*, have been included in previous phylogenetic studies of the family Opecoelidae (e.g., Curran et al. 2007, Bray et al. 2016, Martin et al. 2018, 2019a,b, Sokolov et al. 2020, 2022).

Therefore, the inclusion of data for two species in this work contributes to the understanding of the group by adding a clade where these two taxa are sisters, and exploring the relationships with other genera of the subfamily Opecoelinae. In the present phylogenetic arrangement, the new sequences of *Opegaster mezcal* sp. n. formed a subclade together with an isolate identified as *Opegaster* sp 1. (MK648306) from *Tomicodon* sp. from Barra de Cuatunalco, Oaxaca (Pérez-Ponce de León and Hernández-Mena 2019). The low genetic divergence suggests that this isolate belongs to the new species described herein.

This clade is sister to a subclade formed by two isolates identified as *O. adsphaericus*, forming a well-supported clade associated with another one, which includes sequences of other Opecoelinae. The relation of this clade with remaining terminals and the arrangement of clades of Opecoelidae are, in general, in concordance with proposal by Sokolov et al. (2022), who recently have established the general context of the phylogeny and classification of opecoelids. Further studies, including an increase of the number of analysed sequences, are necessary to elucidate the phylogenetic relationships of opecoelids.

In this study, we observed that the extent of the vitelline follicles consistently facilitates the discrimination of trematodes as members of *Opegaster* or *Opecoelus*, being an easily recognisable characteristic for easy discrimination of taxa in this family (Bray and Justine 2013). Although the use of this feature has been criticised by some authors as insufficient to distinguish between genera (Crowcroft 1947, Cribb 1985, Shimazu 1988, Aken'Ova 2007), we agree with those opinions that consider it to be the main criterion distinguishing between both genera (Manter 1940, 1954, Yamaguti 1958, Banerjee 1965, Bray and Cribb 1989, 2013, Cribb 2005).

In this work, the use of scanning electron microscopy (SEM) allowed us to confirm the absence of papillae on the ventral sucker and to observe other ultrastructural characters that have not been previously mentioned in the descriptions of opecoelid genera, such as papillae observed primarily around the genital pore and the oral sucker, which are only perceptible with this technique. Except for Cribb (1985),

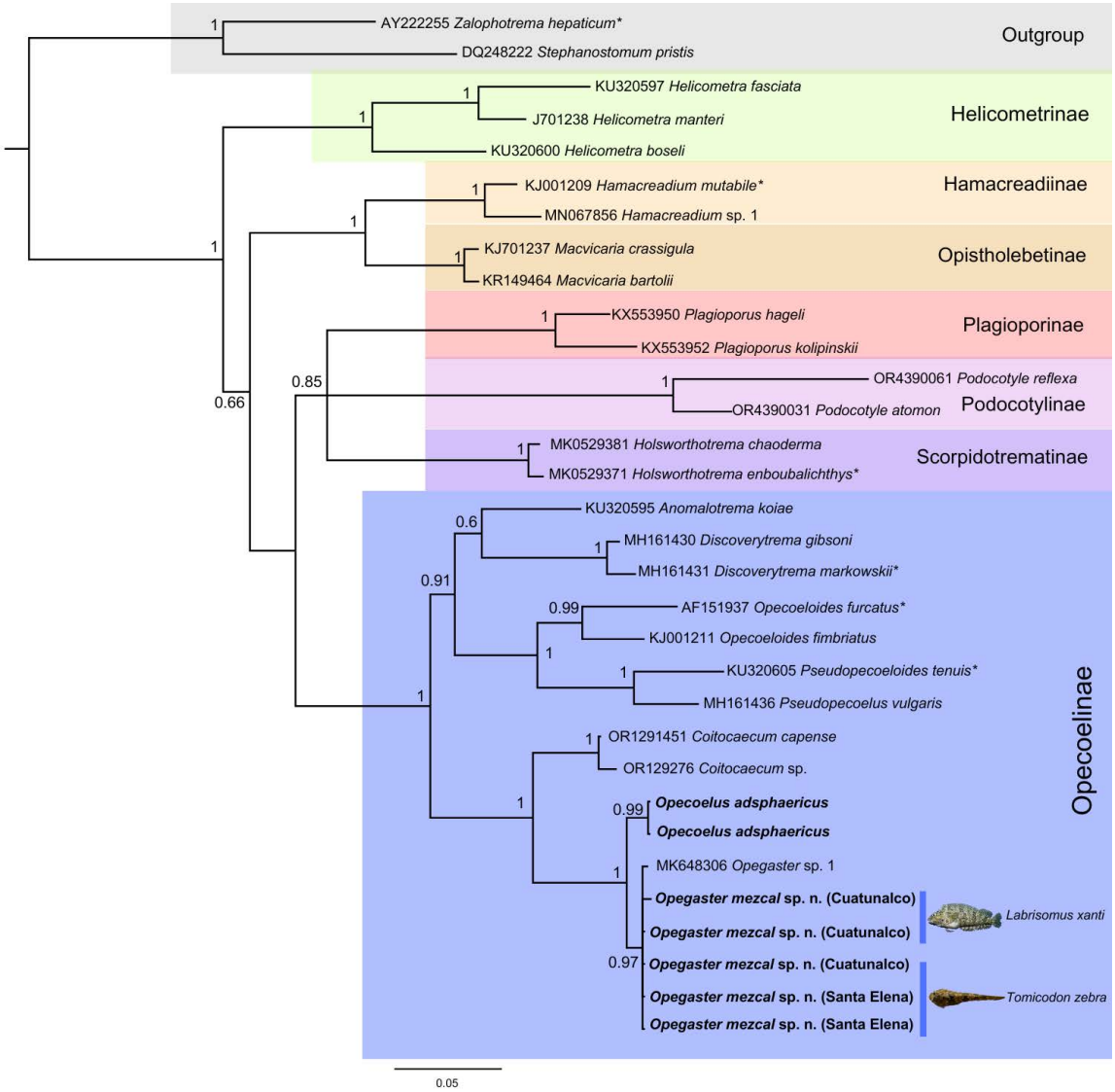

Fig. 4. Bayesian tree of the family Opecoelidae inferred from 28S rDNA sequences. The species *Opegaster mezcal* sp. n. and *Opecoelus adsphaericus* Manter et Van Cleave, 1951, analysed in this study, are shown in bold. Asterisk (*) indicates type species of individual genera.

who analysed the morphological variation of *Opecoelus variabilis* Cribb, 1985, no other studies have used SEM for species of the genera *Opegaster* or *Opecoelus*. Since SEM could contribute to the detection of ultrastructural characters potentially valuable to species delimitation, we consider it a desirable complement to the anatomical study of these morphologically uniform trematodes.

Host specificity in marine helminths is highly variable. While Marcogliese (2002) suggested that helminths in marine environments tend to exhibit low host specificity, more recent studies have demonstrated that many fish trematodes show much higher specificity than previously assumed (Miller et al. 2011). In this context, *O. mezcal* sp. n. has been found in two unrelated species of predatory fish that primarily feed on small crustaceans (Froese and Pauly 2024). Its identification was rigorously conducted using both morphological and molecular data, unequivocally confirming that it is the same species in both hosts. Given that metacercariae of opecoelids are encysted in crustaceans and mollusks as a transmission route to their definitive hosts (Cribb 1985, Cribb et al. 2001), it is plausible to assume that this new species follows a similar mechanism. However, considering that host specificity is difficult to predict (Miller et al. 2011), further studies on diet and transmission are essential to understand better the factors determining transmission in fish hosts.

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Authors' contribution. R.A.A. initiated the study and acquired the funding, R.A.A. and R.I.S.P. participated in the field work. R.I.S.P. processed the samples, performed the morphological analysis and edited the figures. M.G.V. provided material and equipment for the molecular study. M.G.V. and R.I.S.P. performed the molecular and phylogenetic analysis. All authors contributed substantially to the manuscript text, participated in its revision and approved its final draft.

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