
# UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
## POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE BIOLOGÍA

SISTEMÁTICA

## TAXONOMÍA INTEGRATIVA DE HAPLOPÓRIDOS (TREMATODA) PARÁSITOS DE *MUGIL* SPP. (MUGILIDAE) DE LAS COSTAS DE MÉXICO

# TESIS

QUE PARA OPTAR POR EL GRADO DE:

**DOCTOR EN CIENCIAS**

PRESENTA:

**LEOPOLDO ANDRADE GÓMEZ**

**TUTOR PRINCIPAL: DR. MARTÍN GARCÍA VARELA, INSTITUTO DE BIOLOGÍA, UNAM**

**COMITÉ TUTOR: DR. GERARDO PÉREZ-PONCE DE LEÓN, INSTITUTO DE BIOLOGÍA, UNAM**
**DR. JOSÉ JAIME ZUÑIGA VEGA, FACULTAD DE CIENCIAS, UNAM**

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**COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS**

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**ASUNTO: Oficio de Jurado**

**M. en C. Ivonne Ramírez Wence**
**Directora General de Administración Escolar, UNAM**
**P r e s e n t e**

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **18 de octubre de 2021** se aprobó el siguiente jurado para el examen de grado de **DOCTOR EN CIENCIAS** del estudiante **ANDRADE GÓMEZ LEOPOLDO** con número de cuenta **307012052** con la tesis titulada **"Taxonomía integrativa de haploporidos (Trematoda) parásitos de *Mugil* spp. (Mugilidae) de las costas de México",** realizada bajo la dirección del **DR. JOSÉ MARTÍN GARCÍA VARELA,** quedando integrado de la siguiente manera:

| | |
|---|---|
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Sin otro particular, me es grato enviarle un cordial saludo.

**A T E N T A M E N T E**
**"POR MI RAZA HABLARÁ EL ESPÍRITU"**
Ciudad Universitaria, Cd. Mx., a 06 de diciembre de 2021

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Al mejor amigo de vida, el Miller

LFGAZC

***La luz de la Luna siempre brillará intensamente***

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**RESUMEN**

La especie es considerada como la unidad básica en los estudios de biodiversidad y conservación. El concepto de especie puede estar clasificado en dos formas básicas, los conceptos ontológicos y operacionales. Los primeros señalan qué son las especies, como lo son el *Concepto evolutivo de especie*; *Concepto unificado de especie*; y *Concepto general de linaje.* Mientras que los operacionales mencionan cómo reconocer e identificar a las especies y es la suma de la información proporcionada de cada concepto operacional que permite dilucidar a la especie de conceptos ontológicos. En este sentido, la taxonomía integrativa emplea diferentes criterios de información en la delimitación de especies, como son aspectos ecológicos, moleculares, biogeográficos, evolutivos entre otros. Particularmente, en digeneos la utilización de estos caracteres, así como de la taxonomía alfa han sido fundamentales, en la delimitación y descripción de las especies.

Los miembros de la familia Haploporidae (Nicoll, 1914) son tremátodos de 1 a 2 mm que se alojan en el tubo digestivo de peces distribuidos globalmente. Este grupo se caracteriza por presentar un saco hermafrodita y un solo testículo, así como por presentar un tegumento frágil. La familia está comprendida por ocho subfamilias las cuales fueron reconocidas combinando caracteres morfológicos y moleculares. Hasta la fecha se han descrito más de 140 especies de haplopóridos, de las cuales 70 se han reportado en 22 especies de lisas de la familia Mugilidae. En México, se han reportado diez taxones de haplopóridos que corresponden con tres subfamilias, un registro de Hapladeninae, tres de Forticulcitinae, y varios registros de Chalcinotrematinae.

Los mugílidos ofrecen un sistema excelente al ser peces euritermos y eurihalinos para estudiar las relaciones parásito-huésped en sus aspectos geográficos, ecológicos y evolutivos. En las costas de México, se han registrado tres especies de mugílidos del género *Mugil* Linnaeus, 1758, *M. cephalus* Linnaeus, 1758, *M. curema* Valenciennes,

1836, y *M. hospes* Jordan y Culver, 1895; esta última restringida al Golfo de México. Estos peces han sido sujetos a numerosos estudios helmintológicos y una gran diversidad de parásitos se han reportado. Los digéneos son el grupo de helmintos mejor representado con 15 especies, sin embargo, solamente ocho de estas 15 especies son adultos y pertenecen a tres familias, Haplosplanchnidae Poche, 1926, Hemiuridae Looss, 1899, y Haploporidae Nicoll, 1914. Con base en lo anterior, este sistema huésped-parásito es ideal para explorar de forma detallada la composición de haplopóridos que están asociados a mugílidos en ambas costas de México dado que los registros son escasos y dispersos. El objetivo principal de la tesis fue emplear diversos caracteres, como morfológicos, moleculares y biogeográficos para establecer los límites entre especies de haplopóridos que parasitan a lisas (*Mugil* spp.) en regiones costeras de México y describir la diversidad de este grupo de parásitos.

En los años 2018, 2019 y 2020, se muestrearon 101 individuos de *Mugil cephalus*, 135 de *Mugil curema* y 74 de *Mugil* sp. en 42 localidades de ambas costas de México, así como en seis localidades de Centroamérica y cuatro localidades de Venezuela. Se obtuvieron secuencias parciales de dos marcadores moleculares nucleares, el espaciador interno transcrito 2 (ITS2, 113 secuencias) y los dominios D1 + D3 de la subunidad mayor del DNA ribosomal (28S, 204 secuencias) y dos marcadores mitocondriales, el citocromo oxidasa subunidad 1 (*cox1,* 91 secuencias) y la deshidrogenasa subunidad 1 (*nad1,* 119 secuencias).

Con base en los datos generados durante los estudios del doctorado, se publicaron cuatro artículos de investigación. En el primero se realizó la descripción de una nueva especie de haplopórido de la subfamilia Chalcinotrematinae, nombrada *Saccocoelioides macrospinosus* Andrade-Gómez, Sereno-Uribe y García-Varela, 2019., siendo la especie número 24ª que se reconoce dentro del género, y la cuarta especie descrita en México. En

el segundo artículo de investigación se realizó un estudio integrativo del haplopórido *Saccocoelioides lamothei* Aguirre-Macedo y Violante-González, 2008 donde se observó que se encuentra asociado a cinco familias de huéspedes, incluyendo a los mugílidos, quienes parecen ser parte fundamental de su dispersión. Asimismo, *Saccocoelioides lamothei* presenta la mayor variación morfológica, con una amplia distribución geográfica.

En el tercer artículo se analizaron secuencias del DNA ribosomal y caracteres morfológicos y la ultraestructura de diferentes especímenes de haplopóridos colectados en *Mugil curema* y *Mugil cephalus* en 27 localidades de las costas del Océano Pacífico. Asimismo, se obtuvieron fotografías de organismos (*fotogenóforos*) con el objetivo de vincular las secuencias de DNA con cada individuo procesado. La información que se obtuvo permitió describir dos géneros y cuatro especies nuevas de la subfamilia Forticulcitinae.

En el cuarto artículo se describieron tres nuevas especies de la subfamilia Forticulcitinae, *Ekuarhuni mexicanus, Forticulcita macropharyngis,* y *Forticulcita venezuelensis*; que parasitan a dos especies del género *Mugil*. Dos de ellas se distribuyen en el Golfo de México y una tercera en Venezuela. Además, se reconoció un cuarto linaje que se designó como *Overstreetoides* sp. Este linaje no se logró describir por falta de especímenes adultos. Finalmente, se realizó por primera vez una clave taxonómica de la subfamilia Forticulcitinae.

Finalmente, en el presente trabajo se analizó de forma integrativa diferentes fuentes de evidencia para describir la diversidad de haplopóridos en mugilídos. Asimismo, los datos sugieren que los haplopóridos que pertenecen a la subfamilia Chalcinotrematinae han sido transportados por los mugilídos y que mediante transferencia horizontal han podido colonizar nuevos huéspedes y que en ellos han

divergido especies de esta subfamilia. Mientras los datos observados en la presente tesis sugieren que los haplopóridos que pertenecen a la subfamilia Forticulcitinae, podría existir una concordancia filogenética entre mugilídos y forticulcitinos. Es decir, una historia evolutiva conjunta entre estos dos grupos.

**ABSTRACT**

Species are considered both as the basic unit in biodiversity and conservation studies, and as a taxonomic category; yet their definition is a fundamental challenge in biology. Species concepts can be classified in two basic groups: ontological and operational. The former defines what species are, such as the *Evolutionary*; *Unified species*; and *General Lineage concepts*. Meanwhile, the operational definitions mention how to recognize and identify species as the sum of the information provided by each operational concept that allows elucidating the ontological concepts. Integrative taxonomy then uses different information criteria in species delimitation, such as ecological, molecular, biogeographic, and evolutionary aspects among others. Particularly, in parasites such as digeneans, the use of these characters, as well as alpha taxonomy, have been fundamental in the delimitation and description of the species.

Members of Haploporidae Nicoll, 1914 are trematodes of approximately 1 to 2 mm that inhabit the gastrointestinal tract of globally distributed fishes. This group is characterized by a hermaphroditic sac and a single testis, as well as the presence of a fragile tegument. The family is comprised by eight subfamilies that were recognized by combining morphological and molecular characters. Approximately 140 haploporid species have been described, with 70 species reported in 22 fish host species of the mullet family (Mugilidae) alone. In Mexico, ten haploporid taxa have been reported corresponding to three subfamilies, one record in Hapladeninae, three of Forticulcitinae, and several records in Chalcinotrematinae.

Mugilids offer an excellent opportunity to study parasite-host relationships in the context of their geography, ecology, and evolutionary history. In both coasts of Mexico, three mullet species belonging to the genus *Mugil* Linnaeus have been recorded, *M. cephalus* Linnaeus, *M. curema* Valenciennes and *M. hospes* Jordan and Culver, the latter

being restricted to the Gulf of Mexico. These mullets have been subjected to numerous helminthological surveys and a large diversity of parasites has been recorded. Digeneans are the best represented helminth group with 15 species recorded, however, only eight of these are adults belonging to three families, Haplosplanchnidae Poche, 1926, Hemiuridae Looss, 1899, and Haploporidae Nicoll, 1914. Therefore, this host-parasite system is ideal to explore in detail the composition of haploporids that are associated with mullets on both coasts of Mexico since records are scarce and scattered. The main objective of the thesis is to use integrative taxonomy by employing multiple character types to establish species boundaries among haploporids infecting mullets (*Mugil* spp.) in coastal areas of Mexico, and to describe the diversity of this group of parasites.

In 2018, 2019, and 2020, we sampled 101 individuals of *Mugil cephalus*, 135 of *Mugil curema* and 74 of *Mugil* sp. from 42 localities on both coasts from Mexico, as well as in six localities of Central America and four localities of Venezuela. We obtained sequences of two nuclear molecular markers, internal transcribed spacer two (ITS2, 113 sequences) and the D1 + D3 domains of the major subunit of ribosomal DNA (28S, 204 sequences) and two mitochondrial markers, cytochrome oxidase subunit 1 (*cox1*, 91 sequences) and dehydrogenase subunit 1 (*nad1*, 119 sequences).

We published data generated during the current studies in four papers. In the first one, we described a new haploporid species of the subfamily Chalcinotrematinae, named *Saccocoelioides macrospinosus.* Andrade-Gómez, Sereno-Uribe y García-Varela, 2019, being the 24th species recognized within the genus, and the fourth species described in Mexico. In the second manuscript, we carried out an integrative study of the haploporid *Saccocoelioides lamothei* Aguirre-Macedo and Violante-González, 2008; this species was recovered in five host families, including mugilids, which plays a principal

role for its dispersal. Likewise, this species presents the greatest morphological variation, with a wide geographic distribution.

In the third reseach, we analyzed ribosomal DNA sequences and morphological and ultrastructural characters from different haploporid specimens collected from *Mugil curema* and *Mugil cephalus* in 27 localities of the Pacific Ocean coasts. Furthermore, we obtained pictures of organisms (*photogenophores*) to match DNA sequences with each processed individual. The information obtained allowed us to describe two new genera and four species of Forticulcitinae.

In the fourth manuscript, we described three new species of the subfamily Forticulcitinae that parasitize *Mugil* spp., *Ekuarhuni mexicanus, Forticulcita macropharyngis,* and *Forticulcita venezuelensis*. Two of them are distributed in the Gulf of Mexico and a third one in Venezuela. We also recognized and a fourth lineage designated as *Overstreetoides* sp. This lineage could not be described due to the lack of adult specimens. In addition, we elaborated the first taxonomic key to the subfamily Forticulcitinae.

Finally, in the present work, different sources of evidence were analyzed in an integrative way to describe the diversity of haploporids in mugilids. Likewise, the data suggest that the haploporids belonging to the Chalcinotrematinae have been carried out by the mugilidae and that through horizontal transfer they have been able to colonize new hosts and diverged within them. While the data observed in the present thesis suggest that the haploporids that belong to the Forticulcitinae, there could be a phylogenetic concordance between mugilids and forticulcitins. In other words, a close evolutionary history between these two groups.

# I. INTRODUCCIÓN.

## I. I. Taxonomía integrativa

La especie es considerada como la unidad básica y fundamental en los estudios de biodiversidad y conservación; esta es considerada como punto de referencia y comparación en todos los campos del conocimiento biológico (Valdecasas et al. 2013; Sukumaran y Gopalakrishnan 2015). En este sentido, contar con un concepto de *especie* claro y funcional es primordial (Aldhebiani 2018). Sin embargo, el concepto de *especie* en el campo de la biología es uno de los temas con mayor controversia y un vasto número de publicaciones se han generado alrededor de él. El concepto de especie se ha definido al menos un centenar de veces (Zachos 2016). Estas definiciones dependen en su mayoría de la rama biológica en la cual se enfocan, demostrando que no es evidente la forma en cómo definir lo que son las especies.

Mayden (1997) analizó diferentes conceptos de *especie* y concluyó que los conceptos deberían estar clasificados en dos formas básicas: 1) Conceptos ontológicos o primarios; 2) Conceptos operacionales o secundarios (Fig. 1). Los primeros señalan qué son las especies. De los diversos conceptos de *especie* solamente son tres los que definen qué son las especies, *Concepto evolutivo de especie*; *Concepto unificado de especie*; y *Concepto general de linaje* (Mayden 1997, 1999; Wiley y Mayden 2000; de Queiroz 1999, 2007). Estos conceptos tienen como fundamento que las especies son "*linajes*", los cuales se definen como secuencias de entidades biológicas conectadas por relaciones de ascendencia-descendencia que cambian con el tiempo (de Queiroz 1999). Los conceptos ontológicos son considerados bajo el enfoque del *monismo*, es decir, que un concepto aplica para todos los organismos. (Wiley y Mayden 2000; de Queiroz 1999, 2007; Cuadro 1). Estos *linajes* son los que tratamos de encontrar y describir, pero para realizar estas dos tareas requerimos de los conceptos operacionales o secundarios de especie.

Los conceptos operacionales o secundarios de especie mencionan cómo reconocer e identificar a las especies (Mayden 1997, 1999). Estos conceptos señalan de manera práctica cómo reconocer a las especies, sin embargo, no todos los conceptos operacionales o secundarios son aplicables para todos los organismos. Algunos ejemplos de estos conceptos operacionales son el *biológico*, *morfológico*, *filogenético*, *agamoespecie*, *ecológico*, *pragmático* entre otros (ver Mayden 1997, Fig. 1). No obstante, la suma de la información proporcionada de cada concepto operacional permite dilucidar a la especie de conceptos primarios, aquellos que son *linajes*. Esto cobra sentido cuando se habla sobre *taxonomía integrativa*, la cual se define como "una disciplina que ayuda a delimitar las unidades de la diversidad de la vida desde perspectivas múltiples y complementarias" (Dayrat 2005).

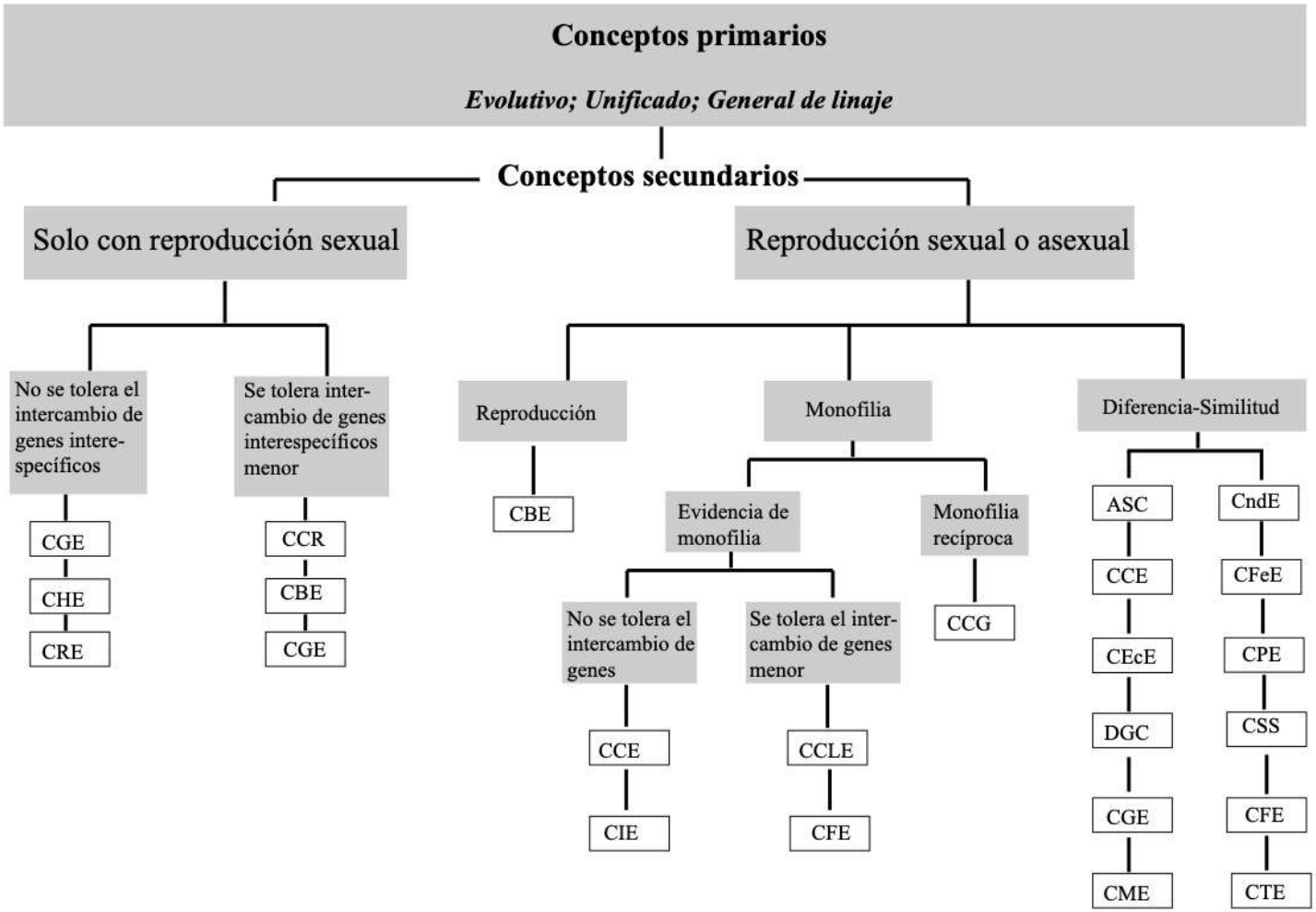
La taxonomía integrativa es una disciplina que se encarga de reconocer, delimitar y clasificar a las especies, así como de nombrarlas. En este sentido, la taxonomía integrativa emplea diferentes criterios de información en la delimitación de especies, como son aspectos ecológicos, moleculares, biogeográficos, evolutivos entre otros, así como de los caracteres morfológicos (Schlick-Steiner et al. 2010).

Particularmente en digéneos (Trematoda) la mayoría de las clasificaciones están sustentadas en la taxonomía alfa de los estadios adultos (Overstreet y Curran 2005). Con la implementación de caracteres moleculares, muchas de las clasificaciones han sido redefinidas y han sufrido cambios taxonómicos importantes como la asignación de nuevas familias, géneros y especies (Blasco-Costa et al. 2009a; Tkach et al. 2016; Pérez-Ponce de León y Hernández-Mena 2019). En años recientes la utilización de los caracteres moleculares, biogeográficos, y ecológicos, así como la taxonomía alfa han sido fundamentales, en la delimitación y descripción de las especies. (Hernández-Cruz et al. 2017; Pérez-Ponce de León et al. 2020).

Cuadro 1. Conceptos ontológicos o primarios.

| | | |
|---|---|---|
| Concepto evolutivo de especie | Las especies son linajes de ancestros-descendientes que evolucionan por separado de otros linajes similares y tienen sus propias tendencias evolutivas y destino histórico. | Wiley y Mayden (2000a) |
| Concepto de linaje general de especie | "Segmentos de linajes evolutivos a nivel de población" | de Queiroz (1999) |
| Concepto de unificado de especie | "Se puede lograr un concepto de especie unificada interpretando la idea fundamental común de ser un segmento de linaje que evoluciona por separado como la única propiedad necesaria de las especies" | de Queiroz (2005b, 2007) |

Fig. 1. Conceptos primarios y secundarios de acuerdo con Mayden (1997)




| Abreviaciones de conceptos secundarios | |
|---|---|
| CGE | Concepto genético de especie |
| CHE | Concepto Hennigiano de especie |
| CRE | Concepto de reconocimiento de especies |
| CCR | Concepto de competencia reproductiva |
| CBE | Concepto biológico de especie |
| CCE | Concepto de cohesión de especie |
| CIE | Concepto de especies internodales |
| CCLE | Concepto cladístico de especie |
| CFE | Concepto filogenético de especie |
| CCG | Concepto de concordancia genealógica |
| ASC | Agamoespecie |
| CEcE | Concepto ecológico de especie |
| DGC | Definición de grupo genotípico |
| CME | Concepto morfológico de especie |
| CndE | Concepto no-dimensional de especie |
| CFeE | Concepto fenético de especie |
| CPE | Concepto politético de especie |
| CSS | Concepto de especies sucesionales |
| CTE | Concepto taxonómico de especie |

## I. II. Familia Haploporidae Nicoll, 1914

Los miembros de la familia Haploporidae Nicoll, 1914 son tremátodos parásitos de tamaño pequeño de aproximadamente 1 a 2 mm. Estos se alojan en el tubo digestivo de peces marinos, dulceacuícolas y estuarinos distribuidos globalmente (Overstreet y Curran 2005). La mayoría de las especies de la familia se caracterizan por presentar un saco hermafrodita y un solo testículo. El órgano denominado saco hermafrodita es una estructura que encierra un ducto hermafrodita, una pars prostática, una vesícula seminal interna, y la porción terminal del útero (Overstreet y Curran 2005; Atopkin et al. 2019). Una de las características de los haplopóridos es la presencia de un tegumento delicado, esto provoca que se degraden cuando el huésped muere (Overstreet y Curran 2005).

Overstreet y Curran (2005) revisaron la taxonomía de la familia utilizando caracteres morfológicos, principalmente la forma y distribución de las glándulas vitelógenas, la distribución del útero, y la forma de la vesícula seminal, reconociendo cuatro subfamilias: Haploporinae Nicoll, 1914; Megasoleninae Manter, 1935; Waretrematinae Srivastava, 1937; y Chalcinotrematinae Overstreet y Curran, 2005. Blasco-Costa et al. (2009a) realizaron el primer trabajo combinando características morfológicas, como la forma y distribución de las glándulas vitelógenas; y moleculares, utilizando la subunidad mayor del DNA ribosomal (28S) así como el segundo espaciador interno transcrito (ITS2). Esto les permitió erigir la subfamilia Forticulcitinae Blasco-Costa, Balbuena, Kostadinova y Olson, 2009. Bray et al. (2014) erigieron la sexta subfamilia, Cadenatellinae (Gibson y Bray, 1982) Bray, Cribb, Waeschenbach y Litlewood, 2014, la cual se caracteriza por la ausencia del saco hermafrodita. Andres et al. (2018) combinaron datos morfológicos y moleculares para erigir la subfamilia Hapladeninae Andres, Pulis Curran y Overstreet, 2018, de peces marinos. Finalmente, Atopkin et al. (2019) emplearon datos moleculares, morfológicos, biogeográficos y

reconocieron la octava subfamilia, Pseudohaploporinae Atopkin, Besprozvannykh, Ha, Nguyen, Nguyen y Chalenko, 2019, basado en especímenes recolectados en tres especies de mugílidos de Vietnam. Con todos estos trabajos mencionados anteriormente la biblioteca genética de los haplopóridos ha incrementado sustancialmente en la última década. Los marcadores moleculares, 28S e ITS2 del DNA ribosomal son el marco de referencia dentro de la familia. Se ha corroborado que estos dos genes tienen la variación suficiente para distinguir entre especies, géneros e incluso a subfamilias de haplopóridos.

En total se han descrito más de 140 especies de haplopóridos, de las cuales 70 se han reportado en 22 especies de peces de la familia Mugilidae (Overstreet y Curran 2005; Blasco-Costa et al. 2009b; Pulis 2013; Besprozvannykh et al. 2017; Atopkin et al. 2019). Es decir, aproximadamente el 50% de las especies de haplopóridos se han reportado en mugílidos, indicando una estrecha asociación ecológica entre mugílidos y haplopóridos. De las ocho subfamilias que se reconocen, cinco (Haploporinae, Waretrematinae, Chalcinotrematinae, Forticulcitinae, y Pseudohaploporinae), tienen especies de haplopóridos que parasitan a especies de mugílidos. Mientras que las otras tres subfamilias, Cadenatellinae, Hapladeninae, Megasoleninae, son exclusivas de peces marinos de diferentes familias (Andres et al. 2018).

## I. III. Ciclo de vida del haplopórido *Xiha fragilis* (Bargiela-Fernández 1987)

El ciclo de vida de los haplopóridos es indirecto, debido a que utilizan a gasterópodos como primeros huéspedes intermediarios y peces como huéspedes definitivos (Overstreet y Curran 2005). El ciclo de vida que se describe a continuación se basa en la especie *Xiha fragilis* reportada en Uruguay (Lado et al. 2013; Fig. 2). Los gusanos adultos viven y se reproducen sexualmente después de 20 días en el intestino del pez *Mugil liza* (Valenciennes, 1836). Los haplopóridos adultos desarrollan huevos, y

dentro de éstos se forman los miracidios, mismos que poseen manchas oculares. Los huevos son liberados al medio acuático a través de las heces donde eclosionan. Esto sugiere que el miracidio de vida libre sería el estadio infectante, penetrando de forma activa al huésped intermediario, el caracol *Heleobia conexa* Gaillard (Cochliopidae: Rissooidea). Dentro del caracol, el miracidio se convierte en redia y de forma asexual se desarrollan las cercarias. Estas son liberadas al medio acuático enquistándose en la vegetación acuática con la ayuda de una estructura denominada "filamento caudal". Los mugilídos juveniles que habitan las lagunas costeras se alimentan de la vegetación junto con las metacercarias, completándose el ciclo de vida de los haplopóridos (Lado et al. 2013).

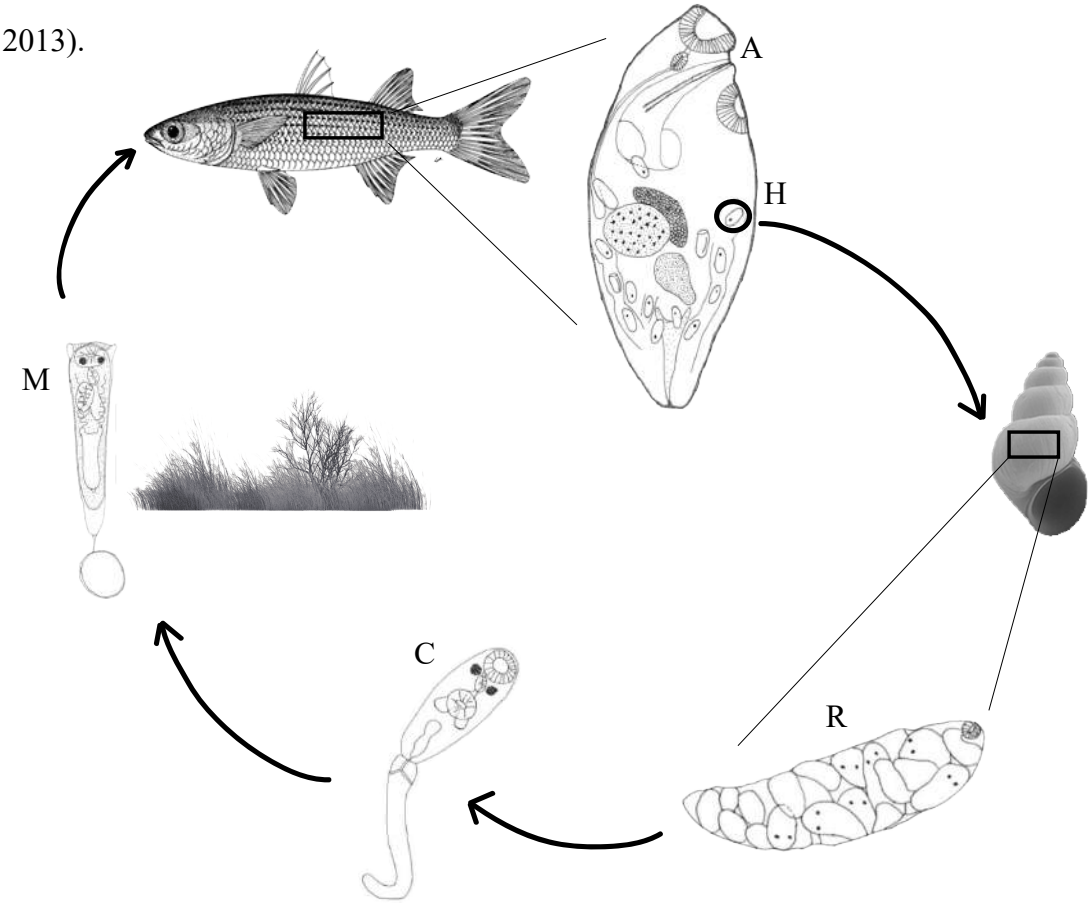



Fig. 2. Ciclo de vida del haplopórido *Xiha fragilis* (Bargiela-Fernández 1987) (Forticulcitinae) parásito de *Mugil liza* (Mugilidae) en Uruguay. Modificado de Lado et al. (2013). A) Adulto; H) Huevo; R) Redia; C) Cercaria; M) Metacercaria

## I. IV. Registros de haplopóridos en México

En México se han reportado ocho especies de haplopóridos y dos especies más sin identificar que corresponden con tres subfamilias, Hapladeninae, Forticulcitinae, y Chalcinotrematinae (Cuadro 2). Los registros son heterogéneos en cada subfamilia, por ejemplo, *Myodera magna* Sogandares-Bernal, 1959 (Hapladeninae), parasita peces marinos tiene un solo registro en Baja California (Cuevas-Macías 1997). Con respecto a la subfamilia Forticulcitinae, que parasita mugílidos, se cuentan con tres registros, uno del género *Xiha* Andres*,* Curran, Fayton, Pulis y Overstreet, 2015 y dos sin identificar a nivel de especie (Lira-Guerrero, 1997; Cabañas-Carranza, 2001). Finalmente, la subfamilia Chalcinotrematinae, que parasita peces dulceacuícolas y estuarinos, es la que presenta el mayor número de registros. El género *Saccocoelioides* Szidat, 1954, que forma parte de Chalcinotrematinae ha sido ampliamente registrado en México con un total de 6 especies reportadas en 6 familias de peces, Mugilidae, Characidae, Goodeidae, Eleotridae, Poeciliidae, y Cichlidae (Cuadro 2).

Cuadro 2. Haplopóridos registrados en México. *Especies registradas en peces del género *Mugil*.

| Especie | Huésped | Familia de Huésped | Estado | Referencia |
|---|---|---|---|---|
| **Hapladeninae** | | | | |
| *Myodera magna* | *Kyphosus elegans* | Kyphosidae | Baja California | Cuevas-Macías, 1997 |
| **Chalcinotrematinae** | | | | |
| *Saccocoelioides chauhani* | *Astyanax aeneus, Bramocharax caballeroi, Dorosoma* sp., *Poecilia catemaconis, Poeciliopsis catemaco, Xiphophorus* sp. | Characidae Poeciliidae | Veracruz | Pérez-Ponce de León et al. 2007 y referencias citadas ahí; Andrade-Gómez et al. 2017 |
| *Saccocoelioides orosiensis* | *Paratheraps bifasciatus, Torichthys helleri* | Cichlidae | Campeche | Andrade-Gómez et al. 2017b lo registró como *Saccocoelioides sogandaresi*. Curran et al. (2018) consideró que los registros de Andrade-Gómez 2017b corresponden con *Saccocoelioides orosiensis.* |
| | *Poeciliopsis balsas, Pseudoxiphophorus* sp. | Poeciliidae | Morelos | |
| | *Poecilia* sp. | | Quintana Roo | |
| | *Poecilia mexicana* | | Oaxaca | |
| | *Poecilia mexicana, Poecilia formosa, Herichthys cyanoguttatus* | Poeciliidae Cichlidae | Tamaulipas | |
| | *Poecilia sphenops, Xiphophorus helleri* | Poeciliidae | Veracruz | |
| *Saccocoelioides olmecae* | *Dormitator maculatus* | Eleotridae | Veracruz | Andrade-Gómez et al. 2017a |
| | *Dormitator maculatus, Gambusia yucatana* | Eleotridae Poeciliidae | Campeche | |
| *Saccocoelioides lamothei* | *Dormitator latifrons* | Eleotridae | Guerrero, Oaxaca | Andrade-Gómez et al. 2017a |

| | | | | |
|---|---|---|---|---|
| *Saccocoelioides overstreeti** | *Mugil cephalus* | Mugilidae | Jalisco | Cabañas-Carranza (2001) lo registró como *Saccocoelioides papernai.* Sin embargo, Curran et al. (2018) mencionaron que *S. papernai* es una sinonimia de *Saccocoelioides overstreeti.* |
| *Saccocoelioides beauforti** | *Mugil curema* | Mugilidae | Tabasco | López-Jiménez (2001) |
| *Saccocoelioides* sp. | *Poecilia sphenops, Poeciliopsis gracilis* | Poeciliidae | Guerrero | Pérez-Ponce et al. 2007 y referencias ahí, lo consideraban como *Saccocoelioides sogandaresi.* No obstante, Curran et al. (2018) menciona que *S. orosiensis* está distribuida solo en Estados Unidos. |
| | *Poecilia mexicana, Poeciliopsis gracilis, Xiphophorus* sp., *Agonostomus monticola, Astyanax aeneus, Cichlasoma istlanum, Oreochromis aureus, Sicydium multipunctatum, Allodontichthys zonistius*, *Ilyodon furcidens*, *Xiphophorus hellerii* | Poeciliidae<br>Mugilidae<br>Cichlidae<br>Gobiidae<br>Goodeidae | Hidalgo | |
| | *Ilyodon whitei, Poecilia sphenops* | Goodeidae<br>Poeciliidae | Morelos | |
| | *Poecilia mexicana, Astyanax aeneus* | Poeciliidae<br>Characidae | Oaxaca | |
| | *Poecilia latipunctata* | Poeciliidae | Quintana Roo | |
| | *Poecilia mexicana* | | San Luis Potosí | |
| | *Poecilia mexicana* | | Tabasco | |

| | | | | |
|---|---|---|---|---|
| | *Poecilia mexicana, Xiphophorus hellerii, Poeciliopsis catemaco, Astyanax aeneus*, *Poecilia mexicana, Xiphophorus hellerii, Poecilia sphenops, Gobiomorus dormitor* | Poeciliidae<br>Characidae<br>Eleotridae | Veracruz | |
| | *Poecilia velifera* | Poeciliidae | Yucatán | |
| **Forticulcitinae** | | | | |
| *Xiha fastigata** | *Mugil cephalus* | Mugilidae | Jalisco | Cabañas-Carranza (2001) lo registró como *Dicrogaster fastigatum.* Sin embargo, Andres et al. (2015) señalaron que *D. fastigatum* es una sinonimia de *Xiha fastigata*. |
| Forticulcitinae gen. sp.* | *Mugil curema* | | Jalisco | Lira-Guerrero (1997) y López-Jiménez (1999) lo identificaron como *Dicrogaster* sp. Sin embargo, *Dicrogaster* se caracteriza por presentar dos glándulas vitelógenas. En el esquema que presenta Lira-Guerrero (1997), se observa una sola glándula vitelógena, carácter diagnóstico de Forticulcitinae (Andres et al. 2015). Por eso consideramos que puede ser un *Forticulcita*, *Xiha* o algún otro linaje sin describir. |
| | *Mugil curema* | | Tabasco | |

## I. V. Mugílidos (Teleostei: Perciformes)

La familia Mugilidae Jarocki, 1822 está compuesta por 77 especies de peces teleósteos clasificados en 20 géneros, comúnmente conocidos como lisas o lebranchas (Eschmeyer y Fong 2017). La mayoría de las especies son marinas y habitan regiones tropicales, subtropicales, así como en regiones templadas (Thomson 1966; Durand et al. 2012). Muchas de estas especies de peces tienen importancia económica en pesquerías y acuicultura, además de tener una función ecológica fundamental, la cual es la conversión de la energía potencial del detritus, en energía aprovechable por otros niveles tróficos. (Thomson 1966; Crosetti y Blaber 2016).

Los mugílidos ofrecen un sistema excelente para estudiar las relaciones parásito-huésped en sus aspectos geográficos, ecológicos y evolutivos. Por ejemplo, al ser peces eurihalinos y euritermos les permiten tolerar una amplia gama de concentraciones de salinidad y temperatura tanto en aguas dulces y marinas sin que su metabolismo se vea afectado. Esto influye en la alimentación a lo largo de su ciclo de vida, cuando son juveniles consumen detritus y en su forma adulta se vuelven preferencialmente carnívoros, esto les permite cambiar su nivel trófico (Ibañez et al. 2012). En costas de México, se han registrado tres especies de lisas del género *Mugil* Linnaeus, *M. cephalus* Linnaeus*, M. curema* Valenciennes y *M. hospes* Jordan y Culver, esta última restringida al Golfo de México (Miller 2005; Ibañez et al. 2011). Estos peces han sido sujetos a numerosos estudios helmintológicos y una gran diversidad de parásitos se ha registrado, con un total de 33 especies de helmintos reportados (Rosas-Valdez et al. 2020). Los digéneos son el grupo de helmintos mejor representado con 15 especies registradas, clasificados en 11 géneros. Sin embargo, solamente ocho de estas 15 especies son adultos y pertenecen a tres familias, Haplosplanchnidae Poche, 1926, Hemiuridae Looss, 1899, y Haploporidae Nicoll, 1914 (Pérez-Ponce de León et al. 1999, 2007).

Con base en lo anterior, estos peces resultan de interés para realizar estudios sobre haplopóridos dado que los registros son escasos y dispersos (Cuadro 2). Además, existe una clara y consistente asociación entre los haplopóridos y sus huéspedes definitivos, los mugílidos (Andres et al. 2018). Por lo tanto, este sistema huésped-parásito es ideal para explorar de forma detallada la composición de haplopóridos que están asociados a mugílidos en ambas costas de México y de esta forma, corroborar las entidades taxonómicas de los parásitos registrados previamente.

## II. OBJETIVO GENERAL

Emplear diversos caracteres para establecer los límites entre especies de haplopóridos que parasitan a lisas (*Mugil* spp.) en regiones costeras de México y describir la diversidad de este grupo de parásitos.

### II. I. Objetivos particulares

1) Identificar a las especies de haplopóridos asociados a las lisas *Mugil* spp. en costas del Golfo de México, Mar Caribe y Océano Pacífico de México.

2) Realizar una revisión taxonómica de la subfamilia Forticulcitinae (Haploporidae).

3) Proponer un esquema filogenético de los haplopóridos basada en caracteres moleculares.

## III. RESULTADOS.

Los resultados de la tesis se presentan en dos capítulos. El primer capítulo está conformado por dos artículos de investigación que corresponden con la subfamilia Chalcinotrematinae (Haploporidae). En el primer artículo se analizaron secuencias del DNA ribosomal y mitocondrial, así como caracteres morfológicos y ultraestructurales de especímenes del género *Saccocoelioides* colectados en *Mugil curema* y *Poecilia catemaconis* Miller en Veracruz, México. Con base en la información analizada, se realizó la descripción de una nueva especie, nombrada *Saccocoelioides macrospinosus* Andrade-Gómez, Sereno-Uribe y García-Varela, 2019, siendo la especie número 24 que se reconoce dentro del género, y la cuarta especie descrita en México. En el segundo artículo de investigación se realizó un estudio integrativo del haplopórido *Saccocoelioides lamothei* Aguirre-Macedo y Violante-González, 2008, donde se analizaron secuencias del DNA ribosomal y mitocondrial, así como la variación morfológica del parásito asociada al huésped que parasita. La información analizada sugiere que este parásito se encuentra asociado en cinco familias de huéspedes, incluyendo a los mugílidos, quienes parecen ser parte fundamental de su dispersión. Asimismo, esta especie es la que presenta la mayor variación morfológica inducida por el huésped, así como una amplia distribución geográfica, desde México hasta Costa Rica.

El segundo capítulo está conformado por dos artículos de investigación que corresponden con la subfamilia Forticulcitinae (Haploporidae). En el primer artículo se analizaron secuencias del DNA ribosomal así como, caracteres morfológicos y ultraestructurales de diferentes especímenes colectados en *Mugil curema* y *Mugil cephalus* en 27 localidades de las costas del Océano Pacífico. Asimismo, se obtuvieron fotografías de cada organismo procesado (*fotogenóforos*) con el objetivo de vincular las secuencias de DNA con la morfología de estos. La información que se obtuvo permitió

describir dos géneros nuevos y cuatro nuevas especies de Forticulcitinae. En el segundo artículo, empleamos características morfológicas, como la forma del cuerpo y de la vesícula excretora; además del tamaño de la faringe y del testículo; y caracteres moleculares del DNA ribosomal para describir tres nuevas especies de la subfamilia Forticulcitinae (*Ekuarhuni mexicanus, Forticulcita macropharyngis,* y *F. venezuelenzis*) que parasitan a *Mugil* spp., Las primeras dos ellas se distribuyen en el Golfo de México y la tercera en Venezuela. Además, se reconoció un cuarto linaje en Veracruz que se designó como *Overstreetoides* sp. Este linaje no se logró describir por falta de especímenes adultos. Finalmente, se realizó por primera vez una clave taxonómica de la subfamilia Forticulcitinae.

## III. I. Chalcinotrematinae (Haploporidae)

### III. I. I. Description of a new species and understanding the genetic diversity of *Saccocoelioides* Szidat, 1954 (Haploporidae) in Middle America using mitochondrial and nuclear DNA sequences. En *Parasitology International.*

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# Description of a new species and understanding the genetic diversity of *Saccocoelioides* Szidat, 1954 (Haploporidae) in Middle America using mitochondrial and nuclear DNA sequences

Leopoldo Andrade-Gómez[a,b], Ana Lucia Sereno-Uribe[a], Martín García-Varela[a,*]

[a] Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, C. P. 04510 Distrito Federal, México

[b] Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, C. P. 04510 Distrito Federal, México

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ABSTRACT

Members of the genus *Saccocoelioides* Szidat, 1954, include endoparasites from freshwater and brackish fishes from the Americas. Adult specimens were collected from the intestines of *Poecilia catemaconis* Miller, 1975, a poeciliid fish endemic to Catemaco Lake, and the white mullet *Mugil curema* Valenciennes, 1836, from Alvarado Lagoon, Veracruz, Mexico. The specimens were sequenced for three molecular markers, internal transcribed spacer 2 (ITS2) and domains D1-D3 from the large subunit (LSU) of nuclear ribosomal DNA and cytochrome *c* oxidase subunit 1 (*cox 1*) from mitochondrial DNA. The newly sequenced specimens were aligned with other sequences downloaded from GenBank. Maximum likelihood and Bayesian inference analyses were inferred with three data sets (a combination of nuclear DNA ITS2 + LSU, *cox 1* alone and the concatenated *cox 1* + ITS2 + LSU). The phylogenetic analyses inferred with the combined data set of the two nuclear molecular markers (ITS2 + LSU) revealed that *Saccocoelioides* is monophyletic and formed 11 independent lineages representing 11 valid species previously recognized plus the new lineage that is herein described as a new species named *Saccocoelioides macrospinosus* n. sp., however, the new species was placed in a basal polytomy in the tree. Therefore, the addition of a mitochondrial gene with a fast rate of substitution was fundamental to clarify the phylogenetic relationships of the new species. The genetic divergences estimated with the *cox 1* gene were high, ranging from 8.3 to 15.5% among *Saccocoelioides macrospinosus* n. sp. and sister taxa. The new species has a slightly elongated body measuring 440–850 μm long and was classified in the diminutive morphotype. In addition, seven adult specimens recovered from the intestines of the banded tetra fish *Astyanax aeneus* Günther, 1860 from Nicaragua and Costa Rica formed a monophyletic clade with other specimens identified previously as *Saccocoelioides tkachi*, expanding its distribution range in other areas of Middle America.

## 1. Introduction

Members of the subfamily Chalcinotrematinae Overstreet and Curran, 2005, include endoparasites from freshwater and occasionally brackish fishes in the Americas. Currently, the subfamily is divided into 6 genera (*Chalcinotrema* Freitas, 1947, *Paralecithobotrys* Teixeira and Freitas, 1948, *Saccocoelioides* Szidat, 1954, *Megacoelium* Szidat, 1954, *Unicoelium* Thatcher and Dossman, 1975, and *Intromugil* Overstreet and Curran, 2005) and is morphologically characterized by having a hermaphroditic sac and numerous vitelline follicles surrounding a single testis [1,2]. The systematics within the subfamily have been explored briefly with morphological or molecular data. Until now, only a few species representing the genera *Intromugil* and *Saccocoelioides* have been analyzed with sequences of the large subunit (LSU) and internal transcribed spacer 2 (ITS2) from nuclear ribosomal DNA (rDNA). The phylogenetic analyses inferred with these molecular markers suggest that *Intromugil* and *Saccocoelioides* share the same ancestor [2–5].

Currently, *Saccocoelioides* is considered the most diverse genus of the subfamily, with 22 described species, 13 of which are distributed in South America, 6 in Middle America, 2 in North America and 1 in Puerto Rico. The taxonomy and systematics of 11 of these 22 species from *Saccocoelioides* were recently evaluated by combining morphological, ecological and molecular characteristics [2]. The authors recognized two morphotypes of *Saccocoelioides*, diminutive (< 1.7 μm) and robust (> 1.7 μm). The first morphotype is distributed along the Americas and includes 9 species (*S. nanii Szidat*, 1954 (Type-species); *S.*

* Corresponding author.
*E-mail address:* garciav@ib.unam.mx (M. García-Varela).

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**Table 1**
Specimens analyzed in this study; host name, localities and GenBank accession numbers of each molecular marker. Sequences in bold were generated in this study.

| Species | Host | Locality | *cox*1 | 28S | ITS2 | References |
|---|---|---|---|---|---|---|
| *Saccocoelioides macrospinosus* n. sp. | *Poecilia catemaconis* Miller | México: Catemaco, Veracruz 18° 25′0″ N 95° 7′0″ W | **MK749565-66** | **MK749164-65** | **MK749181-82** | This study |
| | *M. curema* | Alvarado, Veracruz 18° 46′ 47″ N 95° 44′ 50″ W | **MK749567-70** | **MK749166-69** | **MK749183-86** | This study |
| *Saccocoelioides lamothei* Aguirre-Macedo et Violante-González, 2008 | *Dormitator latifrons* (Richardson) | Mexico: Tres Palos, Guerrero | **MK749571-72** | KU061120-121 | KU061099 | Andrade-Gómez et al. [25] |
| | *P. gillii* | Costa Rica: Rio Tempisque, Guanacaste | - | MG925110 | MG925109 | This study |
| | Unidentified molly (Poeciliidae) | Nicaragua: Campusano River | - | EF032696 | - | Curran et al. [2] |
| *Saccocoelioides cichlidorum* (Aguirre-Macedo and Scholz, 2005) Andrade-Gómez, Pinacho-Pinacho et García-Varela, 2017 | *Paraneetroplus maculicauda* Regan | Nicaragua: Rio Torsuani | **MK749573-74** | KY489644-45 | KY489591-92 | Curran et al. [2] Andrade-Gómez et al. [22] |
| | *Hypsophrys nematopus* Gunter | Costa Rica: Rio Orosí | **MK749575** | KY489634 | KY489581 | This study |
| | *Archocentrus nigrofasciatus* Gunter | Rio Animas | **MK749576** | KY489638 | KY489585 | |
| | *Amatitlania septemfasciatus* (Regan) | | - | MG925106 | MG925105 | Curran et al. [2] |
| *Saccocoelioides tkachi* Curran, Pulis, Andres, et Overstreet 2018 | *Astyanax aeneus* Gunther | Rio Tempisque, Costa Rica | – | MG925122 | MG925121 | Curran et al. [2] |
| | | Nicaragua: Palo de Arquito 11° 7′12” N 84° 36′ 5” W | **MK749577** | **MK749170** | **MK749187** | This study |
| | | Rio Pérez 11° 45′ 0.8” N 84° 14′ 11.4” W | **MK749578-7** | **MK749171-7** | **MK749188-89** | This study |
| | | Rio Torsuani 11° 47′ 06” N 83° 52′ 38” W | **MK749580-81** | **MK749173-74** | **MK749190-91** | This study |
| | | Costa Rica: Rio Entrada Pitahaya 11° 3′ 5” N 85° 24′ 30” W | **MK749582-83** | **MK749175-76** | **MK749192-93** | This study |
| *Saccocoelioides olmecae* Andrade-Gómez, Pinacho-Pinacho, Hernández-Orts, Sereno-Uribe, et García-Varela, 2016 | *Dormitator maculatus* (Bloch) | México: Tamiahua, Veracruz | **MK749584** | KU061128 | KU061109 | Andrade-Gómez et al. [25] |
| | | Rio Palma, Veracruz | **MK749585-86** | KU061131-132 | KU061111-12 | This study |
| *Saccocoelioides chauhani* Lamothe-Argumedo, 1974 | *A. aeneus* | México: Catemaco, Veracruz | **MK749587-89** | KU061117-119 | KU061103-105 | Andrade-Gómez et al. [25] This study |
| *Saccocoelioides sogandaresi* Lumsden, 1963 | *Poecilia latipinna* (Lesueur) | USA: Kleberg County, Texas | – | MG925120 | MG925119 | Curran et al. [2] |
| *Saccocoelioides beauforti* (Hunter et Thomas, 1961) Overstreet, 1971 | *Mugil cephalus* Linnaeus | USA: Masonboro Inlet, North Carolina | – | MG925104 | MG925103 | Curran et al. [2] |
| *Saccocoelioides nanii* Szidat, 1954 | *Prochilodus lineatus* (Valenciennes) | Argentina: Los Talas | – | MG925114 | MG925113 | Curran et al. [2] |
| *Saccocoelioides elongatus* Szidat, 1954 | *P. lineatus* | Argentina: Rio de la Plata | – | MG925108 | MG925107 | Curran et al. [2] |
| *Saccocoelioides magnus* Szidat, 1954 | *Cyphocarynx voga* (Hensel) | Argentina: Rio de la Plata | – | MG925112 | MG925111 | Curran et al. [2] |
| *Saccocoelioides orosiensis* Curran, Pulis, Andres, et Overstreet 2018 | *Poecilia gillii* (Kner) | Costa Rica: | | | | |
| | | Rio Tempisque | - | MG925116 | - | Curran et al. [2] |
| | | Rio Animas | - | MG925118 | - | |
| | | Rio Ciruelas | **MK749590-93** | KY489596 KY489608-610 | KY489545 KY489557-558 | Andrade-Gómez et al. [22] This study |
| | | Rio Las Vueltas | **MK749594-95** | KY489616-617 | KY489563-64 | |
| | | Rio Irigaray | **MK749596-97** | KY489614-615 | KY489561-62 | |
| | | México: | | | | |
| | *Poecilia Formosa* Girard | Rio Purificación, Tamaulipas | **MK749598** | KY489618 | KY489565 | |
| | *Herichthys cyanoguttatus* Baird and Girard | | **MK74959** | KY489621 | KY489568 | |
| | *Pseudoxiphophorus sp.* (Poeciliidae) | Yautepec, Morelos | **MK749600-6001** | KY489606-607 | KY489556 | |
| | *Poecilia sphenops* Valenciennes | Tlacotalpan,Veracruz | **MK749602** | KY489593 | KY489542 | |
| | *Xiphophorus hellerii* Haeckel | Sontecomapan,Veracruz | **MK749603-604** | KY489594-95 | KY489543-44 | |

(*continued on next page*)

**Table 1** (*continued*)

| Species | Host | Locality | *cox*1 | 28S | ITS2 | References |
|---|---|---|---|---|---|---|
| | *M. curema* | México:<br>Montepio, Veracruz<br>18° 38′ 29” N<br>95° 05′ 57” W | **MK749605-607** | **MK749177–79** | **MK749194–96** | This study |
| | *Xiphophorus helleri* Heckel | Rio Palma, Veracruz<br>18° 33′ 21”N<br>95° 2′ 59” W | **MK749608** | – | – | This study |
| **Outgroup** | | | | | | |
| *Forticulcita* sp. | *Mugil curema* Valenciennes | Costa Rica:<br>El Estero<br>9° 13′ 54” N<br>83° 50′ 20” W | **MK749609** | – | – | This study |
| *Intromugil alachuaensis* | *Mugil cephalus* Linnaeus | USA: Florida | – | KC430095 | KC430095 | Pulis et al. [4] |
| *Intromugil mugilicolus* | *M. cephalus* | USA: Louisiana | – | KC430096 | KC430096 | Pulis et al. [4] |

**Table 2**
Comparative morphometric data for species collected in this study of *Saccocoelioides* from Middle America.

| Species | *S. tkachi* | *S. tkachi*<br>Aguirre-Macedo et al. 2001 | *S. tkachi*<br>This study | *S. macrospinosus* n. sp.<br>This study |
|---|---|---|---|---|
| Locality | Guanacaste, Costa Rica | Río Torsuani, Nicaragua | Río Pitahaya, Costa Rica.<br>Palo de Arquito, Nicaragua. | Catemaco, Veracruz |
| Host | *Astyanax aeneus* (Günther, 1860) | *Astyanax aeneus* (Günther, 1860) | *Astyanax aeneus* (Günther, 1860) | *Poecilia catemaconis* (Miller, 1975) |
| No. specimens examined | 5 | 3 | 9 | 22 |
| Body length | 719–1235 | 1070–1210 | 766–1019 | 440–850 |
| Body width | 263–404 | 290–320 | 175–375 | 120–245 |
| Oral sucker length | 91–108 | 90–112 | 74–111 | 67–85 |
| Oral sucker width | 105–134 | 100–120 | 88–120 | 62–102 |
| Ventral sucker length | 99–134 | 105–115 | 82–117 | 67–117 |
| Ventral sucker width | 108–139 | 113–125 | 82–127 | 77–127 |
| Prepharynx length | 55–70 | 45–50 | 28–47 | 8–37 |
| Pharynx length | 57–77 | 70–78 | 43–67 | 35–55 |
| Pharynx width | 65–77 | 63–69 | 55–73 | 35–55 |
| Oesophagus length | | | | 102–158 |
| Hermaphroditic sac length | 142–323 | 274–290 | 105–194 | 70–144 |
| Hermaphroditic sac width | 102–156 | 140–173 | 82–155 | 47–98 |
| External seminal vesicle length | 48–111 | – | 74–96 | 35–81 |
| External seminal vesicle width | 42–89 | – | 33–62 | 27–79 |
| Testis length | 165–346 | 274–290 | 159–254 | 75–186 |
| Testis width | 111–195 | 140–173 | 78–191 | 53–157 |
| Ovary length | 105–167 | 98–105 | 53–101 | 30–79 |
| Ovary width | 57–105 | 80–104 | 46–63 | 24–61 |
| Egg length | 74–91 | 73–75 | 36–78 | 58–103 |
| Egg width | 31–54 | 46–50 | 27–53 | 33–60 |
| % BW/BL | 32[a] | 25[a] | 20–36 | 17–43 |
| Sucker length ratio | 1:1.3[a] | 1:1.1[a] | 1:0.85–1.2 | 1:1.03–1.46 |
| Sucker width ratio | 1:0.9–1.1 | 1:1.1[a] | 1:0.77–1.08 | 1:1–1.5 |
| OS to Pharynx width ratio | 1:0.57[a] | 1:0.66[a] | 1:0.53–0.75 | 1:0.41–0.75 |
| Postesticular space | 81[a] | 200[a] | 134–198 | 59–113 |
| % Postcecal/BL | 22[a] | 24[a] | 28–34 | 30–36 |
| % PostestisS/BL | 6[a] | 18[a] | 15–21 | 8–17 |
| % HS/BL | 26[a] | 27[a] | 13–21 | 12–24 |
| Prostatic bulb long | 23[a] | – | 21–31 | 28–38 |
| Internal seminal vesicle | 73[a] | – | 126–140 | 47–75 |

[a] Measured from the published figure.

*beauforti* Hunter and Thomas, 1961; *S. sogandaresi* Lumsden, 1963; *S. chauhani* Lamothe-Argumedo, 1974; *S. cichlidorum* (Aguirre-Macedo and Scholz, 2005) Andrade-Gómez, Pinacho-Pinacho, and García-Varela, 2017; *S. lamothei* Aguirre-Macedo and Violante-González, 2008; *S. olmecae* Andrade-Gómez, Pinacho-Pinacho, Hernández-Orts, Sereno-Uribe and García-Varela; *S. orosiensis* Curran, Pulis, Andres and Overstreet, 2018; and *S. tkachi* Curran, Pulis, Andres and Overstreet, 2018). The second morphotype is restricted to South America and includes 5 species (*S. elongatus* Szidat, 1954; *S. magnus* Szidat, 1954; *S. szidati* (Szidat, 1954) Travassos, Freitas, and Kohn, 1969; *S. antonioi* Lunaschi, 1984; and *S. guaporense* (Thatcher, 1999) Curran, Pulis, Andres and Overstreet, 2018). The authors analyzed two nuclear genes, the large subunit (LSU) and internal transcribed spacer (ITS2) from rDNA, that have been used to recognize and validate some species of *Saccocoelioides* and other species of the family Haploporidae Nicoll, 1914 [2–5]. Recently, other molecular markers with a high rate of substitution, such as the cytochrome *c* oxidase subunit I (*cox* 1) gene from mitochondrial DNA, have been used successfully to delineate and recognize species within digeneas [6–8]. However, the *cox* 1 gene has never been used to delineate species or genera within the family Haploporidae.

In the current research, we analyzed, for the first time, the *cox* 1 gene to delineate a few species within the genus *Saccocoelioides* distributed in Middle America associated primarily with freshwater fishes. Our analyses inferred with *cox* 1 clearly distinguished species

previously recognized within *Saccocoelioides* with two nuclear molecular markers. The combination of mitochondrial and nuclear markers plus morphological and ecological characteristics allowed us to recognize a new species of *Saccocoelioides* associated with freshwater and brackish fishes from the Gulf of Mexico. In addition, we provide new morphological data and extend the distribution range of *S. tkachi*, a parasite of freshwater fishes from Middle America.

## 2. Materials and methods

### 2.1. Specimen collection

Adult digeneans were collected from the intestines of their definitive hosts in four localities from Mexico, three from Nicaragua, and two from Costa Rica (Table 1). Fishes were collected with seine nets and electrofishing and were kept alive and transported to the laboratory. Each fish was euthanized and immediately examined. Digeneas were preserved either in 100% ethanol for DNA extraction or in hot (steaming) 4% formalin for morphological purposes.

### 2.2. Morphological analyses

Unflattened specimens preserved in formalin were stained with Mayer's paracarmine (Merck, Darmstadt, Germany), dehydrated in a graded ethanol series, cleared with methyl salicylate, and mounted on microscope slides in Canada balsam. Mounted specimens were examined under a bright field Leica DM 1000 LED microscope (Leica, Wetzlar, Germany), and drawings were made using a drawing tube attached to the microscope. Measurements were taken using Leica Application Suite microscope software (Leica) and are given in micrometers (μm). Voucher specimens were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City.

For scanning electron microscopy (SEM), two specimens of *Saccocoelioides* sp. from *Poecilia catemaconis* Miller, 1975, from Catemaco Lake, Veracruz, Mexico, and two specimens identified as *Saccocoelioides tkachi* from *Astyanax aeneus* Günter, 1860, from Palo de Arquito, Nicaragua, were dehydrated with an ethanol series, critical point dried, sputter coated with gold, and examined with a Hitachi Stereoscan Model S-2469 N scanning electron microscope operating at 15 kV from the Instituto de Biología, Universidad Nacional Autónoma de México (UNAM).

### 2.3. Amplification and sequencing of DNA

Each specimen of *Saccocoelioides* spp. was placed individually in tubes and digested overnight at 56 °C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM $Na_2$ EDTA (pH 8.0), 1% sarkosyl, and 0.1 mg/ml proteinase K. Following digestion, DNA was extracted from the supernatant using DNAzol reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions.

Cytochrome *c* oxidase subunit 1 (*cox 1*) of mitochondrial DNA was amplified using polymerase chain reaction (PCR) with forward (MplatCOX1dF, 5´-TGTAAAACGACGGCCAGTTTWCITTRGATCATAAG-3′) and reverse (MplatCOX1dR, 5´-CAGGAAACAGCTAT GACTGAAAYAAYAIIGGATCICCACC-3′) primers [9]. In addition, the ITS2 region and D1–D3 domains of LSU from rDNA were amplified using forward (5´-GAACATCGACATCTTGAACG-3′) [10] and reverse ( 5′-CAGCTATCCTGAGGGAAAC-3′) primers [11]. PCRs (25 μl) consisted of 1 μl of each primer (10 μM), 2.5 μl of 10× PCR Rxn buffer, 1.5 μl of 2 mM $MgCl_2$, 0.5 μl of dNTPs (10 mM), 16. 375 μl of water, 2 μl of genomic DNA and 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil). PCR cycling parameters for rDNA amplifications included denaturation at 94 °C for 1 min, followed by 35 cycles at 94 °C for 1 min, annealing at 48 °C for *cox 1* and at 50 °C for ITS2 + LSU for 1 min, and extension at 72 °C for 1 min, followed by postamplification incubation at 72 °C for 10 min. Sequencing reactions were performed using the initial primers for *cox 1*, ITS2 and LSU plus four internal primers, 504 (5′–CGTCTTGAAACACGGACTAAGG–3′), 502 (5′–CAAGTACCGTGAGGGAAAGTTGC–3′) [11], 503 (5′–CCTTGG TCCGTGTTTCAAGACG–3′) [12], and BD2 (5′–TATGCTTAAATTCAGC GGGT–3′) [13], with ABI Big Dye (Applied Biosystems, Boston, Massachusetts) terminator sequencing chemistry, and reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contigs were assembled and base-calling differences resolved using Codoncode Aligner version 5.0.2 (Codoncode Corporation, Dedham, Massachusetts).

### 2.4. Alignments and phylogenetic analyses

Newly obtained sequences for *cox 1*, ITS2 and LSU of *Saccocoelioides* spp. were aligned with other congeneric sequences downloaded from the GenBank data set plus other sequences of the genera *Forticulcita* Overstreet, 1982 and *Intromugil* generated in this study and were used as outgroups (Table 1). The alignment of *cox 1* and the nuclear combination of ITS2 + LSU and the concatenated *cox 1* + ITS2 + LSU data sets were constructed using the software Clustal W [14], with default parameters and adjusted manually with the Mesquite program [15]. The best fit model was identified with the Akaike information criterion (AIC) using the jModelTest v0.1.1 program [16]. The best model for *cox 1* was GTR + I + G; that for the nuclear combined (ITS2 + LSU) was TVM + I; and that for the concatenated of the *cox 1* + ITS2 + LSU data set was GTR + I + G. For the ML analyses, the program RAxML v7.0.4 [17] was used with the GTR + I + G model for all the data sets. To support each node, 10,000 bootstrap replicates were run. Bayesian analyses were inferred with the program MrBayes 3.1.2 [18] with the models previously estimated with the jModelTest v0.1.1 program [16]. Settings included 2 simultaneous runs of the Markov chain (MCMC) for 10 million generations, sampling every 1000 generations, a heating parameter value of 0.2, and a "burn-in" of 25%. Trees were drawn using FigTree version 1.3.1 [19]. The genetic divergence among species of *Saccocoelioides* and between genera *Intromugil* and *Forticulcita* was estimated using uncorrected "*p*" distances with the program MEGA version 6 [20].

## 3. Results

### 3.1. Morphological description of Saccocoelioides macrospinosus n. sp. (based on 22 mature measured whole mounts)

Measurements of holotype are given (ranges from paratype are in parentheses).

Body slightly elongate, 705 (440–850) long, widest at first third of body, 236 (120–245) wide, representing 33% (17–43%) of BL (Table 2). Eyespot remnants scattered in forebody extending to level posterior of pharynx (Figs. 1A, 2A). Tegumental spines conspicuous, covering entire body surface (Figs. 2A, C). Oral sucker spherical, terminal (2A), 81 (67–85) long, 84 (62–100) wide. Ventral sucker subspherical, with tiny spines inside, 84 (67–117) long, 103 (77–127) wide (Figs. 1A, 2D). Ratio of oral sucker to ventral sucker widths 1: 1.22 (1: 1–1.5). Ratio of oral sucker to ventral sucker lengths 1: 1.03 (1: 1.03–1.46). Prepharynx 28 (8–37) long. Pharynx globular, 49 (39–55) long, 55 (35–55) wide. Ratio of oral sucker to pharyngeal widths 1: 0.65 (1: 0.41–0.75). Oesophagus, 136 (102–158) long, approximately 2.7–3.4 times pharynx length, extending to approximately at slightly anterior to middle of body. Intestinal bifurcation, immediately posterior to ventral sucker, dorsal to hermaphroditic sac. Caeca sac-shaped, approximately twice as long as wide, vacuolar, terminating blindly at anterior to testis; postcaecal space, representing 35% (30–36%) of BL. Testis single, subspherical, 186 (75–186) long, 133 (53–157) wide, located in posterior of body but no reaching the end of body. Posttesticular space, 59

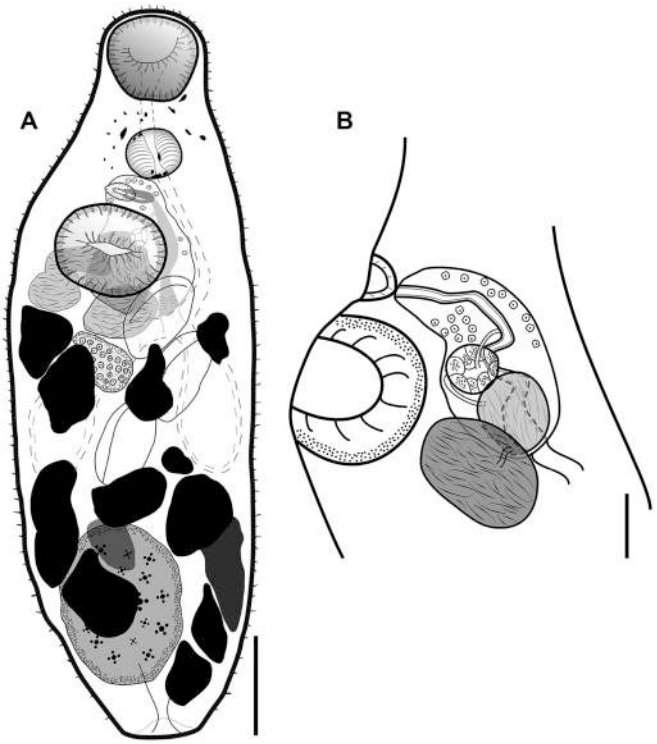


**Fig. 1.** *Saccocoelioides macrospinosus* n. sp., from *Poecilia catemaconis* (A) whole worm, holotype, ventral view; (B) Hermaphroditic sac, paratype, ventral view; Scale bars = 100 μm (A); 50 μm (B).

(59–113) representing 8% (8–17%) of BL. External seminal vesicle, spherical to elongated, 73 (35–81) long, 46 (27–79) wide, dorsal to ventral sucker, contiguous with the hermaphroditic sac. Hermaphroditic sac, oval to ellipsoidal, the posterior zone of the hermaphroditic sac is wider than anterior, 139 (70–144) long, 97 (47–98) wide, representing 19% (12–24%) of BL, containing terminal genitalia; internal seminal vesicle, 47 (47–75) long, in posterior portion, subspherical to elongate; prostatic bulb 28 (28–38) long, swollen; male duct short, uniting with female duct in midlevel of sac; hermaphroditic duct muscular, eversible, as intromittent organ. Genital pore medial, anterior to anterior margin of ventral sucker (Fig. 1B). Ovary subspherical to elongate, 72 (30–79) long, 54 (24–61) wide, located approximately in middle of body contiguous with intestinal bifurcation. Laurer's canal not observed. Seminal receptacle, slightly elongated, located anterior of ovary, dorsally to ventral sucker. Numerous vitelline follicles elongated, irregular, distributed in poorly-differentiated lateral fields surrounding gonads and ceca, extending from middle of body to posterior end of body. Uterus, occupying from level of ventral sucker opening to hermaphroditic sac extending anterior of testis, metraterm thick walled. Eggs 3 (2–8) in distal portion of uterus, 91–95 (58–103) long, 46–53 (33–60) wide. Excretory vesicle, Y-shaped. Pore terminal covered by spines (Figs. 1A, 2E).

Type host: *Poecilia catemaconis* Miller, 1975

Additional host: *Mugil curema* Valenciennes, 1836

Type locality: Catemaco Lake, Veracruz (18° 25′ 0″ N, 95° 7′0″ W)

Additional localities: Alvarado Lagoon, Veracruz (18° 46′ 47″ N; 95° 44′ 50″W).

Site of infection: Intestine

Type Material: Holotype CNHE 11129; paratype: CNHE No. 11130

Sequence deposited: *cox 1* from mitochondrial DNA, LSU and ITS2 from nuclear DNA.

sequences GenBank Accession Nos. MK749565 – 70, MK749164 – 69, MK749181 – 86, respectively.

Etymology: The specific epithet refers to the presence of conspicuous spines that cover the entire surface of the tegument.

### 3.2. Remarks

*Saccocoelioides macrospinosus* n. sp. is the 23rd species described from the Americas and the fifth species described from Mexico. This species was found in *Poecilia catemaconis* and *Mugil curema* from Veracruz state. In a review of the genus *Saccocoelioides*, Curran et al. [2], mentioned that only a few morphological features are useful for distinguishing species and that molecular data are essential to delimit the proper species. Those authors divided the genus *Saccocoelioides* morphologically into 2 distinct morphotypes. One morphotype consists of 17 diminutive species that have relatively small bodies (< 1.7 mm long). *Saccocoelioides macrospinosus* n. sp. belongs to this group because it has a slightly elongated body measuring 440–850 long. With the inclusion of the newly identified species in Middle America, this biogeographical region now harbors 7 species: *Saccocoelioides macrospinosus* n. sp., *S. chauhani*, *S. lamothei*, *S. olmecae*, *S. orosiensis*, *S. cichlidorum* and *S. tkachi*.

*Saccocoelioides macrospinosus* n. sp. can also be differentiated from the other 6 congeneric species distributed in Middle America because it has a smaller body size than *S. tkachi*, a parasite that infects characid fishes from Nicaragua and Costa Rica (440–850 long in *S. macrospinosus vs* 719–1235 in *S. tkachi*). *Saccocoelioides olmecae* infects an eleotrid fish in the Gulf of Mexico, and it has a smaller body size than the new species (340–527 *vs* 440–850 in *S. macrospinosus*). *Saccocoelioides lamothei* infects eleotrid fish in the Pacific coast of Mexico and is wider in size than the new species (240–510 *vs* 120–245 in *S. macrospinosus*). *Saccocoelioides cichlidorum* infects cichlid fishes in Nicaragua and Costa Rica, and it is slightly shorter in length than the new species (448–680 *vs* 440–850 in *S. macrospinosus*); moreover, its sucker ratio is less size than that of the new species (1: 0.97–1.2 *vs* 1: 1.03–1.4 in *S. macrospinosus*). *Saccocoelioides orosiensis* mostly infects poeciliid fishes and is slightly wider than the new species (204–359 *vs* 120–245 in *S. macrospinosus*). Finally, *Saccocoelioides chauhani* infects characid fishes in Catemaco Lake, Veracruz, and it has a body that is wider than the new species (198–418 *vs* 120–245 in *S. macrospinosus)* and an oral sucker that is slightly longer than the new species (70–112 *vs* 67–85 in *S. macrospinosus*) [2,21,22].

### 3.3. Morphological description

*Saccocoelioides tkachi* Curran, Pulis, Andres and Overstreet, 2018.

Our specimens collected in Palo de Arquito, Nicaragua and Rio Pitahaya, Costa Rica from *A. aeneus* were identified as *S. tkachi* by having features that are consistent with the diagnosis of the original description [2]. Tegument entirely covered by minute spines (Fig. 3B–F). Eye-spot remnants present in anterior of body reaching half of pharynx (Fig. 3A). Oral sucker subterminal. Ventral sucker slightly anterior to middle of body. Prepharynx short. Pharynx oval to spherical. Oesophagus long. Caeca sac-shaped but elongated, terminating in posterior half of hindbody. Testis oval to subspherical, longer than wide, in middle of hindbody. External seminal vesicle small sac- shaped continuous to hermaphroditic sac. Hermaphroditic sac oval, dorsal to ventral sucker. Internal seminal vesicle elongated sac-shaped. Genital pore opening medially, anterior to ventral sucker. Ovary subglobular. Laurer's canal not observed, Mehlis' gland not observed. Uterus confined between hermaphroditic sac and testis, with well-developed metraterm entering posterior end of hermaphroditic sac. Vitelline follicles elongated, irregular, distributed in lateral fields from level of posterior of hermaphroditic sac to posterior of testis surrounding gonads and ceca, but not confluent at the end of the body. Eggs operculate. Miracidia not observed. Excretory vesicle Y-shaped. Excretory pore terminal (Fig. 3A).

Type host: *Astyanax aeneus* Günther, 1860.

Additional host: *Astyanax fasciatus* Cuvier, 1819.

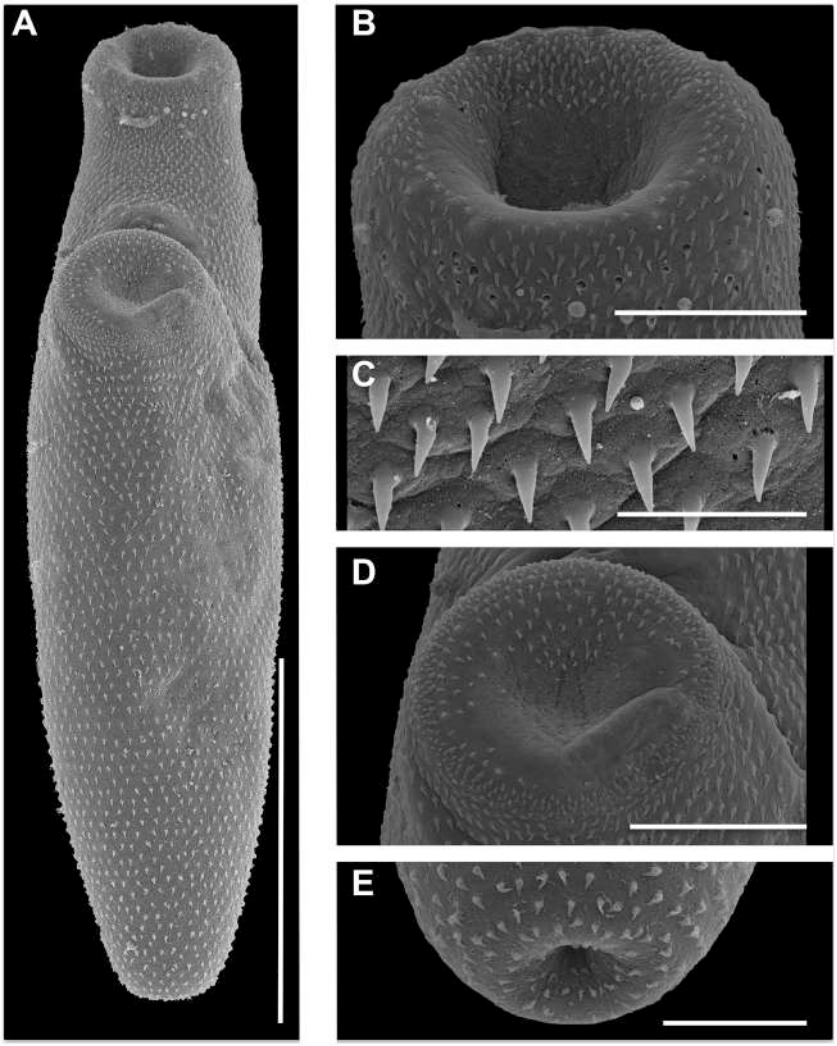


**Fig. 2.** Scanning electron micrographs of paratype of *Saccocoelioides macrospinosus* n. sp., from *Poecilia catemaconis* (A), Whole worm; (B) Oral sucker; (C) Tegumental spines; (D) Ventral sucker; (E) Pore terminal. Scale bars = 200 μm (A); 40 μm (B); 10 μm (C); 50 μm (D); 30 μm (E).

Type locality: Rio Animas (tributary of Rio Sapoa), Guanacaste, Costa Rica (11° 02′ 54″N, 85° 35′09″W).

Additional localities: Rio Tempisque (and tributaries), Guanacaste Costa Rica (10° 47′ 21”N, 85° 33′03″W). Rio Pitahaya, Costa Rica (11° 03′ 05”N, 85° 24′30″W). Palo de Arquito, Nicaragua (11° 07′ 12.3″ N, 84° 36′ 5.3″ W). Rio Torsuani, Nicaragua (11° 47′ 06″ N, 83° 52′ 38″W). Rio Perez, Nicaragua (11° 45′ 0.8″ N, 84° 14′ 11.4″W).

Site of infection: Intestine

Voucher material: CNHE 11130.

Sequence deposited: *cox 1,* LSU and ITS2 rDNA gene sequences GenBank Accession Nos.

MK749577–83, MK749170–76, MK749187–93, respectively.

### 3.4. Remarks

Our specimens identified as *S. tkachi* show certain level of morphological variability (Table 2). For instance, the meristic data of newly collected material provide lower limits for the following characteristics: maximum body width (175–375 this study *vs* 263–404 original description), oral sucker length (74–111 *vs* 91–108) and width (88–120 *vs* 105–134), prepharynx length (28–47 *vs* 55–70), hermaphroditic sac length (105–194 *vs* 142–323), ovary length (53–101 *vs* 105–167) and width (46–63 *vs* 57–105), and egg length (53–101 *vs* 105–167). In addition, we considered that *Saccocoelioides* sp. 1 recorded by Aguirre-Macedo et al. [23], from banded Astyanax fish (*A. fasciatus*) in Rio Torsuani, Nicaragua, belongs to *S. tkachi*.

### 3.5. Phylogenetic analyses

#### 3.5.1. Nuclear genes

The combined data set (ITS2 + LSU) included 1741 characters. The phylogenetic analyses inferred with ML and BI showed that the genus *Saccocoelioides* is monophyletic, with strong support of bootstrap and Bayesian posterior probabilities (100/1) (Fig. 4). The phylogenetic trees were subdivided into two major clades. The first contained *S. elongatus* (GenBank MG925108) plus *S. magnus* (GenBank MG925112) from South America and was recognized as the robust form (*sensu* Curran et al. [2]). The second clade contained nine valid species and was recognized as the diminutive form (*sensu* Curran et al. [2]), plus the new species, *Saccocoelioides macrospinosus* n. sp. was recovered from the poeciliid fish *Poecilia catemaconis* and white mullet *Mugil cuema* from the Gulf of Mexico. In addition, seven adult specimens of *Saccocoelioides*

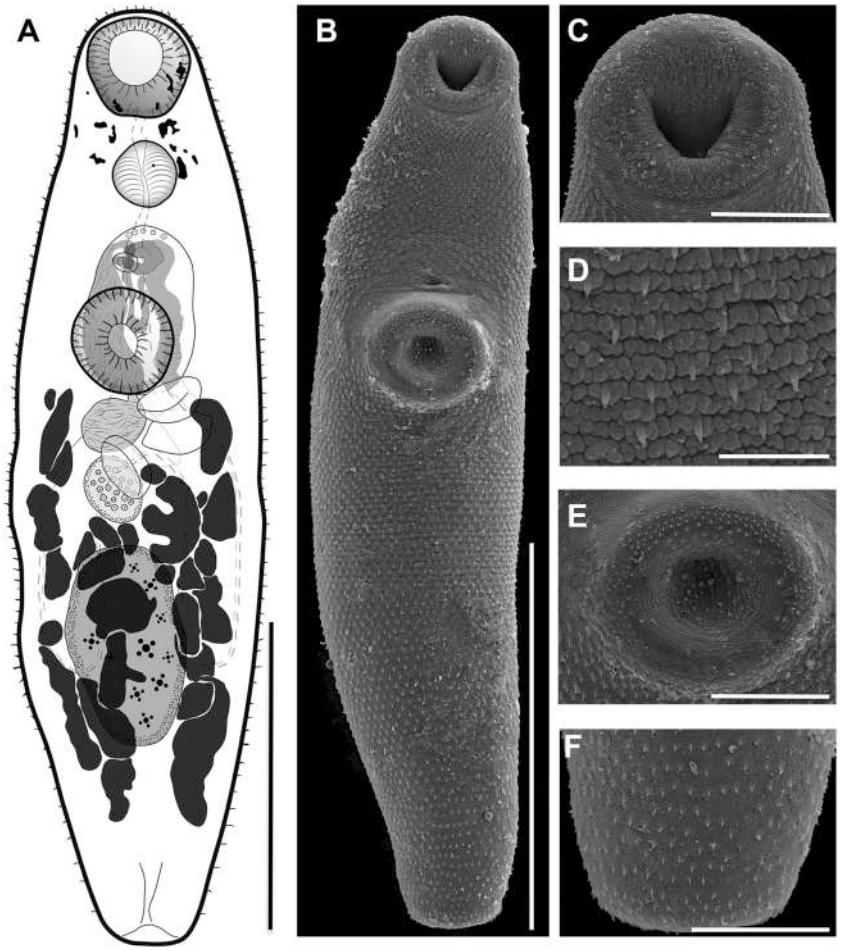


**Fig. 3.** *Saccocoelioides tkachi* from *Astyanax aeneus* (A) whole worm voucher, ventral view; Scanning electron micrographs of voucher (B), Whole worm; (C) Oral sucker; (D) Tegumental spines; (E) Ventral sucker; (F) Posterior region. Scale bars = 300 μm (A–B); 50 μm (C); 10 μm (D); 50 μm (E–F).

spp. collected from the banded tetra fish (*A aeneus*) in two countries (Nicaragua and Costa Rica) from Middle America were identified as *S. tkachi*. These seven sequences form a subclade together with a specimen previously identified as *S. tkachi* (GenBank MG925122) from banded tetra fish from Tempisque River, Costa Rica [2]. Three specimens recovered from white mullet fish (*M. curema*) from the Gulf of Mexico were identified as *S. orosiensis* and were nested in a single subclade with 14 other specimens (GenBank, KY489593–96, KY489606–10, KY489614–18, and KY489621) collected from poeciliid and cichlid fishes from Mexico and Costa Rica [22], together with two other sequences (GenBank, MG925116 and MG925118) collected from the intestines of a poeciliid fish from Costa Rica [2] (Fig. 4). The genetic divergence estimated with the combined data set (ITS2 + LSU) from rDNA among species of *Saccocoelioides* ranged from 0 to 5%. The lowest divergence found was between *S. beauforti* and *S. olmecae* (0–0.1%), and the highest divergence found was between *S. elongatus* and *S. lamothei* (4.8–5%) (see Table 3), whereas the genetic divergence among *S. macrospinosus* n. sp. with the other 11 congeneric species ranged from 0.2 to 4.1% (see Table 3). The genetic intraspecific divergence in *S. macrospinosus* n. sp., was 0–0.5%.

### 3.5.2. Mitochondrial gene

The *cox 1* data set included 623 characters with 45 sequences. The phylogenetic analyses inferred with ML and BI recovered seven subclades representing seven species of *Saccocoelioides* from Middle America, with strong bootstrap support and Bayesian posterior probabilities (Fig. 5). However, the phylogenetic relationships among the species received weak nodal bootstrap support and Bayesian posterior probabilities (Fig. 2). The six specimens representing the species *Saccocoelioides macrospinosus* n. sp. recovered from poeciliid fish *P. catemaconis* and white mullet *M. curema* form a subclade that is closely related to other subclades formed by *S. orosiensis* collected from poeciliid and cichlid fishes from Mexico and Costa Rica plus *S. lamothei*, a parasite from the Pacific fat sleeper fish *Dormitator latifrons* (Richardson, 1844) from Tres Palos, Guerrero, Mexico [24,25]. The genetic divergence estimated with the *cox 1* data set among the seven species of *Saccocoelioides* ranged from 8.3 to 17%, and among *Saccocoelioides macrospinosus* n. sp. and its closely related species, *i.e.*, *S. orosiensis* and *S. lamothei*, the genetic divergence ranged from 8.7 to 11.3% (see Table 3). Intraspecific variation of *Saccocoelioides macrospinosus* n. sp. was low, ranging from 0 to 3.3 (see Table 3).

### 3.5.3. Nuclear and mitochondrial genes

The concatenated data set of three molecular markers (*cox 1* + ITS2 + LSU) included 2360 characters with 44 terminals. The phylogenetic analyses inferred with ML and BI showed similar topologies to that of the *cox 1* tree (Fig. 6), including the seven subclades that represent the seven species of *Saccocoeloides*, with strong bootstrap support and Bayesian posterior probabilities. The subclade formed by *Saccocoelioides macrospinosus* n. sp. is sister to four other congeneric

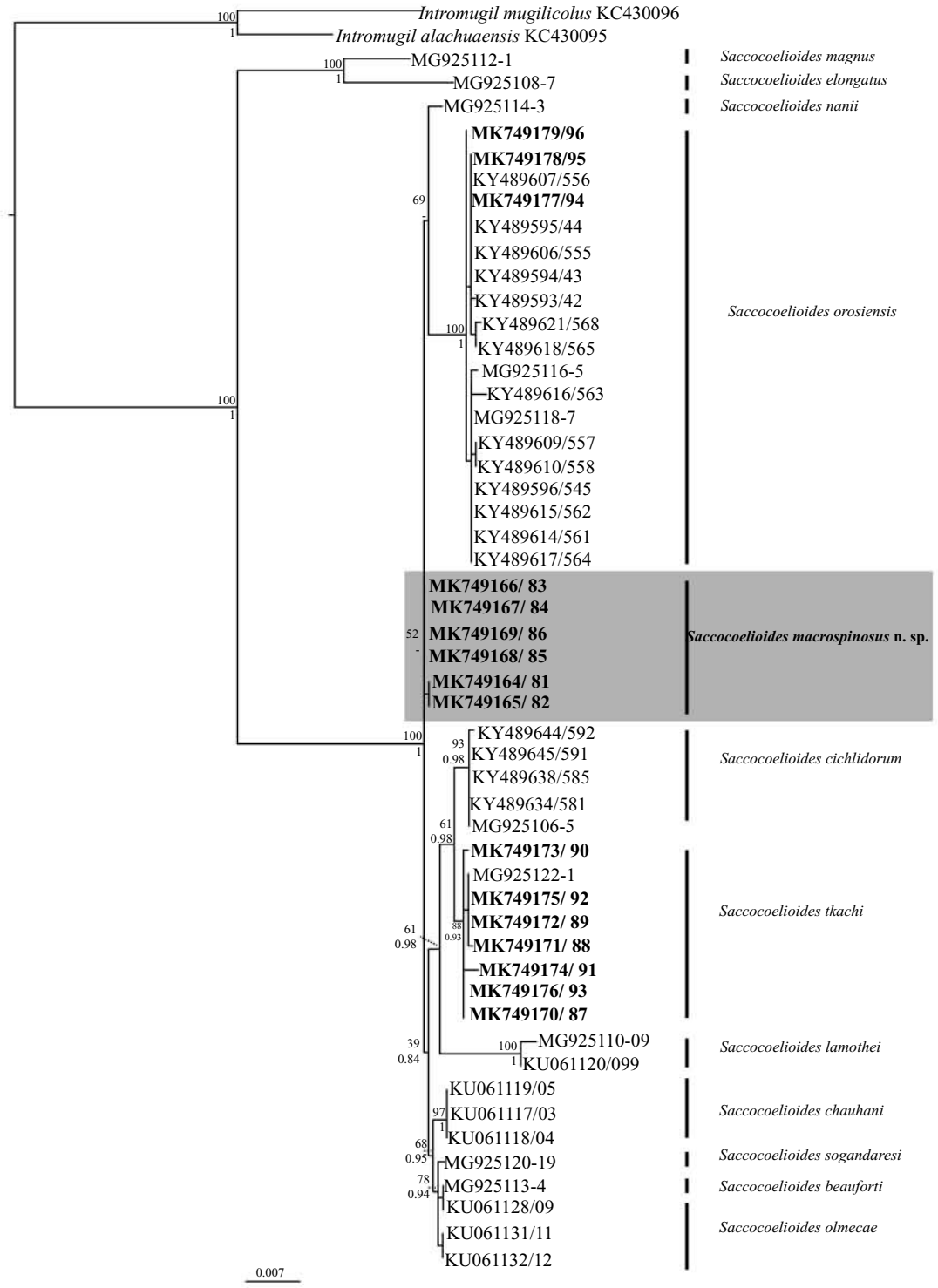


**Fig. 4.** Maximum likelihood tree and consensus Bayesian Inference trees inferred with the combined (ITS2 + LSU) data set; numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI).

species, *i.e.*, *S. cichlidorum*, *S. tkachi*, *S. olmecae* and *S. chauhani* (Fig. 6).

## 4. Discussion

*Saccocoelioides macrospinosus* n. sp. represents the fifth species of the genus described in the Neotropical region of Mexico. This species was found in the intestines of *P. catemaconsis*, a poeciliid fish endemic to Catemaco Lake, and the white mullet (*M. curema*) from Alvarado Lagoon, Veracruz. Both aquatic systems are part of the Papaloapan river basin and are considered the second largest hydrobiological system in Mexico [26]. In the same hydrobiological system, two other species of the genus *Saccocoelioides*, *i.e.*, *S. olmecae* and *S. chauhani* occur sympatrically with *Saccocoelioides macrospinosus* n. sp., although *S. olmecae* only infects the fat sleeper fish *Dormitator maculatus* (Bloch, 1972) in some localities from the Gulf of Mexico. In contrast, *S. chauhani* only infects the banded tetra fish in Catemaco Lake and appears that this trematode is part of the core helminth parasite fauna of the banded tetra fish [25]. Our study also showed that three isolated of

**Table 3**
Pairwise nucleotide sequence comparisons between taxa for the aligned ITS2 + LSU rDNA sequences ($N$ = 1741 nt) (below the diagonal) and for the aligned cox1 sequences ($N$ = 623 nt) (above the diagonal). In bold is represented the genetic intraspecific divergence.

| | *S. magnus* | *S. elongatus* | *S. beauforti* | *S. sogandaresi* | *S. nanii* | *S. lamothei* | *S. chauhani* | *S. orosiensis* | *S. olmecae* | *S. cichlidorum* | *S. tkachi* | *S. macrospinosus* n. sp. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. magnus* | **0/–** | – | – | – | – | – | – | – | – | – | – | – |
| *S. elongatus* | 1.8 | **0/–** | – | – | – | – | – | – | – | – | – | – |
| *S. beauforti* | 4.5 | 4.7 | **0/–** | – | – | – | – | – | – | – | – | – |
| *S. sogandaresi* | 4 | 4.2 | 0.1 | **0/–** | – | – | – | – | – | – | – | – |
| *S. nanii* | 4 | 4.2 | 0.5 | 0.5 | **0/–** | – | – | – | – | – | – | – |
| *S. lamothei* | 4.6–4.8 | 4.8–5 | 1.5–1.7 | 1.3–1.5 | 1.4–1.6 | **0.2/0** | 14.2–14.6 | 14.8–17 | 14.4–15.9 | 15.1–15.5 | 14.6–16.2 | 14.8–15.5 |
| *S. chauhani* | 4 | 4.3 | 0.3 | 0.3 | 0.5 | 1.3–1.5 | **0/0.3–0.5** | 7.8–9.7 | 8.6–9.9 | 9.8–10.9 | 9.8–11.2 | 8.7–9.7 |
| *S. orosiensis* | 4.1–4.5 | 4.4–4.7 | 0.8–1.3 | 0.8–1 | 0.7–1 | 1.5–2 | 0.7–1 | **0–0.4/0–1.9** | 9.2–11.8 | 9.9–12 | 9.2–11.4 | 8.7–11.3 |
| *S. olmecae* | 4 | 4.1 | 0–0.1 | 0.1 | 0.5 | 1.2–1.5 | 0.3 | 0.7–1 | **0–0.1/ 0.5–1.9** | 9–11.4 | 8.3–11.6 | 9.3–11.4 |
| *S. cichlidorum* | 4.1 | 4.4 | 0.7 | 0.7 | 0.8 | 1.1–1.3 | 0.7 | 1–1.4 | 0.6–0.7 | **0–0.05/0–0.4** | 7.7–8.8 | 10.5–11.6 |
| *S. tkachi* | 3.9–4 | 4.3–4.4 | 0.7–0.9 | 0.6–0.8 | 0.7–0.9 | 1.2–1.5 | 0.6–0.8 | 1–1.4 | 0.6–0.7 | 0.3–0.4 | **0–0.2/ 0–3.1** | 10.5–12.2 |
| *S. macrospinosus* n. sp. | 3.8 | 4–4.1 | 0.3 | 0.3 | 0.2–0.3 | 1.2–1.4 | 0.3 | 0.5–0.7 | 0.2 | 0.5–0.6 | 0.5–0.7 | **0–0.05/0–3.3** |

*Saccocoelioides* collected from white mullet fish from the Gulf of Mexico correspond to *S. orosiensis* [2], because those species form a clade with other sequences of specimens previously identified as *S. sogandaresi* (GenBank, KY489593–96, KY489606–10, KY489614–18, and KY489621) by Andrade-Gómez et al. [22]. However, all these sequences herein are transferred to *S. orosiensis*, expanding the geographical distribution and host range in four countries from Middle America, Costa Rica, Nicaragua, Honduras and Mexico. Curran et al. [2], conducted a comprehensive molecular phylogenetic analysis of the genus *Saccocoelioides* that included species from North, Middle and South America. The authors mentioned that *S. sogandaresi* is a species limited geographically to estuarine regions of the northwestern Gulf of Mexico. In this study, all the phylogenetics analyses inferred with the ITS2 + LSU and *cox 1* data sets revealed that all the isolates of *S. orosiensis* nested in a reciprocal monophyletic clade with very low genetic divergence, varying from 0 to 0.4% for ITS2 + LSU and from 0 to 1.9% for *cox 1* (see Table 3). The low level of genetic divergence found with the nuclear molecular markers among specimens is consistent with previous studies. For instance, the genetic divergence among 11 isolates of *S. olmecae* ranged from 0 to 1% [25] and among 2 isolates of *S. beauforti*; 6 of *S. cichlidorum*; 2 of *S. elongatus*; 6 of *S. lamothei*; 9 of *S. nanii*; 5 of *S. sogandaresi*; and 5 from *S. orosiensis*, the genetic divergence was zero [2].

Seven adult specimens collected from the intestines of the banded tetra fish *A. aeneus* from Palo de Arquito, Nicaragua and Rio Pitahaya, Costa Rica, formed a monophyletic clade with other specimens of the species *S. tkachi* (GenBank, MG925121–2) (Fig. 4). The genetic divergence among the 8 isolates was very low, ranging from 0% to 0.2% for ITS2 + LSU and from 0% to 3.1% for *cox 1* (Table 3). The current record expands the geographical distribution range of *S. tkachi* in other areas of Middle America since the species was originally described from Costa Rica [2].

The phylogenetic analyses inferred with the combined data set of the two nuclear molecular markers (ITS2 + LSU) revealed that *Saccocoelioides* forms 12 independent lineages representing 12 valid species (Fig. 4). However, *Saccocoelioides macrospinosus* n. sp. was placed in a basal polytomy, possibly because ITS2 and LSU rDNA are conserved regions with a low rate of substitution. Therefore, the addition of a mitochondrial gene with a fast rate of substitution was fundamental to clarify the phylogenetic relationships of the new species (Fig. 5). In addition, the genetic divergence estimated with the *cox 1* gene among the 7 species of *Saccocoelioides* was high, ranging from 8.3 to 17%; and between *Saccocoelioides macrospinosus* n. sp. and *S. orosiensis* ranged from 8.7 to 11.3%; and between *Saccocoelioides macrospinosus* n. sp. and *S. lamothei* ranged from 14.8 to 15.5% (see Table 3). These high levels of genetic divergence are similar to other species of trematodes [6–8].

Curran et al. [2], discussed that the species diversity of *Saccocoelioides* in the Americas should be very different from what we know today, mainly because a large proportion of the species is currently distinguished based only on morphological characteristics. The entire genus of *Saccocoelioides* requires a deep taxonomic revision and, most importantly, new sequences from nuclear and mitochondrial genes as well as information from other congeneric species distributed in South America that are key to better understanding the phylogenetic relationships among species.

## 5. Conclusions

*Saccocoelioides macrospinosus* n. sp. is the fifth species of the genus described in the Neotropical region of Mexico and is associated with a poeciliid fish endemic to Catemaco Lake and the white mullet from Alvarado Lagoon. Both hydrobiological systems belong to the Papaloapan river basin in the state of Veracruz. Morphologically, the new species is distinguished from other congeneric species from Middle America by having a tegument covered with large spines, small body

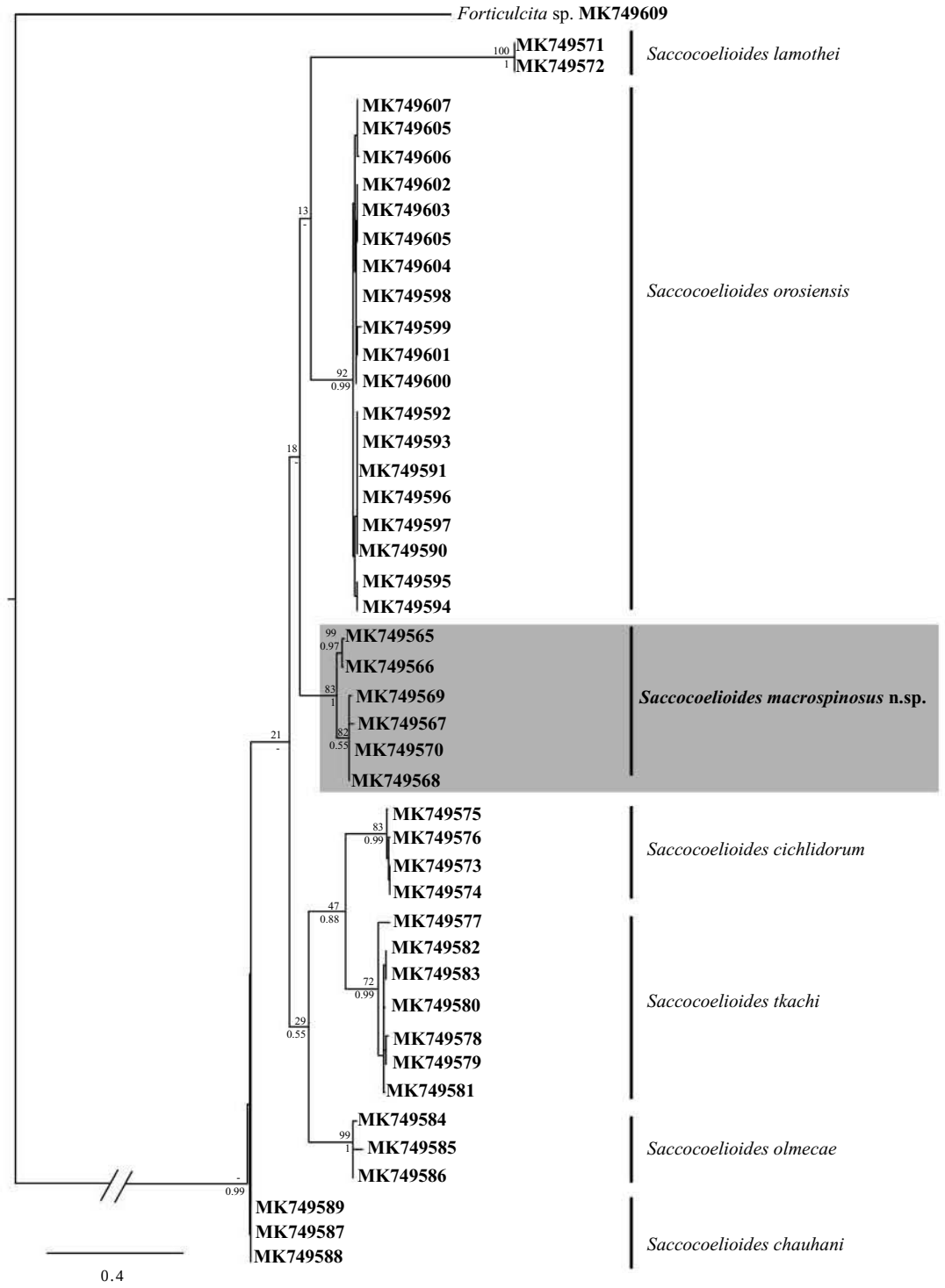


**Fig. 5.** Maximum likelihood tree and consensus Bayesian Inference trees inferred with *cox 1* data set; numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI).

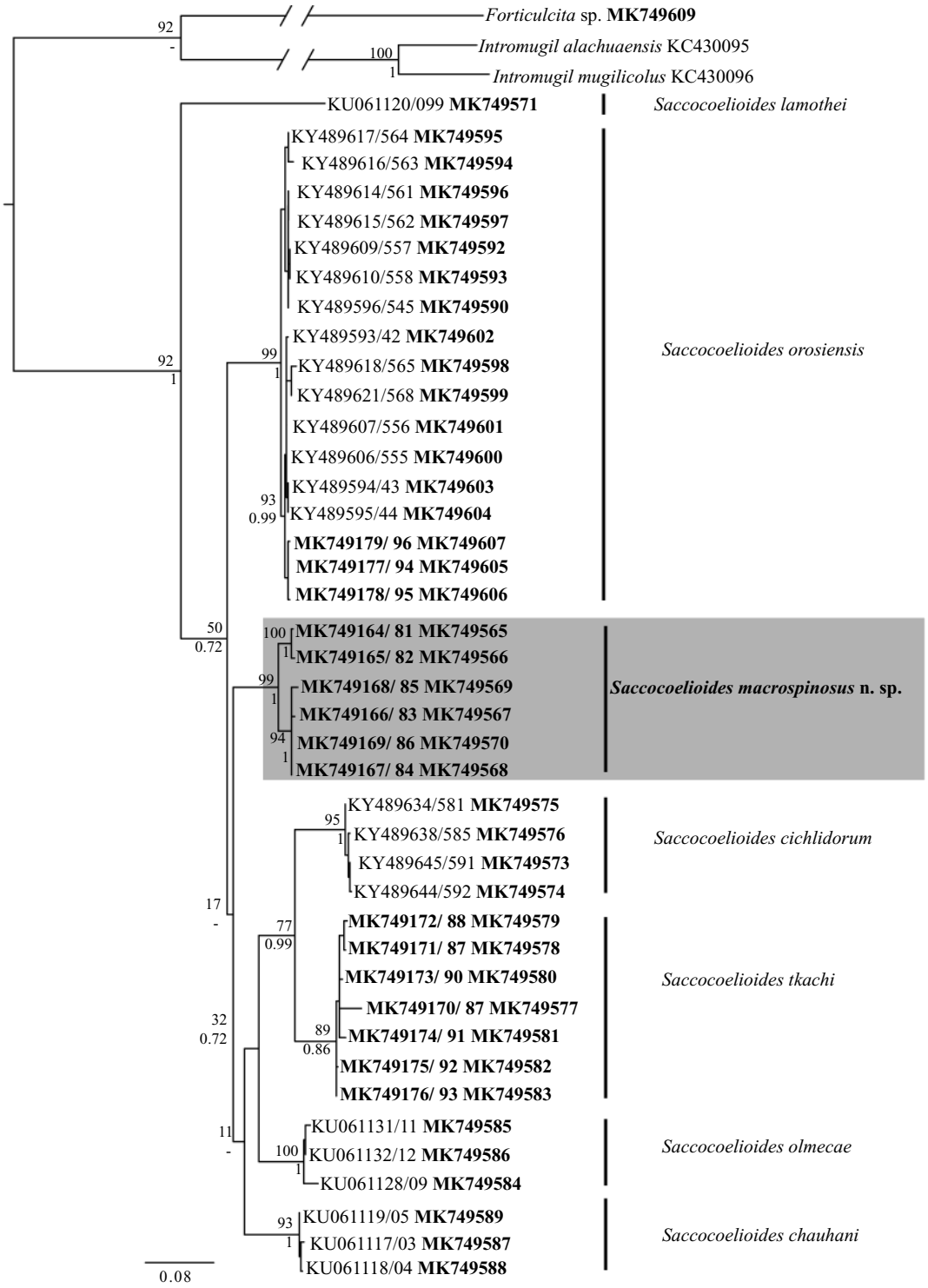


**Fig. 6.** Maximum likelihood tree and consensus Bayesian Inference trees inferred with the concatenated (*cox 1* + ITS2 + LSU) data set; numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI).

size, and small oral sucker. These morphological distinctions were demonstrated with phylogenetic analyses inferred with three molecular markers. We found that the *cox 1* gene is a complementary molecular marker than together with nuclear molecular markers are useful to delimitate species within the genus *Saccocoelioides*.

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## Conflict of interest

None.

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**III. I. II. Host-induced phenotypic plasticity in *Saccocoelioides lamothei* Aguirre-Macedo and Violante-González, 2008 (Digenea: Haploporidae) a parasite of freshwater, brackish and marine fishes from Middle America. En *Parasitology.***

Marcelo Tonatiuh González-García, **Leopoldo Andrade-Gómez**, Carlos Daniel Pinacho-Pinacho, Ana Lucia Sereno-Uribe, Martín García-Varela

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*Parasitology*

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# Host-induced phenotypic plasticity in *Saccocoelioides lamothei* Aguirre-Macedo and Violante-González, 2008 (Digenea: Haploporidae) a parasite of freshwater, brackish and marine fishes from Middle America

Marcelo Tonatiuh González-García[1], Leopoldo Andrade-Gómez[1,2], Carlos Daniel Pinacho-Pinacho[3], Ana Lucia Sereno-Uribe[1] and Martín García-Varela[1]

[1]Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, C.P. 04510, Ciudad de México, México; [2]Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, C. P. 04510, Ciudad de México, México and [3]Investigador Cátedras CONACyT, Instituto de Ecología, A.C., Red de Estudios Moleculares Avanzados, Km 2.5 Ant. Carretera a Coatepec, Xalapa, Veracruz 91070, México

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**Author for correspondence:**
Martín García-Varela,
E-mail: garciav@ib.unam.mx

## Abstract

*Saccocoelioides* is a genus of trematodes associated with fishes from the Americas. In the current research, morphologically distinct specimens of *Saccocoelioides* spp. were collected from six countries in Middle America. Specimens were sequenced using three molecular markers, the domains D1–D3 of the large subunit (LSU) from the nuclear rDNA, the cytochrome c oxidase subunit 1 (*cox1*) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad1*) from mitochondrial DNA. A total of 74 new sequences were compared and aligned with other sequences available in GenBank. Maximum likelihood and Bayesian inference analyses were inferred from the LSU and *cox1* datasets, revealing unequivocally that all the specimens correspond to *S. lamothei*. A haplotype network was built with 119 sequences of the *nad1* gene. The network detected 57 distinct haplotypes divided into three haplogroups. To explore morphological differences among samples of *S. lamothei*, 17 morphological features were measured from 53 specimens from three fish families: Eleotridae, Mugilidae and Gobiidae. Principal component analysis yielded three main polygons that corresponded with each family analysed, suggesting host-induced phenotypic plasticity. The current evidence suggests that *S. lamothei* infects at least five fish families along the Pacific coasts of Mexico, Guatemala, El Salvador, Honduras, Nicaragua and Costa Rica.

## Introduction

Phenotypic plasticity is the ability of a single species to produce multiple distinct phenotypes in response to the environmental conditions (Miner *et al.*, 2005). In parasites, with complex life cycles, different environmental conditions include (1) the host's immune system, (2) different host species and (3) the geographical distribution of the definitive hosts. All of these factors are correlated with the phenotypic plasticity, mainly in the body size or fecundity of parasites (Poulin, 2007).

Trematodes species are characterized mainly using morphological features, such as body size, proportion, shape and location of internal organs. Understanding the phenotypic plasticity in populations is essential and key to defining, recognizing and delineating species (Hildebrand *et al.*, 2015). Parasite taxonomy has heeded the call to shift toward 'integrative taxonomy', i.e. the use of multiple and complementary sources (Dayrat, 2005). The recent application of molecular markers in species identification has uncovered an extraordinary genetic richness in parasites, revealing many times more species than presently described (Poulin, 2011; Pérez-Ponce de León and Nadler, 2011). In addition, integrative taxonomy has helped to define, recognize, delineate and better understand the intraspecific variation that can be attributed to differences in the development and phenotypic plasticity of parasites (Hildebrand *et al.*, 2015; Poulin and Presswell, 2016).

*Saccocoelioides* Szidat, 1954 is the most diverse genus of trematodes belonging to the subfamily Chalcinotrematinae, and includes 24 recognized species, of which 14 are distributed in South America, seven in Middle America, two in North America and one in Puerto Rico; all these species are associated with freshwater, brackish and marine fishes from 10 families (Curran *et al.*, 2018; Andrade-Gómez *et al.*, 2019; Gallas and Utz, 2019). The genetic library of species of *Saccocoelioides* has increased significantly in the last few years. Curran *et al.* (2018) and Andrade-Gómez *et al.* (2019) evaluated the systematics of the genus

*Saccocoelioides* by combining nuclear and mitochondrial molecular markers, and ecological and morphological characteristics, detecting an extraordinary diversity in the Americas. Currently, Middle America harbours seven species of *Saccocoelioides*, *S. macrospinosus* Andrade-Gómez *et al.*, 2019; *S. orosiensis* Curran *et al.*, 2018; *S. tkachi* Curran *et al.*, 2018; *S. olmecae*, Andrade-Gómez *et al.*, 2016; *S. cichlidorum* (Aguirre-Macedo and Scholz, 2005) Andrade-Gómez *et al.*, 2017; *S. chauhani* Lamothe-Argumedo, 1974; and *S. lamothei* Aguirre-Macedo and Violante-González, 2008.

*Saccocoelioides lamothei* was described from the Pacific fat sleeper fish, *Dormitator latrifons* Richardson, 1844, from coastal lagoons of Guerrero state, Mexico (Aguirre-Macedo and Violante-González, 2008), and was subsequently reported as being associated with four fish species from three families (Poeciliidae, Profundulidae and Gobiidae) along the Pacific coasts of Middle America (Aguirre-Macedo and Violante-González, 2008; Pinacho-Pinacho *et al.*, 2015; Andrade-Gómez *et al.*, 2016, 2017, 2019; Curran *et al.*, 2018). Curran *et al.* (2018) noted that the biodiversity of *Saccocoelioides* in Middle America is far from well-known and that parasitological studies that combine morphological and molecular data are necessary to documenting its diversity in this biogeographical region.

The aim of the present study was to combine morphological and molecular characteristics to investigate the specific status of *Saccocoelioides* spp. in association with five fish families: Eleotridae, Mugilidae, Gobiidae, Poeciliidae and Profundulidae distributed along the Pacific coasts of Mexico, Guatemala, El Salvador, Honduras, Nicaragua and Costa Rica in Middle America. Sequences of three molecular markers were generated: the domains D1–D3 of the large subunit (LSU) from nuclear ribosomal DNA, the cytochrome c oxidase subunit 1 (*cox1*) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad1*) from mitochondrial DNA.

## Materials and methods

### Specimen collection

Digeneans were collected from 2010 through 2019 from the intestines of their definitive hosts in 29 localities from Middle America, 19 from Mexico, three from Guatemala, three from El Salvador, two from Nicaragua, one from Honduras and one from Costa Rica (see Fig. 1; Table 1). Fishes were collected with seine nets, cast nets and electrofishing, kept alive and transported to the laboratory. Each fish was euthanized and immediately examined. The collected digeneans were preserved either in 100% ethanol for DNA extraction or in hot (steaming) 4% formalin for morphological examination. Fishes were identified following the keys of Miller *et al.* (2005).

### Amplification, sequencing of DNA, phylogenetic analyses and haplotype network

Each specimen of *Saccocoelioides* spp. was placed individually in tubes and digested overnight at 56°C in a solution containing 10 mM Tris–HCl (pH 7.6), 20 mM NaCl, 100 mM $Na_2$ EDTA (pH 8.0), 1% Sarkosyl and 0.1 mg/ml proteinase K. Following digestion, DNA was extracted from the supernatant using the DNAzol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The domains D1–D3 of the LSU of nuclear ribosomal DNA plus two partial regions of mitochondrial DNA, cytochrome c oxidase subunit 1 (*cox1*) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad1*) were amplified using polymerase chain reaction (PCR). Domains D1–D3 from LSU were amplified using forward 5′–AGCGGAGGAAAAGAAACTAA–3′ (Nadler *et al.*, 2000) and reverse 5′–CAGCTATCCTGAGGGAAAC–3′ primers (García-Varela and Nadler, 2005). Two new primers were designed for the fragment of the *cox1*, forward primer SaccoF, 5′–TGTAAAACGACGGCCAGTTTWCITTRGATCATAAG–3′ and reverse primer SaccoR, 5′–TAAAGAAAGAACATAATGAAAA TG–3′. Finally, the gene *nad1* was amplified using forward 5′–AGATTCGTAAGGGGCCTAATA–3′ (Morgan and Blair, 1998) and reverse 5′–CTTCAGCCTCAGCATAAT–3′ primers (Kostadinova *et al.*, 2003). PCR reactions (25 μL) consisted of 1 μL of each primer (10 μM), 2.5 μL of 10× buffer, 1.5 μL of 2 mM $MgCl_2$, 0.5 μL of dNTPs (10 mM), 16.375 of water, 2 μL of genomic DNA and 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil). PCR cycling condition amplifications included denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, annealing at 40°C for *cox1* and 50°C for *nad1* and LSU for 1 min, and extension at 72°C for 1 min, followed by postamplification incubation at 72°C for 10 min. The sequencing reactions were performed using the initial primers *cox1*, *nad1* and LSU plus two internal primers: forward 5′–CCTTGGTCCGTGTTTCAAGACG–3′ and reverse 5′–CGT CTTGAAACACGGACTAAGG–3′ primers (García-Varela and Nadler, 2005), for LSU with ABI Big Dye (Applied Biosystems, Boston, MA, USA) terminator sequencing chemistry, and reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contigs were assembled and base-calling differences resolved using CodonCode Aligner v 9.0.1 (CodonCode Corporation, Dedham, MA, USA). Sequences obtained in the current research for LSU and *cox1* were aligned with sequences of *Saccocoelioides* spp., downloaded from GenBank (Table 1). In addition, sequences obtained for *nad1* of *Saccocoelioides* spp., and *S. lamothei* from type host and type locality, were aligned.

Sequences of each molecular marker were aligned separately using the software SeaView v.4 (Gouy *et al.*, 2010) and adjusted with the Mesquite program (Maddison and Maddison, 2011). The nucleotide substitution model was selected for each molecular marker using jModelTest v0.1.1 (Posada, 2008) and applying the Akaike information criterion; for the LSU dataset, the selected model was TVM + I, and for *cox1* TrN + I + G. The phylogenetic analyses were performed with LSU and *cox1* using maximum likelihood (ML) and Bayesian inference (BI) methods, using the online interface Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway v3.3 (Miller *et al.*, 2010). The ML analyses were carried out with RAxML v.7.0.4 (Silvestro and Michalak, 2011), and 10 000 bootstrap replicates were run to assess nodal support. The BI analyses were inferred with MrBayes v.3.2.7 (Ronquist *et al.*, 2012), with two simultaneous runs of the Markov Chain Monte Carlo (MCMC) for 10 million generations, sampled every 1000 generations, a heating parameter value of 0.2 and a 'burn-in' of 25%. Trees were drawn using FigTree v.1.3.1 (Rambaut, 2012). The genetic divergence among taxa was estimated using uncorrected '*P*' distances with the program MEGA version 6 (Tamura *et al.*, 2013).

In order to examine the relationships among *Saccocoelioides* spp., the *nad1* haplotype frequency was estimated, an unrooted statistical network was constructed using the program NETWORK version 5.0 (www.fluxus-engineering.com) keeping the $\varepsilon = 0$. This method starts with minimum spanning trees combined within a single network and then, to reduce tree length, median vectors (consensus sequences) are added. Such vectors can be interpreted as possibly extant unsampled sequences or extinct ancestral sequence (Bandelt *et al.*, 1999). In addition, the median-joining algorithm was employed to build the network.

### Morphometrics analysis

Unflattened specimens preserved in formalin were stained with Mayer's paracarmine, dehydrated in a graded ethanol series,

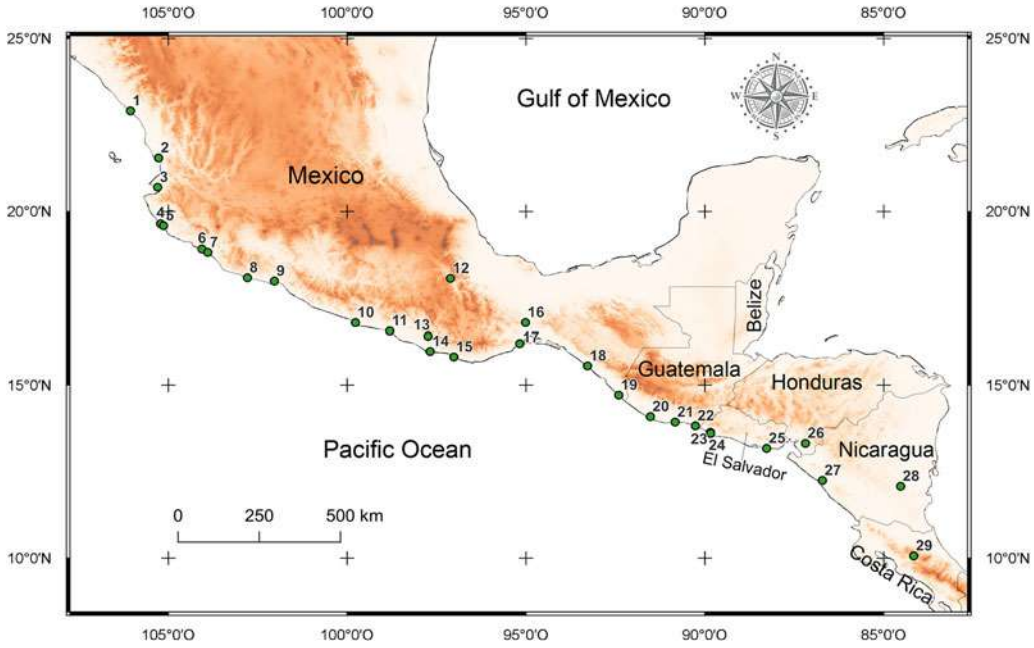


**Fig. 1.** Map indicating the 29 localities for *Saccocoelioides* spp., in Middle America; localities correspond with Table 1.

cleared with methyl salicylate and mounted on permanent slides with Canada balsam. Specimens collected in the present study were compared with the specimens of *S. lamothei* deposited at the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad de México, México. All the specimens were examined using a bright-field Leica DM1000 LED compound microscope (Leica, Wetzlar, Germany) and new specimens were deposited in CNHE.

Drawings were made using a drawing tube attached to the microscope. A total of 17 morphological features (BL, body length; BW, maximum body width; OSL, oral sucker length; OSW, oral sucker width; VSL, ventral sucker length; VSW, ventral sucker width; P, prepharynx; PHL, pharynx length; PHW, pharynx width; HSL, hermaphroditic sac length; HSW, maximum hermaphroditic sac width; TL, testis length; TW, testis width; OL, ovary length; OW, ovary width; EL, egg length; EW, egg width) were measured of 53 individuals with the Leica Application Suite microscope software. All measurements are in micrometres ($\mu$m). A principal component analysis (PCA) was implemented to explore and describe the patterns of morphological variation of the specimens. PCA was conducted using R-packages ggplot2, ggfortify, cluster and alpha implemented in R (R Core Team, 2016). For the analysis, 21 specimens identified as *S. lamothei* from CNHE (4349, 4350, 4906, 6671, 9370–9373), and 32 newly collected individuals from *Saccocoelioides* spp. were selected. PCA included the 17 characteristics; BL, BW, OSL, OSW, VSL, VSW, P, PHL, PHW, HSL, HSW, TL, TW, OL, OW, EL, EW.

## Results

### Phylogenetic trees

The LSU dataset included 1271 characters and the best model obtained was TVM + I. The alignment included a total of 91 sequences, 74 new sequences of *Saccocoelioides* spp., plus six sequences of *S. lamothei* (KU061120–KU061124, MG925110) and one sequence each of *S. chauhani* (KU061119), *S. macrospinosus* (MK749169), *S. olmecae* (KU061136), *S. orosiensis* (MG925108), *S. tkachi* (MG925122), *S. cichlidorum* (MG925106), *S. sogandaresi* (MG925120), *S. magnus* Szidat, 1954 (MG925112), *S. elongatus* Szidat, 1954 (MG925108), *S. nanii* Szidat, 1954 (MG925114) and *S. beauforti* Hunter and Thomas, 1961 (MG925104) (see Table 1). The phylogenetic analyses inferred with ML and BI yielded several clades. The base of the tree was formed by a polytomy that contained two species from South America, *S. magnus* and *S. elongatus*. Another clade was formed by *S. orosiensis* and *S. nanii* and an unresolved branch belonging to *S. macrospinosus*. Other clade was formed by four species (*S. sogandaresi*, *S. chauhani*, *S. olmecae* and *S. beauforti*). A clade was formed by *S. tkachi* and *S. cichlidorum* which was supported with a good bootstrap value and Bayesian posterior probabilities (Fig. 2). The remaining new sequences of *Saccocoelioides* spp. formed a clade that included six sequences previously identified as *S. lamothei*, two (KU061120–KU061121) from the type host and type locality; others from Chacahua, Oaxaca, Mexico (KU01122–KU01124) and one (MG925110) from Río Tempisque, Costa Rica. This clade received strong support from the bootstrap and Bayesian posterior probabilities (100/1) (Fig. 2).

The genetic divergence estimated using the LSU dataset from rDNA among the new sequences of *Saccocoelioides* spp. and *S. lamothei* ranged from 0 to 0.31%. The monophyly of the new sequences and the six sequences previously identified as *S. lamothei*, in combination with the low genetic divergence among the sequences, suggests that all new sequences belong to *S. lamothei*, expanding its distribution range and host spectrum along the Pacific coasts of Middle America.

A second dataset for the *cox1* gene included 588 characters, and the best selected model was TrN + I + G. The alignment included 57 terminals, 47 new sequences of *S. lamothei*, plus 10 sequences of the genus *Saccocoelioides*, two of *S. lamothei* (MK749571–72) from the type host and type locality, two of *S. orosiensis* (MK749590 and MK749602), two of *S. tkachi* (MK749577–78) and one of *S. cichlidorum* (MK749574), *S. olmecae* (MK749584), *S. macrospinosus* (MK749566) and *S. chauhani* (MK749589) (Table 1). The phylogenetic analyses inferred with ML and BI from the *cox1* dataset showed that the specimens collected in this study formed a clade with specimens previously identified as *S. lamothei* with strong support from the bootstrap and Bayesian posterior probabilities (100/1) (Fig. 3), suggesting that all of the sequences belonged to the

**Table 1.** Specimens analysed in this study; localities (in parenthesis are localities sampled in this study and correspond with Fig. 1); host species; host family and GenBank accession numbers of each molecular marker. Sequences in bold were generated in this study

| Species | Locality | Host | Family | LSU | *cox1* | *nad1* | References |
|---|---|---|---|---|---|---|---|
| *Saccocoelioides olmecae* | Tamiahua, Veracruz, México 21°15′49″N, 97°27′41″W | *Dormitator maculatus* | Eleotridae | KU061128 | MK749584 | – | Andrade-Gómez *et al.* (2016)/ Andrade-Gómez *et al.* (2019) |
| *Saccocoelioides chauhani* | Catemaco, Veracruz, México 18°25′0″N, 95°7′0″W | *Astyanax aeneus* | Characidae | KU061117 | MK749589 | – | |
| *Saccocoelioides macrospinosus* | Catemaco, Veracruz, México | *Poeciliopsis catemaco* | Poeciliidae | – | MK749566 | – | Andrade-Gómez *et al.* (2019) |
| | Alvarado, Veracruz, México 18°46′47″N, 95°44′50″W | *Mugil curema* | Mugilidae | MK749169 | – | – | |
| *Saccocoelioides orosiensis* | Tlacotalpan, Veracruz, México 18°36′0″N, 95°39′0″W | *Poecilia sphenops* | Poeciliidae | – | MK749602 | – | |
| *Saccocoelioides orosiensis* | Río Ciruelas, Costa Rica 10° 3′38″N, 84°45′31″W | *Poecilia gilii* | Poeciliidae | – | MK749590 | – | |
| *Saccocoelioides cichlidorum* | Río Torsuani, Nicaragua 11° 47′06″N, 83°52′38″W | *Paraneetroplus maculicauda* | Cichlidae | – | MK749574 | – | |
| *Saccocoelioides tkachi* | Palo de Arquito, Nicaragua 11°7′12″N, 84°36′5″W | *Astyanax aeneus* | Characidae | – | MK749577 | – | |
| *Saccocoelioides tkachi* | Río Pérez, Nicaragua 11° 45′0.8″N, 84°14′11.5″W | *Astyanax aeneus* | Characidae | – | MK749578 | – | |
| *Saccocoelioides tkachi* | Río Tempisque, Costa Rica 10°47′21″N, 85°33′03″W | *Astyanax aeneus* | Characidae | MG925122 | – | – | Curran *et al.* (2018) |
| *Saccocoelioides cichlidorum* | Río Animas, Costa Rica 11° 02′54″N, 85°35′09″W | *Archocentrus nigrofasciatus* | Cichlidae | MG925106 | – | – | |
| *Saccocoelioides orosiensis* | Río Tempisque, Costa Rica | *Poecilia gilii* | Poeciliidae | MG925116 | – | – | |
| *Saccocoelioides magnus* | Río de la Plata, Argentina 34° 48′48″S, 57°58′25″W | *Cyphocarynx voga* | Characidae | MG925112 | – | – | |
| *Saccocoelioides elongatus* | Río de la Plata, Argentina | *Prochilodus lineatus* | Prochilodontidae | MG925108 | – | – | |
| *Saccocoelioides nanii* | Los Talas, Argentina 34° 53′56″S, 57°48′17″W | *Prochilodus lineatus* | Prochilodontidae | MG925114 | – | – | |
| *Saccocoelioides sogandaresi* | Texas, United States 27° 30′29″N, 97°50′08″W | *Poecilia latipinna* | Poeciliidae | MG925120 | – | – | |
| *Saccocoelioides beauforti* | North Carolina, United States 34°11′05″N, 77° 48′52″W | *Mugil cephalus* | Mugilidae | MG925104 | – | – | |
| *Saccocoelioides lamothei* | Río Tempisque, Costa Rica 10°47′21″N, 85°33′03″W | *Poecilia gilii* | Poeciliidae | MG925110 | – | – | |
| *Saccocoelioides lamothei* | (10) Tres Palos, Guerrero, México 16°48′0″N, 99°47′0″W | *Dormitator latifrons* | Eleotridae | KU061120–21 | MK749571–72 | **MW287784–85** | Andrade-Gómez *et al.* (2016)/ Andrade-Gómez *et al.* (2019)/ This study |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Saccocoelioides lamothei* | (13) Chacahua, Oaxaca, México 15°58′4.73″N, 97° 40′55.44″W | *Dormitator latifrons* | Eleotridae | KU061122–24 **MW282051–53** | **MW287346–47** | **MW287786–95** | Andrade-Gómez *et al.* (2016)/ This study |
| *Saccocoelioides lamothei* | (1) El Huizache, Sinaloa, México 22°54′29″N, 106° 3′39.48″W | *Mugil cephalus* | Mugilidae | **MW282054** | **MW283184** | **MW287796–98** | This study |
| *Saccocoelioides lamothei* | (2) La Tovara, Nayarit, México 21°32′43.6″N, 105° 16′24.1″W | *Dormitator latifrons*<br>*Mugil cephalus* | Eleotridae<br>Mugilidae | **MW282055**<br>**MW282056** | – | **MW287799–802**<br>**MW287803–10** | |
| *Saccocoelioides lamothei* | (3) Nuevo Vallarta, Nayarit, México 20°41′57″N, 105° 17′56.7″W | *Dormitator latifrons* | Eleotridae | **MW282057–58** | **MW283185–87** | **MW287811–15** | |
| *Saccocoelioides lamothei* | (4) Quémaro, Jalisco, México 19°38′40.2″N, 105°12′55.5″W | *Dormitator latifrons*<br>*Mugil* sp. | Eleotridae<br>Mugilidae | **MW282059–60**<br>**MW282061–62** | **MW283188–89**<br>**MW283190** | **MW287816–18**<br>**MW287819–20** | |
| *Saccocoelioides lamothei* | (5) Playa Punta Pérula, Jalisco, México 19°35′16.7″N, 105°8′7.526″W | *Mugil* sp. | Mugilidae | **MW282063** | – | **MW287821** | |
| *Saccocoelioides lamothei* | (6) Cuyutlán, Colima, México 18°54′45.39″N, 104° 3′36.74″W | *Dormitator latifrons* | Eleotridae | **MW282064–65** | **MW283191–94** | **MW287822–26** | |
| *Saccocoelioides lamothei* | (7) Estero Tecuanillo, Colima, México 18°49′2.34″N, 103° 53′54.70″W | *Mugil* sp. | Mugilidae | **MW282066** | **MW283195** | **MW287827** | |
| *Saccocoelioides lamothei* | (8) Barra de Nexpa, Michoacán, México 18° 5′0.24″N, 102°47′18.366″W | *Mugil* sp. | Mugilidae | **MW282067–68** | – | **MW287828–31** | |
| *Saccocoelioides lamothei* | (9) Playa Las Peñitas, Guerrero, México 17° 59′16.718″N, 102°2′5.01″W | *Mugil* sp. | Mugilidae | **MW282069–79** | **MW283196–203** | **MW287832–43** | |
| *Saccocoelioides lamothei* | (11) Marquelia, Guerrero, México 16°33′19.75″N, 98° 48′38.89″W | *Mugil curema* | Mugilidae | **MW282080–81** | – | **MW287844–45** | |
| *Saccocoelioides lamothei* | (12) Río Salado, Oaxaca, México 18°3′52.7″N, 97° 6′58.399″W | *Poeciliopsis gracilis*<br>*Poecilia sphenops* | Poeciliidae | **MW282082–84**<br>**MW282085** | **MW283204**<br>**MW283205** | **MW287846–47**<br>**MW287848** | |
| *Saccocoelioides lamothei* | (14) San José de las Flores, Oaxaca, México 16° 24′21.5″N, 97°44′22.599″W | *Sicydium multipunctatum*<br>*Profundulus sp.* | Gobiidae<br>Profundulidae | **MW282090–91**<br>**MW282086–88** | **MW283206–07**<br>– | **MW287849–51**<br>**MW287852-54** | |
| *Saccocoelioides lamothei* | (15) Barra de Navidad, Oaxaca, México 15°48′39″N, 97°1′10.999″W | *Mugil cephalus* | Mugilidae | **MW282092–93** | **MW283208** | **MW287855–56** | |
| *Saccocoelioides lamothei* | (16) Matías Romero, Oaxaca, México 16°47′30.8″N, 95° 0′59″W | *Poecilia mexicana* | Poeciliidae | **MW282094** | **MW283209** | **MW287857** | |

(*Continued*)

**Table 1.** (*Continued.*)

| Species | Locality | Host | Family | LSU | *cox1* | *nad1* | References |
|---|---|---|---|---|---|---|---|
| *Saccocoelioides lamothei* | (17) La Ventosa, Oaxaca, México 16°11′42.67″N, 95° 10′40.79″W | *Mugil cephalus* | Mugilidae | – | **MW283210** | **MW287859–61** | |
| *Saccocoelioides lamothei* | (18) Pijijiapán, Chiapas, México 15°33′12″N, 93° 16′55.999″W | *Dormitator latifrons* | Eleotridae | – | **MW283211** | **MW287862–63** | |
| *Saccocoelioides lamothei* | (19) Puerto Chiapas, Chiapas, México 14° 42′36.46″N, 92°24′30.91″W | *Mugil* sp. | Mugilidae | **MW282095** | – | **MW287864–68** | |
| *Saccocoelioides lamothei* | (20) Río Nahualate, Guatemala 14°5′19.039″N, 91°31′28.87″W | *Poecilia mexicana*<br>*Poeciliopsis gracilis* | Poeciliidae | **MW282096–97**<br>**MW282098–99** | –<br>**MW283212** | **MW287869–70**<br>**MW287871–72** | |
| *Saccocoelioides lamothei* | (21) Puerto San José, Guatemala 13°55′49.375″N, 90°50′2.98″W | *Mugil curema* | Mugilidae | **MW282100–01** | **MW283213–14** | **MW287873–74** | |
| *Saccocoelioides lamothei* | (22) Las Lisas, Guatemala 13° 49′19.942″N, 90°16′2.23″W | *Dormitator latifrons* | Eleotridae | **MW282102** | **MW283215** | **MW287875–76** | |
| *Saccocoelioides lamothei* | (23) Río Sunza, El Salvador 13°38′14″N, 89°50′40.99″W | *Sicydium* sp.<br>*Poecilia mexicana* | Gobiidae<br>Poeciliidae | **MW282103–04**<br>**MW282105** | –<br>**MW283216** | **MW287877–78**<br>**MW287879–80** | |
| *Saccocoelioides lamothei* | (24) Río Banderas, El Salvador 13°36′2.618″N, 89° 50′26.19″W | *Awaous banana*<br>*Dajaus monticola* | Gobiidae<br>Mugilidae | **MW282106–07**<br>**MW282108–09** | **MW283217**<br>**MW283218–19** | **MW287881–82**<br>**MW287883–85** | |
| *Saccocoelioides lamothei* | (25) Bahía de San Antonio, El Salvador 13°10′12.65″N, 88° 16′29.87″W | *Dormitator latifrons* | Eleotridae | **MW282110–12** | **MW283220–22** | **MW287886–88** | |
| *Saccocoelioides lamothei* | (26) Río Choluteca, Honduras 13°18′57″N, 87° 11′23.999″W | *Poecilia mexicana* | Poeciliidae | **MW282113–14** | **MW283223** | **MW287889–90** | |
| *Saccocoelioides lamothei* | (27) Río Tamarindo, Nicaragua 12°14′33.82″N, 86° 42′57.26″W | *Sicydium* sp.<br>*Dajaus monticola*<br>*Poecilia gillii* | Gobiidae<br>Mugilidae<br>Poeciliidae | **MW282115–16**<br>**MW282117**<br>**MW282118-19** | **MW283224–25**<br>–<br>**MW283226** | **MW287891–93**<br>**MW287894–95**<br>**MW287896–97** | |
| *Saccocoelioides lamothei* | (28) Río Mico, Nicaragua 12° 4′32.016″N, 86°31′47.95″W | *Poecilia gillii* | Poeciliidae | **MW282120** | **MW283227** | **MW287898–900** | |
| *Saccocoelioides lamothei* | (29) Río Ciruelas, Costa Rica 10°3′51.576′N, 84°9′49.646″W | *Sicydium salvini*<br>*Poecilia gillii* | Gobiidae<br>Poeciliidae | **MW282121–22**<br>**MW282123** | –<br>**MW283228** | **MW287901–02**<br>**MW287903–04** | |

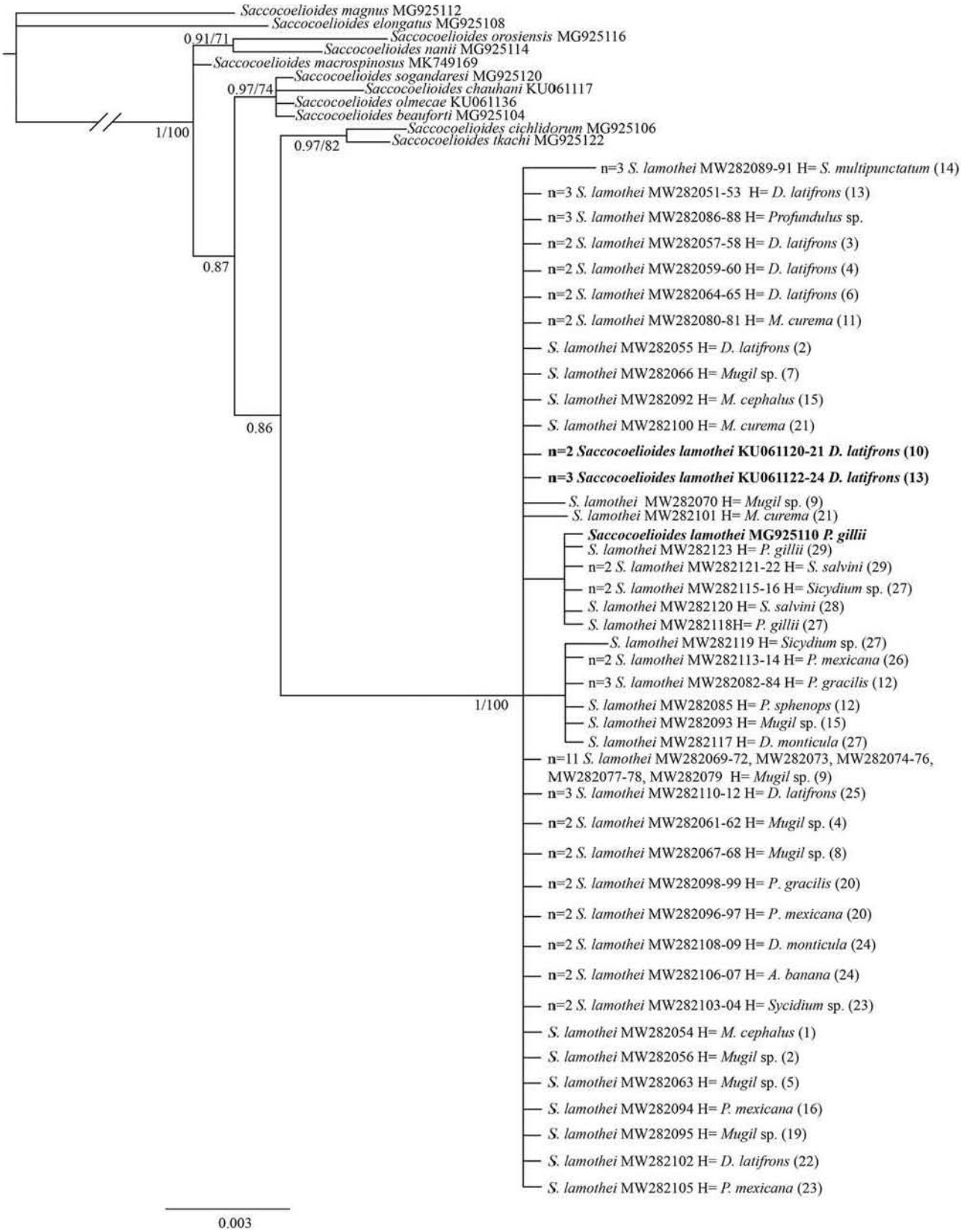


**Fig. 2.** Maximum likelihood tree and consensus Bayesian inference trees inferred from the LSU dataset. Numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI). *n*, number of identical sequences; H, host name. In parenthesis, number of locality (Table 1). Sequences in bold of *Saccocoelioides lamothei* were downloaded from GenBank

same lineage. The genetic intraspecific divergence estimated with the *cox1* dataset among the new sequences and two sequences of *S. lamothei* from the type host and type locality ranged from 0 to 6.62%.

### Haplotype network

A haplotype network was built using the *nad1* gene. This dataset was formed by 119 specimens with 485 characters and 57

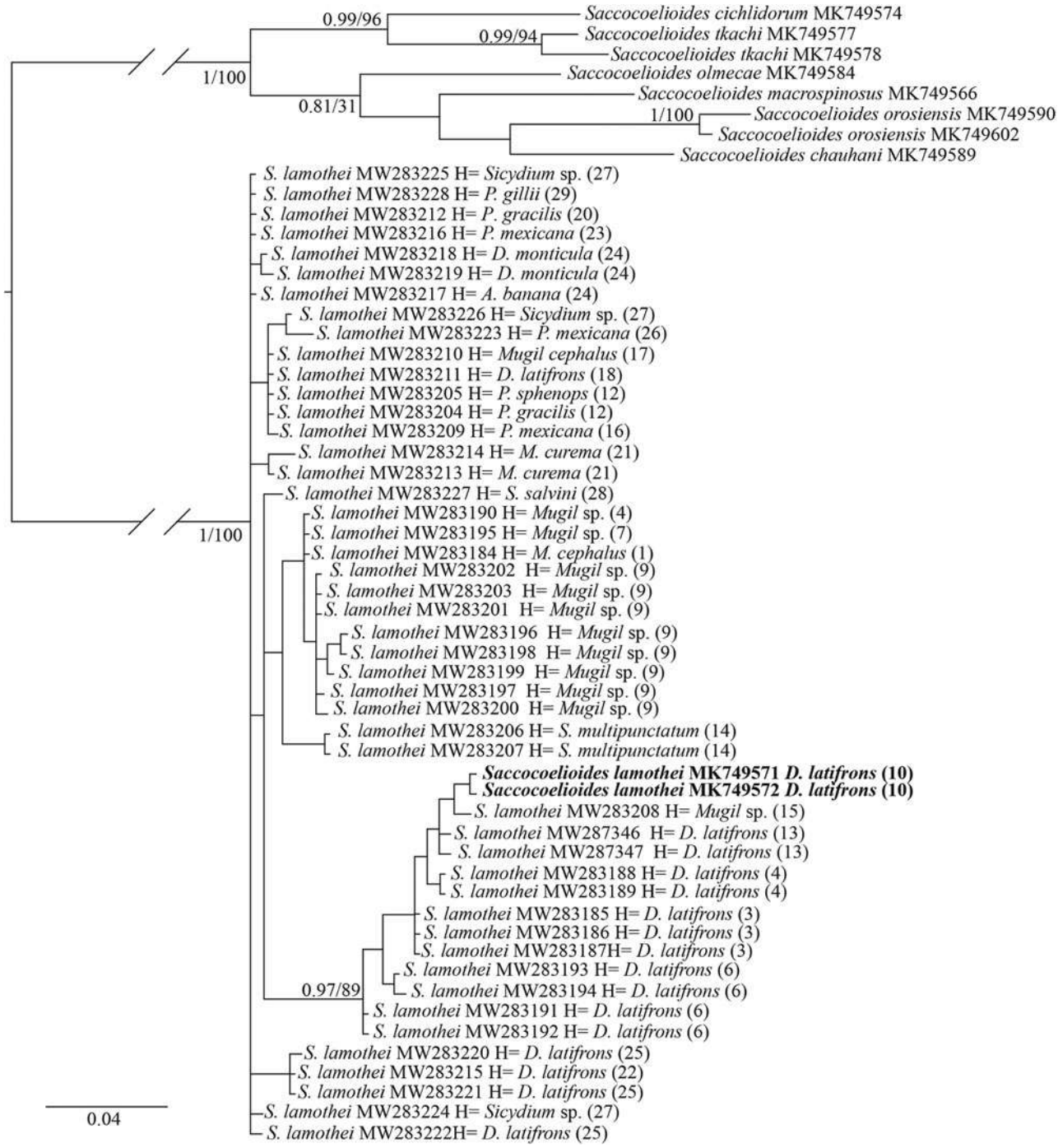


**Fig. 3.** Maximum likelihood tree and consensus Bayesian inference trees inferred with *cox1* dataset; numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI). H, host. In parenthesis, number of locality (Table 1). Sequences in bold of *Saccocoelioides lamothei* were downloaded from GenBank.

haplotypes were detected. The haplotype network yielded three haplogroups separated by a few mutational steps (no more than 12 steps) (Fig. 4). The first haplogroup contained 17 distinct haplotypes from 32 specimens from eleotrid and mugilid fishes. The second haplogroup contained three haplotypes (BS, BU and BT) from mugilid fishes. Only the third haplogroup contained 37 distinct haplotypes from 84 individuals from five host families (see Table S1). The most frequent haplotype was CB, which was formed by 13 specimens from four localities (4, 5, 9, 25 see Fig. 5; Table S1) in two countries, Mexico and El Salvador, from eleotrid and mugilid fishes. Most of the haplotypes had a restricted geographic distribution. For instance, five localities (3, 7, 10, 23, 29) showed a unique haplotype (a single haplotype per locality; AD, BJ, BO, CD, CF), and 15 localities (2, 4, 6, 9, 11, 13–15, 18–20, 24–27) had 45 exclusive haplotypes. The remaining nine localities (1, 5, 8, 12, 16, 17, 21, 22, 28) shared haplotypes (Fig. 5; Table S1).

### Morphometric analyses

Morphometric analysis was conducted to corroborate that the morphological differences among the isolates of *S. lamothei* were associated with their different hosts. A total of 17 variables were considered from 53 specimens (Table 2). PCA was used to classify host species (Fig. 6A). The measurements of the specimens from *D. latifrons* formed a separate polygon. However,

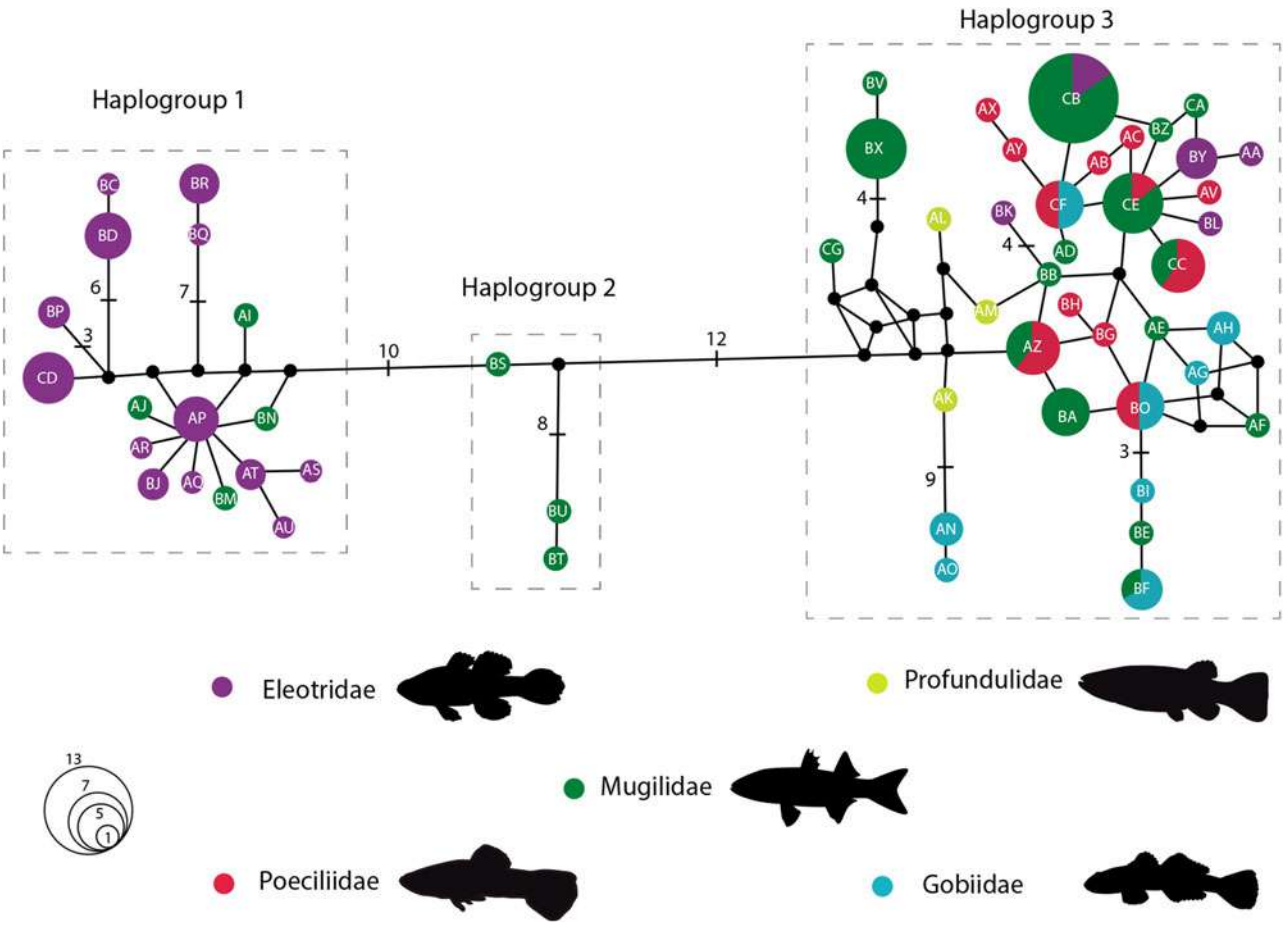


**Fig. 4.** Haplotype network of samples of *Saccocoelioides lamothei*, build with the gene nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad1*). Each circle represents a haplotype, with size proportional to the haplotype's frequency in the populations. The haplotype name is indicated in letters and corresponds with Table S1.

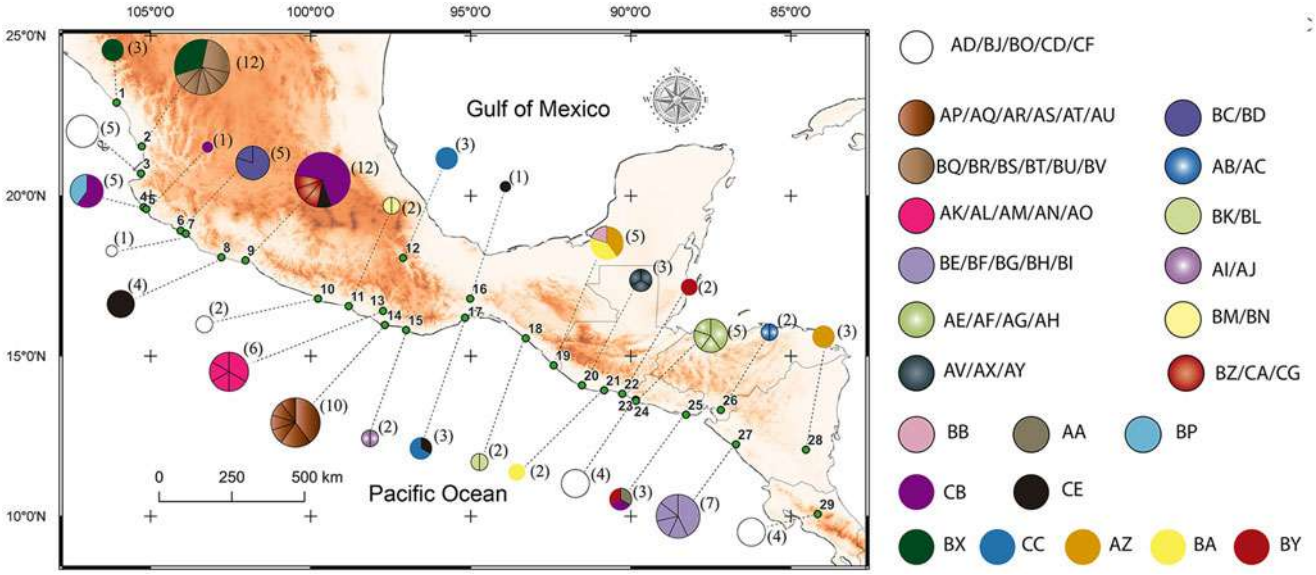


**Fig. 5.** Distribution of haplotypes in the localities sampled of *Saccocoelioides lamothei*. Size corresponds to the number of specimens sampled (see Table S1).

the measurements of the other specimens from different host species overlapped with each other (Fig. 6A). Later, the measurements of specimens from each host species were clustered by host families (Eleotridae, Mugilidae and Gobiidae). PCA showed three polygons representing each definitive host family (Fig. 6B). Those three polygons relate to each morphotype found (Fig. 7).

### *Morphological description*

*Saccocoelioides lamothei* Aguirre-Macedo and Violante-Gónzalez, 2008.

Based on 53 specimens studied from *Dormitator latifrons*, *Mugil curema*, *Mugil* sp., *Sicydium multipunctatum*, *Sicydium* sp.

**Table 2.** Comparative morphometric data for the three morphotypes of *Saccocoelioides lamothei.*

| Host | *Dormitator latifrons* (*n* = 15) | *Mugil curema* (*n* = 7) and *Mugil* sp. (*n* = 8) | *Sicydium multipunctatum* (*n* = 4), *S. salvini* (*n* = 7), *Sicydium* sp. (*n* = 9) and *Awaous banana* (*n* = 3) | |
|---|---|---|---|---|
| Host family | Eleotridae | Mugilidae | Gobiidae | |
| Locality | México: Tres Palos, Coyuca and Chacahua | México: Puerto Chiapas Guatemala: Puerto San José | México: San José de las Flores El Salvador: Río Sunza and Río Banderas Nicaragua: Río Tamarindo Costa Rica: Río Ciruelas | Total (*n* = 53) |
| BL | 420–850 | 533–906 | 371–876 | 371–906 |
| BW | 240–510 | 206–441 | 134–438 | 134–510 |
| OSL | 63–155 | 68–100 | 51–129 | 51–155 |
| OSW | 77–127 | 91–119 | 54–135 | 54–135 |
| VSL | 71–131 | 78–130 | 62–123 | 62–131 |
| VSW | 82–151 | 96–143 | 61–185 | 61–185 |
| P | 7.5–23 | 12–29 | 6–26 | 6–29 |
| PHL | 52–112 | 54–93 | 52–106 | 52–112 |
| PHW | 55–115 | 52–95 | 46–117 | 46–117 |
| HSL | 99–215 | 96–181 | 59–170 | 59–215 |
| HSW | 36–130 | 51–107 | 40–107 | 36–130 |
| TL | 90–207 | 107–213 | 52–164 | 52–213 |
| TW | 67–166 | 75–144 | 48–134 | 48–166 |
| OL | 31–79 | 42–77 | 28–80 | 28–80 |
| OW | 11–51 | 27–63 | 17–63 | 11–63 |
| EL | 80–135 | 55–94 | 47–92 | 47–135 |
| EW | 50–80 | 33–58 | 38–69 | 33–80 |

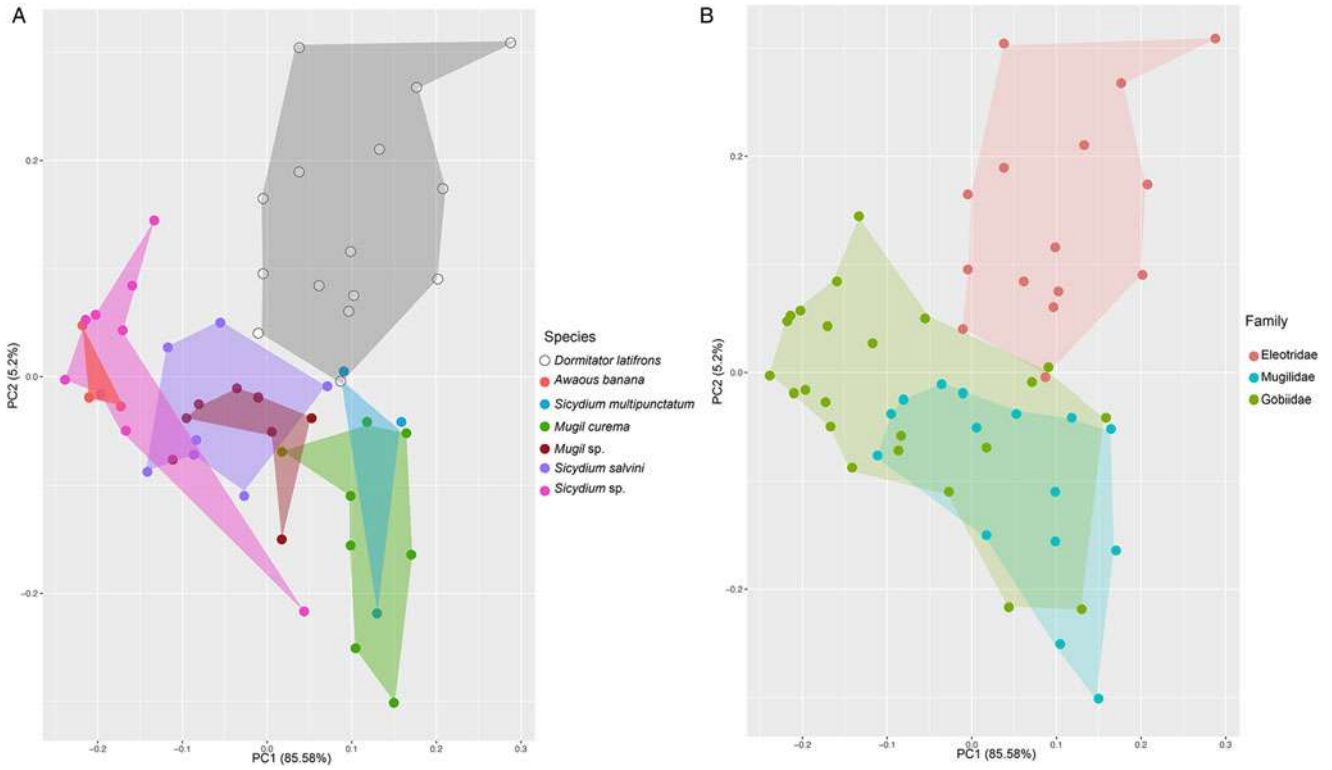


**Fig. 6.** Principal component analysis conducted with 17 morphometric variables from 53 specimens of *Saccocoelioides lamothei*. (A) Specimens analysed by host species. (B) Specimens analysed by host families.

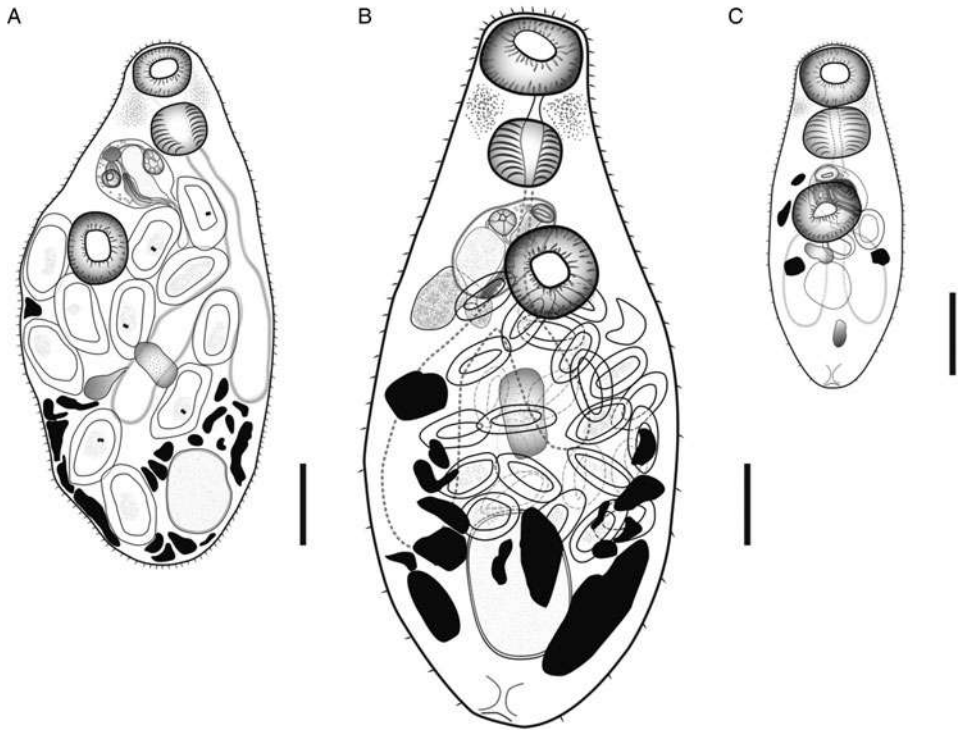


**Fig. 7.** Morphotypes of *Saccocoelioides lamothei*: (A) from *Dormitator latifrons*, Tres Palos, Guerrero, México; (B) from *Mugil curema*, Puerto San José, Guatemala; (C) from *Sycidium* sp., Río Tamarindo, Nicaragua. Scale bars = 100 $\mu$m

and *Awaous banana* from 10 localities distributed in Mexico, Guatemala, El Salvador, Nicaragua and Costa Rica (Table 2).

Tegument entirely covered by minute spines, being scatter in the posterior end in mugilid and gobiid fishes (Fig. 7A–C). Eye-spot remnants present in the anterior of the body reaching half of the pharynx (Fig. 7). Oral sucker subterminal. Ventral sucker slightly anterior to middle of the body or at the middle body in gobiid fishes (Fig. 7C). Prepharynx present or absent in gobiid fishes (Fig. 7C). Pharynx oval to spherical. Oesophagus long. Caeca sac-shaped elongated, terminating in hindbody. Testis oval to subspherical, in the middle of hindbody or the posterior end of hindbody in eleotrid fishes (Fig. 7A). External seminal vesicle small, contiguous to hermaphroditic sac. Hermaphroditic sac oval to spherical, at the level of ventral sucker, or anterior to ventral sucker in eleotrid fishes (Fig. 7A). Internal seminal vesicle elongated to spherical. Genital pore opening anterior to ventral sucker. Ovary elongated at the middle of the body. Laurer's canal not observed, Mehlis' gland not observed. Uterus confined between hermaphroditic sac and testis (Fig. 7B–C) or filling the entire body in eleotrid fishes (Fig. 7A), with the metraterm entering the posterior end of hermaphroditic sac. Vitelline follicles elongated, irregular, distributed in lateral fields from the level of the hermaphroditic sac to the posterior of the testis, anterior in gobiid fishes (Fig. 7C). Eggs operculate. Miracidia observed in eleotrid fishes. Excretory vesicle Y-shaped. Excretory pore terminal (Fig. 7; see Table 2).

### *Taxonomic summary*

*Saccocoelioides lamothei* Aguirre-Macedo and Violante-González, 2008.

Type-host: *Dormitator latifrons* (Eleotridae).

Other hosts: *Dajaus monticola*, *Mugil cephalus*, *M. curema*, *Mugil* sp. (Mugilidae), *Poecilia gillii*, *Poecilia mexicana*, *Poecilia sphenops*, *Poeciliopsis gracilis* (Poeciliidae), *A. banana*, *S. multipunctatum*, *Sicydium salvini*, *Sicydium* sp. (Gobiidae) y *Profundulus* sp. (Profundulidae).

Type-locality: Tres Palos, Guerrero, México.

Other localities: México: El Huizache, Sinaloa; La Tovara and Nuevo Vallarta, Nayarit; Quémaro and Playa Punta Pérula, Jalisco; Cuyutlán and Estero Tecuanillo, Colima; Barra de Nexpa, Michoacán; Playa las Peñitas and Marquelia, Guerrero; Río Salado, San José de las Flores, Chacahua, Barra Navidad, Matías Romero and Ensenada la Ventosa, Oaxaca; Pijijiapán and Puerto Chiapas, Chiapas. Guatemala: Río Nahualate, Puerto San José and Las Lisas. El Salvador: Río Sunza, Río Banderas and Bahía de San Antonio. Honduras: Río Choluteca. Nicaragua: Río Tamarindo. Costa Rica: Río Ciruelas.

Site in host: Intestine.

## Discussion

The phylogenetic analyses inferred with the LSU unequivocally placed all the new sequences from the Pacific coasts of Middle America into a monophyletic clade together with six sequences previously identified as *S. lamothei*, including specimens from the type host and type locality (see Fig. 2). The genetic divergence estimated among the 12 species of the genus *Saccocoelioides* ranged from 0.2 to 5.7% and its range was similar than found previously by Andrade-Gómez *et al.* (2019), who reported a range of genetic divergence from 0 to 4.8%. The intraspecific genetic divergence among the isolates of *S. lamothei* ranged from 0 to 0.31% for LSU. The intraspecific divergence found herein is similar to the LSU reported previously for *S. tkachi* (0–0.2%) and *S. orosiensis* (0–0.4%) (Andrade-Gómez *et al.*, 2019). The phylogenetic analysis inferred with the *cox1* clearly distinguished species previously recognized within *Saccocoelioides* (see Fig. 3). The genetic divergence estimated with *cox1* dataset among the seven species of *Saccocoelioides* ranged from 9.7 to 17% and its range (from 8.3 to 17%) was similar than reported previously (Andrade-Gómez *et al.*, 2019). The *cox1* tree placed all the isolates of *S. lamothei* in a monophyletic subclade. From the 49 isolates of *S. lamothei*, 35 were recorded on four fish families (Eleotridae, Poeciliidae, Gobiidae and Mugilidae), with a sympatrical

distribution. The remaining 14 isolates were found in six localities (3, 4, 6, 10, 13 and 15; Fig. 3; Table 1) associated with the Pacific fat sleeper (*D. latifrons*) and a mullet fish (*Mugil* sp.). The fat sleeper is an amphidromous species distributed from Northern Mexico to Ecuador (Galván-Quesada *et al.*, 2016). Meanwhile, mullets are distributed in freshwater, brackish and marine habitats in the Pacific coasts (Colín *et al.*, 2020). The intraspecific genetic divergence among the isolates of *S. lamothei* ranged from 0 to 6.62% for *cox1* [as observed between one specimen from *Mugil* sp. in Barra de Navidad, Oaxaca, Mexico (MW283208), and one from *P. mexicana* Steindachner, 1863, in Río Choluteca, Honduras (MW283223); localities 15 and 26, respectively; Fig. 1; Table 1]. The intraspecific divergence of *cox1* found herein is higher than reported previously; for example, the intraspecific divergence of *S. tkachi* ranged from 0 to 3.1% and for *S. macrospinosus* ranged from 0 to 3.3% (Andrade-Gómez *et al.*, 2019). The current research confirmed that the *cox1* gene is a good molecular marker that allows delineating species and populations within *Saccocoelioides*.

The haplotype network analysis of *nad1* detected 57 distinct haplotypes obtained from 119 individual sequences, which divided into three haplogroups separated by a few mutational steps (fewer than 12 steps) (Fig. 4). The three haplogroups were found in mugilid fishes from 18 localities from four countries (Mexico, Guatemala, El Salvador and Nicaragua). The distribution pattern of *S. lamothei* along the Pacific coasts of Middle America may have been formed by a combination of environmental factors and those related to the biology of the intermediate and definitive hosts, it is well known that adult mullets (*M. cephalus* and *M. curema*) have been found in sympatry (Ibañez *et al.*, 2012; Nirchio *et al.*, 2017; Colín *et al.*, 2020). Both mullet species live and mature sexually in the open sea, where they migrate to different regions following marine currents (Funicelli *et al.*, 1989; Thomson, 1997). Mullets spawn offshore, and larval stages migrate from the open sea to the estuaries and lagoons near their nursery grounds (De Silva, 1980; Thomson, 1997). The life cycle of three species of the genus *Saccocoelioides* (*S. tilapiae* Nasir and Gómez, 1976; *S. carolae* Lunaschi, 1984; *S. tarpazensis* Díaz and González, 1990) are well known (see Martorelli, 1986; Díaz and González, 1990; Díaz *et al.*, 2009).

Adult worms live and reproduce sexually in the digestive tracts of freshwater fishes, which serve as definitive hosts. Eggs are expelled into the environment in the feces of their host. Then, the eggs develop into miracidia, ciliate free-swimming larval forms that search for and penetrate snails of the genus *Pyrgophorus* Ancey, 1888, which serves as the intermediate host and in which the parasites develop into cercariae. Cercariae emerge from snails and are encysted on the water surface where they develop into metacercariae. Metacercariae are frequently found on aquatic vegetation that is ingested by their definitive hosts (Martorelli, 1986; Díaz and González, 1990; Díaz *et al.*, 2009). Mullets feed on aquatic vegetation, and their life cycle is completed in the open sea, estuaries and lagoons along the Pacific coasts of Middle America (Ibañez *et al.*, 2012). Andres *et al.* (2018) noted that mugilid fishes act as 'ecological bridges' between marine, estuarine and freshwater habitats and can disperse parasites along their range of distribution.

Morphometric analyses of the 53 specimens of *S. lamothei* recovered from three host families, exhibited remarkable morphological differences (Fig. 6B). PCA considered 17 variables (Table 2) and clearly showed three polygons corresponding to specimens recovered from the families Eleotridae, Mugilidae and Gobiidae, which sympatrically inhabit the Pacific coasts of Middle America, suggesting host-induced phenotypic plasticity. For example, *S. lamothei* associated with eleotrids has the widest body (240–510 $\mu$m); those associated with mugilids have the longest body (533–906 $\mu$m); those associated with gobiids have the smallest testis (52–164 $\mu$m) (see Table 2; Fig. 7). The phenotypic plasticity found in *S. lamothei* along its distribution range is consistent with a previous study of *Saccocoelium tensum* Looss, 1902 (a haloporid that parasitizes two mugilids, *Liza ramada* Risso, 1827 and *Liza aurata* Risso, 1810, from the Mediterranean Sea coasts of Spain) which has four morphotypes (Blasco-Costa *et al.*, 2010). Morphological plasticity has also been documented in numerous trematodes species, and its variation has been linked to its definitive hosts (Blankespoor, 1974; Pérez-Ponce de León, 1995; Blasco-Costa *et al.*, 2010). Many morphologically distinct taxa of trematodes are assumed to represent several species or complexes of species, but they have been resolved into a single species under molecular and morphometric analyses, showing that parasites can alter their morphology depending on their host. This trait allows them to utilize a wide variety of definitive hosts.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S0031182020002334

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**Conflict of interest.** None.

**Ethical standards.** Not applicable.

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**Table 1S**. Specimens of *Saccocoelioides lamothei* analyzing; locality, host, number of specimens, GenBank accession number, and haplotype code.

| Locality | Host | n | GenBank access | Haplotype |
|---|---|---|---|---|
| **MÉXICO** | | | | |
| 1. El Huizache, Sinaloa. | *Mugil cephalus* | 3 | MW287796 | BX |
| | | | MW287797 | BX |
| | | | MW287798 | BX |
| 2. La Tovara, Nayarit. | *Dormitator latifrons* | 4 | MW287799 | BQ |
| | | | MW287800 | BR |
| | | | MW287801 | BR |
| | | | MW287802 | BR |
| | *Mugil cephalus* | 8 | MW287803 | BS |
| | | | MW287804 | BT |
| | | | MW287805 | BU |
| | | | MW287806 | BX |
| | | | MW287807 | BV |
| | | | MW287808 | BX |
| | | | MW287809 | BX |
| | | | MW287810 | BX |
| 3. Nuevo Vallarta, Nayarit. | *Dormitator latifrons* | 5 | MW287811 | CD |
| | | | MW287812 | CD |
| | | | MW287813 | CD |
| | | | MW287814 | CD |
| | | | MW287815 | CD |
| 4. Quémaro, Jalisco. | *Dormitator latifrons* | 3 | MW287816 | BP |
| | | | MW287817 | BP |
| | | | MW287818 | CB |
| | *Mugil* sp. | 2 | MW287819 | CB |
| | | | MW287820 | CB |
| 5. Playa Punta Pérula, Jalisco. | *Mugil* sp. | 1 | MW287821 | CB |
| 6. Cuyutlán, Colima. | *Dormitator latifrons* | 5 | MW287823 | BC |
| | | | MW287822 | BD |
| | | | MW287824 | BD |
| | | | MW287825 | BD |
| | | | MW287826 | BD |
| 7. Estero Tecuanillo, Colima. | *Mugil* sp. | 1 | MW287827 | AD |
| 8. Barra de Nexpa, Michoacán. | *Mugil* sp. | 4 | MW287831 | CE |
| | | | MW287828 | CE |

| | | | | |
|---|---|---|---|---|
| | | | MW287829 | CE |
| | | | MW287830 | CE |
| 9. Playa Las Peñitas, Guerrero. | *Mugil* sp. | 12 | MW287834 | CE |
| | | | MW287832 | BZ |
| | | | MW287835 | CG |
| | | | MW287838 | CA |
| | | | MW287836 | CB |
| | | | MW287837 | CB |
| | | | MW287833 | CB |
| | | | MW287839 | CB |
| | | | MW287840 | CB |
| | | | MW287841 | CB |
| | | | MW287842 | CB |
| | | | MW287843 | CB |
| 10. Tres Palos, Guerrero. | *Dormitator latifrons* | 2 | MW287784 | BJ |
| | | | MW287785 | BJ |
| 11. Marquelia, Guerrero. | *Mugil curema* | 2 | MW287844 | BM |
| | | | MW287845 | BN |
| 12. Río Salado, Oaxaca. | *Poeciliopsis gracilis* | 2 | MW287846 | CC |
| | | | MW287847 | CC |
| | *Poecilia sphenops* | 1 | MW287848 | CC |
| 13. San José de las Flores, Oaxaca. | *Profundulus* sp. | 3 | MW287852 | AK |
| | | | MW287853 | AL |
| | | | MW287854 | AM |
| | *Sicydium multipunctatum* | 3 | MW287850 | AO |
| | | | MW287849 | AN |
| | | | MW287851 | AN |
| 14. Chacahua, Oaxaca. | *Dormitator latifrons* | 10 | MW287786 | AP |
| | | | MW287787 | AP |
| | | | MW287788 | AP |
| | | | MW287790 | AP |
| | | | MW287789 | AQ |
| | | | MW287791 | AR |
| | | | MW287792 | AS |
| | | | MW287794 | AU |
| | | | MW287793 | AT |
| | | | MW287795 | AT |
| 15. Barra de Navidad, Oaxaca. | *Mugil cephalus* | 2 | MW287855 | AI |
| | | | MW287856 | AJ |
| 16. Matías Romero, Oaxaca. | *Poecilia mexicana* | 1 | MW287857 | CE |

| | | | | |
|---|---|---|---|---|
| 17. La Ventosa, Oaxaca. | *Mugil cephalus* | 3 | MW287861 | CE |
| | | | MW287859 | CC |
| | | | MW287860 | CC |
| 18. Pijijiapan, Chiapas. | *Dormitator latifrons* | 2 | MW287862 | BK |
| | | | MW287863 | BL |
| 19. Puerto Chiapas, Chiapas. | *Mugil* sp. | 5 | MW287864 | AZ |
| | | | MW287865 | AZ |
| | | | MW287866 | BA |
| | | | MW287868 | BA |
| | | | MW287867 | BB |
| **GUATEMALA** | | | | |
| 20. Río Nahualate. | *Poeciliopsis gracilis* | 2 | MW287871 | AV |
| | | | MW287872 | AX |
| | *Poecilia mexicana* | 1 | MW287870 | AY |
| 21. Puerto San José. | *Mugil curema* | 2 | MW287873 | BA |
| | | | MW287874 | BA |
| 22. Las Lisas. | *Dormitator latifrons* | 2 | MW287875 | BY |
| | | | MW287876 | BY |
| **EL SALVADOR** | | | | |
| 23. Río Sunza. | *Poecilia mexicana* | 2 | MW287880 | CF |
| | | | MW287879 | CF |
| | *Sicydium* sp. | 2 | MW287877 | CF |
| | | | MW287878 | CF |
| 24. Río Banderas. | *Agonostomus monticola* | 2 | MW287883 | AE |
| | | | MW287884 | AF |
| | *Awaous banana* | 3 | MW287881 | AG |
| | | | MW287882 | AH |
| | | | MW287885 | AH |
| 25. Bahía de San Antonio. | *Dormitator latifrons* | 3 | MW287886 | AA |
| | | | MW287887 | BY |
| | | | MW287888 | CB |
| **HONDURAS** | | | | |
| 26. Río Choluteca, Honduras. | *Poecilia mexicana* | 2 | MW287889 | AB |
| | | | MW287890 | AC |
| **NICARAGUA** | | | | |
| 27. Río Tamarindo, Nicaragua. | *Agonostomus monticola* | 2 | MW287894 | BE |
| | | | MW287895 | BF |
| | *Poecilia gillii* | 2 | MW287896 | BG |
| | | | MW287897 | BH |
| | *Sicydium* sp. | 3 | MW287891 | BF |

| | | | | |
|---|---|---|---|---|
| | | | MW287893 | BF |
| | | | MW287892 | BI |
| 28. Río Mico, Nicaragua. | *Poecilia gillii* | 3 | MW287898 | AZ |
| | | | MW287899 | AZ |
| | | | MW287900 | AZ |
| **COSTA RICA** | | | | |
| 29. Río Ciruelas. | *Sicydium salvini* | 2 | MW287901 | BO |
| | | | MW287902 | BO |
| | *Poecilia gillii* | 2 | MW287903 | BO |
| | | | MW287904 | BO |

## III. II. Forticulcitinae (Haploporidae)

### III. II. I. Unexpected morphological and molecular diversity of trematode (Haploporidae: Forticulcitinae) parasites of mullets from the ocean Pacific coasts in Middle America. En *Parasitology Research.*

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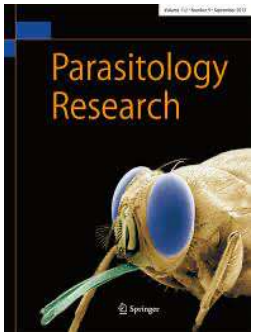

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FISH PARASITOLOGY - ORIGINAL PAPER

# Unexpected morphological and molecular diversity of trematode (Haploporidae: Forticulcitinae) parasites of mullets from the ocean Pacific coasts in Middle America

L. Andrade-Gómez[1,2] · M. García-Varela[1]

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## Abstract

Two new genera and four new species from subfamily Forticulcitinae are described from the intestines of white mullet (*Mugil curema*) and flathead grey mullet (*Mugil cephalus*) collected in 27 localities across a wide geographical range on Pacific Ocean slopes comprising three countries in Middle America: Mexico, Guatemala and Costa Rica. The new genera *Ekuarhuni* n. gen. and *Overstreetoides* n. gen. had to be erected to accommodate two new species, *Ekuarhuni papillatum* n. sp. and *Overstreetoides pacificus* n. sp., with unique morphological traits that differentiate them from the two genera described previously. In addition, two new species, *Forticulcita minuta* n. sp. and *Forticulcita isabelae* n. sp., were described, which were characterized as exhibiting a small body size (< 1100 μm long). These new species were classified as the diminutive morphotype, together with three other congeneric species of *Forticulcita*. *Forticulcita minuta* n. sp. is distinguished by being the smallest species within the genus (< 305 μm). Meanwhile, *Forticulcita isabelae* n. sp. is distinguished by its body size and testis length. In specimens of the four new species, sequencing was performed with two molecular markers, the large subunit (LSU) and the internal transcribed spacer 2 (ITS2) of nuclear rDNA, and the results were compared with other sequences available in GenBank. Phylogenetic analyses performed with the combined dataset of the two nuclear molecular markers (LSU + ITS2) placed all the analysed species within the clade of Forticulcitinae with strong bootstrap support (100%) and a high Bayesian posterior probability (1.0). The four new species showed differences in abundance in their definitive hosts and were widely distributed along the Pacific Ocean coasts of Mexico, Guatemala and Costa Rica, in Middle America.

**Keywords** Digenea · Forticulcitinae · *Mugil* · Nuclear markers · Taxonomy · Middle America

## Introduction

The members of Haploporidae Nicoll, 1914 are digenean endoparasites of marine, estuarine and freshwater teleost fishes that share the following traits: a hermaphroditic sac that encloses the terminal portion of the male and female genitalia and a single testis (Overstreet and Curran 2005). Overstreet and Curran (2005) reviewed the taxonomy of Haploporidae using morphological traits and recognized four subfamilies: Haploporinae Nicoll, 1914; Megasoleninae Manter, 1935; Waretrematinae Srivastava, 1937; and Chalcinotrematinae Overstreet and Curran, 2005. Blasco-Costa et al. (2009a) erected the subfamily Forticulcitinae Blasco-Costa, Balbuena, Kostadinova & Olson, 2009, combining morphological and molecular characteristics. Bray et al. (2014) erected the subfamily Cadenatellinae (Gibson & Bray, 1982) Bray, Cribb, Waeschenbach & Litlewood, 2014. Andres et al. (2018) combined morphological and molecular data to erect the subfamily Hapladeninae Andres, Pulis Curran & Overstreet, 2018. Finally, Atopkin et al. (2019) performed one of the most exhaustive phylogenetic analyses of Haploporidae using molecular data and recognized a new

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✉ M. García-Varela
garciav@ib.unam.mx

[1] Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, CP. 04510 Mexico City, Mexico

[2] Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, CP 04510 Mexico City, Mexico

subfamily, Pseudohaploporinae Atopkin, Besprozvannykh, Ha, Nguyen, Nguyen & Chalenko, 2019, based on specimens collected in three fish species from Vietnam.

The Forticulcitinae subfamily is a group of digenean endoparasites of mullet fishes distributed around the world including seven species classified into two genera: *Forticulcita* Overstreet, 1982 and *Xiha* Andres, Curran, Fayton, Pulis & Overstreet, 2015 (Andres et al. 2015). The species of the subfamily are morphologically diagnosed by the presence of a single large spherical to subtriangular compact mass of small vitelline follicle at the level of or posterior to the gonads (Blasco-Costa et al. 2009a; Andres et al. 2015). Currently, *Forticulcita* comprises five species distributed worldwide. *Forticulcita glabra* Overstreet, 1982 (type species) was described from the bluespot mullet, *Moolgarda seheli* (Forsskål), and *Forticulcita mugilis* Hassanine, 2007 was isolated from the fringelip mullet, *Crenimugil crenilabis* (Forsskål); both species are distributed in the Red Sea (Overstreet 1982; Hassanine 2007). *Forticulcita gibsoni* Blasco-Costa, Montero, Balbuena, Raga & Kostadinova, 2009 was described from the striped mullet, *Mugil cephalus* Linnaeus, in Santa Pola, Spain (Blasco-Costa et al. 2009b). Finally, Andres et al. (2015) described two species in the Americas: *Forticulcita platana* Andres, Curran, Fayton, Pulis & Overstreet, 2015 from the lebranche mullet, *Mugil liza* Valenciennes, in Río la Plata and Río Salado, Argentina, and *Forticulcita apiensis* Andres, Curran, Fayton, Pulis & Overstreet, 2015 from the striped mullet, *M. cephalus*, in Salt Springs, St. Johns River, Marion County, Florida, USA.

In the Americas, two species of the genus *Xiha* Andres, Curran, Fayton, Pulis & Overstreet, 2015 have been recorded. *Xiha fastigata* (Thatcher & Sparks, 1958) Andres, Curran, Fayton, Pulis & Overstreet, 2015 (type species) was described from the intestine of the striped mullet *M. cephalus* in Grand Isle, Louisiana, USA. Finally, *Xiha fragilis* (Fernández-Bargiela, 1987) Andres, Curran, Fayton, Pulis & Overstreet, 2015 was described from *M. cephalus* in Concepción, Chile (Fernández-Bargiela 1987).

During a helminthological expedition for helminth parasites of mullet fishes, specimens belonging to Forticulcitinae were collected in 27 localities across a wide geographical range on Pacific Ocean slopes comprising three countries: Mexico, Guatemala and Costa Rica, in Middle America. Our extensive sampling allowed us to reassess the relationships among members of the subfamily Forticulcitinae. The results of phylogenetic analysis and morphological studies including the examination of the ultrastructure of the body surface using scanning electron microscopy (SEM) required a revision of the classification and nomenclature of the group; two new genera and species had to be erected to accommodate species with unique morphological traits that differed from the two genera previously described. We also describe two new species of the genus *Forticulcita*. Additionally, we updated the phylogenetic tree of the family Haploporidae on the basis of LSU and ITS2 sequences from ribosomal rDNA and discuss some aspects of the classification of the subfamily Forticulcitinae, including the systematic position of *Forticulcita* and the two new genera.

## Materials and methods

### Sample collection and species identification

A total of 204 individuals of *Mugil* spp., 93 from *Mugil cephalus* Linnaeus, 50 of *Mugil curema* Valenciennes and 61 of *Mugil* sp., were collected in 27 localities across a wide geographical range in the Pacific Ocean slopes comprising three countries: Mexico, Guatemala and Costa Rica in Middle America from January 2018 to December 2019 (Fig. 1, Table 1). Hosts were maintained alive, transported to the laboratory and searched for helminths a few hours after capture. Definitive hosts were identified using the field guide of Miller et al. (2005). Individual fish were sacrificed by spinal severance (pithing) following the American Veterinary Medical Association (AVMA 2013). Haploporids were recovered from the intestine of mullets and were fixed in distilled water and preserved in 100% ethanol for morphological and molecular studies.

### Morphological analyses

Unflattened specimens preserved in ethanol 100% were hydrated in graded ethanol and stained with Mayer's paracarmine (Merck, Darmstadt, Germany), cleared with methyl salicylate and mounted on microscope slides in Canada balsam. Mounted specimens were examined under a bright field Leica DM 1000 LED microscope (Leica, Wetzlar, Germany), and drawings were made using a drawing tube attached to the microscope. Measurements were taken using Leica Application Suite microscope software (Leica) and are given in micrometres (μm). Holotype measurements are presented in description and metrical features are shown in Table 2. Holotypes and paratypes were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City. Images of holotypes and paratypes are shown in supplementary file 1. For SEM, specimens were dehydrated in a graded ethanol series, critical point dried, sputter coated with gold and examined with a Hitachi Stereoscan Model S-2469 N scanning electron microscope operating at 15 kV from the Instituto de Biología, Universidad Nacional Autónoma de México.

**Table 1** Species of Forticulcitinae recorded in this study with localities, hosts and GenBank accession number

| | Locality | Coordinates | Host | Host infected/ examined | Species | LSU | ITS2 |
|---|---|---|---|---|---|---|---|
| 1 | Bacochibampo, Sonora, Mexico | 27° 55′ 37.65″ N, 110° 56′ 50.43″ W | *Mugil cephalus* | 0/11 | | – | – |
| 2 | El Empalme, Sonora, Mexico | 27° 57′ 20.4″ N, 110° 49′ 38.19″ W | *M. cephalus* | 2/7 | *Forticulcita isabelae* n. sp. | MT957774-76 | MT957635 |
| 3 | Topolobampo, Sinaloa, Mexico | 23° 34′ 51.5″ N, 109° 6′ 57.95″ W | *M. cephalus* | 2/6 | *Forticulcita isabelae* n. sp. | MT957777 | |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957673 | |
| | | | *Mugil* sp. | 3/7 | *Forticulcita minuta* n. sp. | MT957796 | |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957674-6 | |
| 4 | Las Arenitas, Sinaloa, Mexico | 24° 22′ 18.12″ N, 107° 32′ 19.52″ W | *M. cephalus* | 1/13 | *Ekuarhuni papillatum* n. g. n. sp. | MT957677 | |
| 5 | Camino Las Arenitas, Sinaloa, Mexico | 24° 20′ 42.96″ N, 107° 29′ 23.32″ W | *Mugil curema* | 3/4 | *Forticulcita isabelae* n. sp. | MT957778-82 | |
| 6 | Cerritos, Sinaloa, Mexico | 23° 18′ 15″ N, 106° 29′ 1.72″ W | *M. cephalus* | 3/15 | *Forticulcita isabelae* n. sp. | MT957783-84 | MT957636-37 |
| 7 | El Huizache, Sinaloa, Mexico | 22° 53′ 4.65″ N, 106° 3′ 39.15″ W | *M. cephalus* | 2/7 | *Forticulcita minuta* n. sp. | MT957797-99 | |
| | | | | | *Overstreetoides pacificus* n. g. n. sp. | MT957741 | |
| 8 | La Tovara, Nayarit, Mexico | 21° 32′ 43.66” N 105° 16′ 24.12” W | *M. cephalus* | 10/11 | *Forticulcita minuta* n. sp. | MT957800-803 | |
| | | | | | *Overstreetoides pacificus* n. g. n. sp. | MT957742-44 | |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957678-86 | |
| 9 | Quémaro, Jalisco, Mexico | 19° 38′ 40.26″ N, 105° 12′ 55.55″ W | *Mugil* sp. | 1/2 | *Ekuarhuni papillatum* n. g. n. sp. | MT957687-88 | MT957587-88 |
| 10 | Playa Punta Pérula, Jalisco, Mexico | 19° 35′ 16.78″ N, 105° 8′ 7.52″ W | *Mugil* sp. | 5/6 | *Forticulcita isabelae* n. sp. | MT957785 | MT957638 |
| 11 | Barra de Chamela, Jalisco, Mexico | 19° 31′ 56.49″ N, 105° 4′ 50.98″ W | *M. curema* | 8/10 | *Forticulcita minuta* n. sp. | MT957804-07 | MT957642-45 |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957689-94 | MT957589-93 |
| 12 | Río Cuitzmala, Jalisco, Mexico | 19° 27′ 35.8″ N, 104° 56′ 11.4″ W | *Mugil* sp. | 2/2 | *Forticulcita minuta* n. sp. | MT957808 | MT957646 |
| | | | | | *Overstreetoides pacificus* n. g. n. sp. | MT957745 | MT957621 |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957695-96 | MT957594-95 |
| 13 | Manzanillo, Colima, Mexico | 19° 0′ 35.28″ N, 104° 14′ 41.28″ W | *Mugil* sp. | 0/5 | | – | – |
| 14 | Estero Tecuanillo, Colima, Mexico | 18° 48′ 49.38″ N, 103° 53′ 54.7″ W | *Mugil* sp. | 5/8 | *Forticulcita minuta* n. sp. | MT957809 | MT957647 |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957697-98 | MT957596-97 |
| 15 | Boca de Apiza, Michoacán, Mexico | 18° 41′ 5.26″ N, 103° 44′ 12.89″ W | *M. curema* | 2/3 | *Ekuarhuni papillatum* n. g. n. sp. | MT957699-MT957704 | MT957598-MT957602 |
| 16 | Barra de Nexpa, Michoacán, Mexico | 18° 5′ 0.28″ N, 102° 47′ 18.36″ W | *Mugil* sp. | 1/1 | *Ekuarhuni papillatum* n. g. n. sp. | MT957705-07 | MT957603-05 |
| 17 | Playa las Peñitas, Guerrero, Mexico | 17° 59′ 16.72″ N, 102° 2′ 5.01″ W | *Mugil* sp. | 6/6 | *Forticulcita minuta* n. sp. | MT957810-21 | MT957648-59 |
| | | | | | *Overstreetoides pacificus* n. g. n. sp. | MT957746-51 | MT957622-7 |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957708-16 | MT957606-13 |
| 18 | Barra de Coyuca, Guerrero, Mexico | 16° 56′ 56.3″ N, 100° 6′ 33.2″ W | *M. curema* | 1/5 | *Ekuarhuni papillatum* n. g. n. sp. | MT957717 | MT957614 |

**Table 1** (continued)

| | Locality | Coordinates | Host | Host infected/ examined | Species | LSU | ITS2 |
|---|---|---|---|---|---|---|---|
| 19 | Barra Vieja, Guerrero, Mexico | 16° 41′ 22.5″ N, 99° 37′ 20.37″ W | *M. curema* | 4/7 | *Forticulcita isabelae* n. sp. | MT957786-88 | MT957639-40 |
| | | | | | *Overstreetoides pacificus* n. g. n. sp. | MT957752-54 | MT957628-30 |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957718-19 | MT957615 |
| 20 | Marquelia, Guerrero, Mexico | 16° 33′ 19.75″ N, 98° 48′ 38.89″ W | *M. cephalus* | 6/11 | *Forticulcita isabelae* n. sp. | MT957789-90 | MT957641 |
| | | | | | *Forticulcita minuta* n. sp. | MT957822-23 | MT957660-61 |
| | | | | | *Overstreetoides pacificus* n. g. n. sp. | MT957755-57 | MT957631-32 |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957720-21 | MT957616 |
| 21 | Laguna de Chacahua, Oaxaca, Mexico | 15° 57′ 58.5″ N, 97° 40′ 41.6″ W | *Mugil* sp. | 1/5 | *Ekuarhuni papillatum* n. g. n. sp. | MT957722-23 | MT957617-18 |
| 22 | Barra de Navidad, Oaxaca, Mexico | 15° 48′ 39″ N, 97° 1′ 11″ W | *M. curema* | 7/10 | *Overstreetoides pacificus* n. g. n. sp. | MT957758-64 | |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957724-27 | |
| 23 | Salina Cruz, Oaxaca, Mexico | 16° 11′ 42.67″ N, 95° 10′ 40.79″ W | *M. cephalus* | 7/7 | *Forticulcita isabelae* n. sp. | MT957791-92 | |
| | | | | | *Forticulcita minuta* n. sp. | MT957824-30 | |
| | | | | | *Overstreetoides pacificus* n. g. n. sp. | MT957765 | |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957728-29 | |
| 24 | Pijijiapán, Chiapas, Mexico | 15° 33′ 12″ N, 93° 16′ 56″ W | *Mugil* sp. | 8/9 | *Forticulcita minuta* n. sp. | MT957831-33 | |
| | | | | | *Overstreetoides pacificus* n. g. n. sp. | MT957766-69 | MT957633 |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957730-33 | |
| 25 | Puerto Chiapas, Chiapas, Mexico | 14° 42′ 36.46″ N, 92° 24′ 30.91″ W | *M. cephalus* | 3/5 | *Forticulcita minuta* n. sp. | MT957834 | |
| | | | | | *Overstreetoides pacificus* n. g. n. sp. | MT957770-71 | MT957634 |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957734-35 | |
| | | | *Mugil* sp. | 5/10 | *Forticulcita minuta* n. sp. | MT957835-36 | |
| | | | | | *Overstreetoides pacificus* n. g. n. sp. | MT957772-73 | |
| 26 | Puerto de San José, Guatemala | 13° 55′ 11.7″ N, 90° 48′ 40.6″ W | *M. curema* | 1/4 | *Forticulcita isabelae* n. sp. | MT957793 | |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957736-37 | MT957619 |
| 27 | El Estero, Costa Rica | 9° 13′ 54″ N, 83° 50′ 20.7″ W | *M. curema* | 1/7 | *Forticulcita isabelae* n. sp. | MT957794-95 | |
| | | | | | *Ekuarhuni papillatum* n. g. n. sp. | MT957738-40 | MT957620 |

## Amplification and sequencing of DNA

Prior to extraction of the genomic DNA, each unflattened specimen was mounted on microscope slides and some images were taken as reference with the bright field Leica DM 1000 LED microscope (Leica, Wetzlar, Germany). Each image is link with its genomic DNA. Herein, we denominated "*photogenophore*" and consider that is an

analogue word to *hologenophores* (Astrin et al. 2013). *Photogenophores* are shown in supplementary file 2. Each specimen was removed from the microscope slide and was placed individually in tubes and digested overnight at 56 °C. The digestion solution contained 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM $Na_2$ EDTA (pH 8.0), 1% sarkosyl and 0.1 mg/ml proteinase K. Following digestion, DNA was extracted from the supernatant using DNAzol reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. For amplification, the ITS2 region and D1–D3 domains of LSU from the rDNA were amplified using forward (5′-GAACATCGACATCT TGAACG-3′) (Hernández-Mena et al. 2014) and reverse (5′-CAGCTATCCTGAGGGAAAC-3′) primers (García-Varela and Nadler 2005). PCRs (25 μl) consisted of 1 μl of each primer (10 μM), 2.5 μl of 10X PCR Rxn buffer, 1.5 μl of 2 mM $MgCl_2$, 0.5 μl of dNTPs (10 mM), 16. 375 μl of water, 2 μl of genomic DNA and 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil). PCR cycling parameters for rDNA amplifications included denaturation at 94 °C for 1 min, followed by 35 cycles at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min, followed by postamplification incubation at 72 °C for 10 min. Sequencing reactions were performed using the initial primers plus four internal primers, 504 (5′–CGTCTTGAAACACGGACTAAGG–3′), 502 (5′–CAAGTACCGTGAGGGAAAGTTGC–3′) (García-Varela and Nadler 2005), 503 (5′–CCTTGGTCCGTGTTTCAAGA CG–3′) (Stock et al. 2001) and BD2 (5′–TATGCTTA AATTCAGCGGGT–3′) (Luton et al. 1992), with ABI Big Dye (Applied Biosystems, Boston, MA) terminator sequencing chemistry, and reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contigs were assembled and base-calling differences resolved using Codoncode Aligner version 5.0.2 (Codoncode Corporation, Dedham, MA).

### Alignments and phylogenetic analyses

Sequences obtained in the current research from LSU and ITS2 from rDNA were aligned separately with sequences from another haploporid species downloaded from the GenBank dataset, plus three species from the family Atroctrematidae that were used as the outgroup. The alignment consisted of 205 sequences with 1261 nucleotides for the LSU and 108 sequences with 403 nucleotides for the ITS2. The combined alignment contained 108 sequences with 1664 nucleotides. The alignment was constructed using the software Clustal W (Thompson et al. 1997) with default parameters and adjusted manually with the Mesquite program (Maddison and Maddison 2011). The best model of nucleotide substitution for each dataset was estimated with the Akaike information criterion (AIC) implemented in jModelTest v0.1.1 (Posada 2008). The phylogenetic analyses were performed using maximum likelihood (ML) and Bayesian inference (BI) methods. The ML was carried out with the RAxML version 7.0.4 (Silvestro and Michalak 2011) and BI analyses were inferred with MrBayes version 3.2.7 (Huelsenbeck and Ronquist 2001) using the online interface: Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway v3.3 (Miller et al. 2010). The best model for each dataset was GTR + I + G for the LSU and TVM + I + G for ITS2. The ML analysis was inferred with models previously estimated for each molecular marker. To support each node, 10,000 bootstrap replicates were run. BI analyses included Markov chain Monte Carlo (MCMC) searches two simultaneous runs for 10 million generations, sampling every 1000 generations, a heating parameter value of 0.2 and a "burn-in" of 25%. Trees were drawn using FigTree program v.1.3.1 (Rambaut 2012). The genetic divergence among taxa were estimated using uncorrected "*p*" distances with the program MEGA version 6 (Tamura et al. 2013).

## Results

Two new genera and four new species of Forticulcitinae were found parasitizing the intestine of mullets distributed along the Pacific coasts in Middle America. *Mugil curema* was the most infected with 54% followed by *M. cephalus* with 39% and *Mugil* sp. with 37% (Table 1).

***Morphological description***

Class Trematoda Rudolphi, 1808

Order Plagiorchiida La Rue, 1957

Suborder Haploporata Pérez-Ponce de León & Hernández-Mena, 2019

Family Haploporidae Nicoll, 1914

Subfamily Forticulcitinae Blasco-Costa, Balbuena, Kostadinova & Olson, 2009

***Ekuarhuni*** gen. n. (Fig. 2a–g)

Diagnosis. Body fusiform. Tegument spinous, scatter at end of body. Eyespot pigment dispersed, mostly at pharynx level. Oral sucker subspherical, subterminal with papillae. Conspicuous glands distributed from pharynx level and crossing dorsally through oral sucker. Ventral sucker larger than oral sucker. Forebody approximately half than hindbody. Body width at level of ventral sucker. Prepharynx present. Pharynx well developed. Oesophagus approximately as long as hermaphroditic sac. Caeca saccular, terminating blindly at middle of body. Testis spherical to elongate. External seminal vesicle present. Hermaphroditic sac slightly elongate, longer than ventral sucker. Hermaphroditic duct strongly muscular, likely eversible as intromittent organ. Ovary spherical, located at middle of body. Vitellarium single mass, elongate to

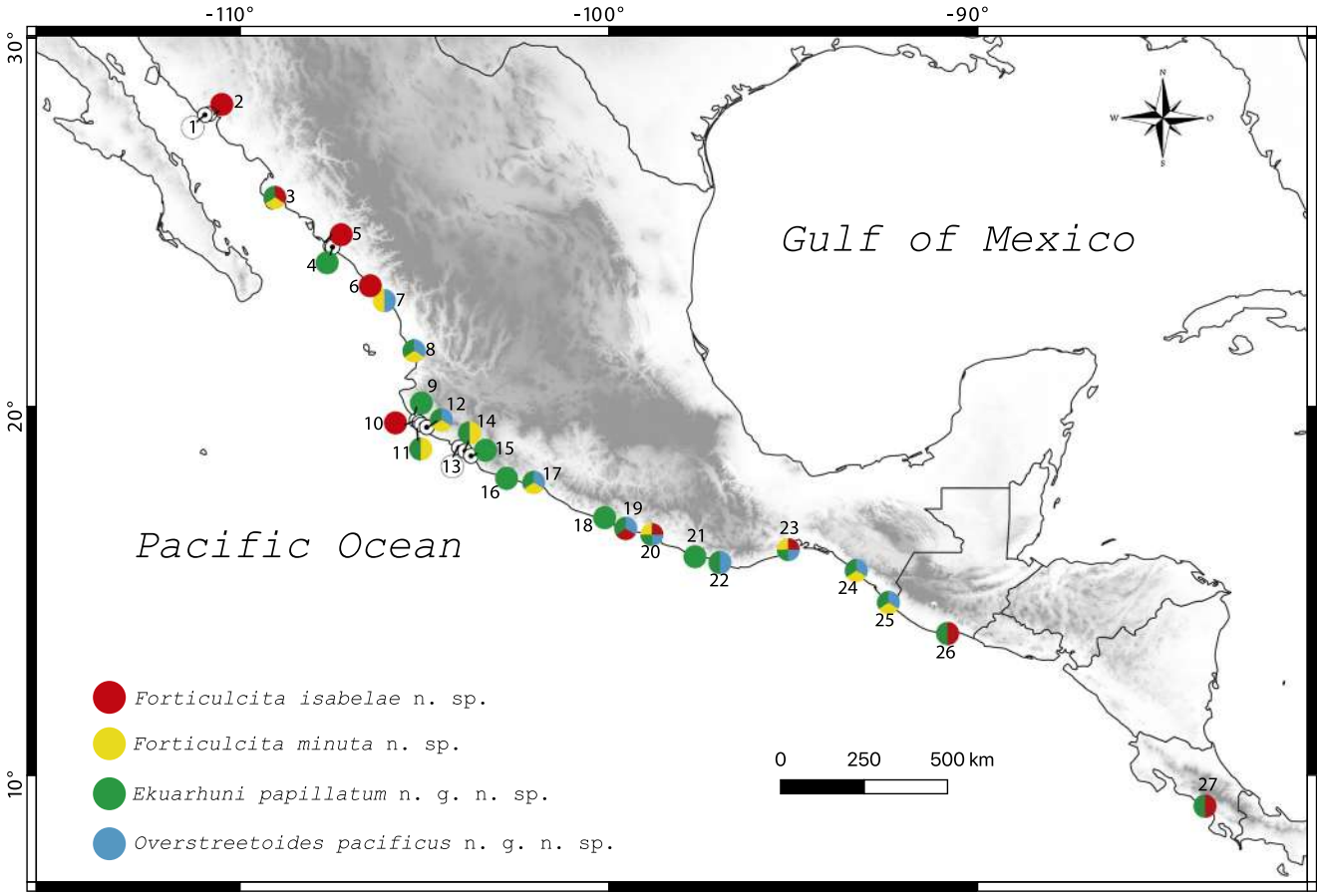


**Fig. 1** Map indicating the 27 sampled localities for species of Forticulcitinae in Middle America. The colours represent the species recovered; in white, the infections were negative

spherical. Eggs with developed oculate miracidia. Excretory vesicle Y-shaped; pore terminal.

*Type species*: *Ekuarhuni papillatum* n. sp.

*Etymology*: *Ekuarhuni* (pronounced i'kuar'ōnē) comes from the Purépecha language and refers to "veins on the forehead". The name refers to the glands in the forebody. We consider the gender as masculine.

*Taxonomic remarks*

*Ekuarhuni* n. gen. is placed within Forticulcitinae based on the presence of a vitellarium being comprised of a single elongate to subspherical mass (Blasco-Costa et al. 2009a; Andres et al. 2015). The new genus is morphologically similar to *Forticulcita* and *Xiha* in the body shape and size. However, *Ekuarhuni* n. gen. can be distinguished from other genera from subfamily by possessing conspicuous glands in the forebody, mainly at pharynx level, crossing dorsally to oral sucker. In addition, *Ekuarhuni* n. gen. lacks spines in the hermaphroditic sac as *Xiha* (Andres et al. 2015).

Class Trematoda Rudolphi, 1808

Order Plagiorchiida La Rue, 1957

Suborder Haploporata Pérez-Ponce de León & Hernández-Mena, 2019

Family Haploporidae Nicoll, 1914

Subfamily Forticulcitinae Blasco-Costa, Balbuena, Kostadinova & Olson, 2009a

Genus *Ekuarhuni* n. gen.

***Ekuarhuni papillatum*** n. gen. n. sp. (Fig. 2a–g)

Description (based on 12 individuals mature; measurements are given in micrometres (μm)). Body fusiform, 637 long, widest at posterior level of ventral sucker, 141 wide, representing 22% of BL. Forebody, 143, representing 22% of BL. Hindbody 410 long, representing 64% of BL. Eyespot pigment dispersed mainly at pharynx level (Fig. 2a). Tegumental spines becoming sparse in posterior of body (Fig. 2c, f, g). Oral sucker subspherical, subterminal, 67 long, 72 wide, bearing seven papillae in the upper part of the oral sucker (Fig. 2d). Ventral sucker spherical, 84 long, 84 wide (Fig. 2e). Ratio of oral sucker to ventral sucker lengths 1:1.25. Ratio of oral sucker to ventral sucker widths 1:1.16. Prepharynx short 11 long. Pharynx muscular, subspherical, 46 long, 57 wide. Ratio of oral sucker width to pharynx width 1:0.79. Oesophagus 105 long, approximately as long as the hermaphroditic sac. Intestinal bifurcation typically well posterior to ventral sucker. Caeca sac-like at middle of body, in some specimens present asymmetrical length of caeca, postcaecal space 296 long, representing 46% of BL. Testis single located at middle of

**Table 2** Comparative metrical data for species of Forticulcitinae

| | *Forticulcita glabra* (type species) | *F. mugilis* | *F. gibsoni* | *F. platana* | *F. apiensis* | *F. isabelae* n. sp. | *F. minuta* n. sp. | *Ekuarhuni papillatum* n. g. n. sp. | *Overstreetoides pacificus* n. g. n. sp. | *Xiha fastigata* | *Xiha fragilis* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Reference | Overstreet, 1982 | Hassanine, 2007 | Blasco-Costa et al., 2009 | Andres et al., 2015 | Andres et al., 2015 | This study | This study | This study | This study | Overstreet, 1971; Andres et al., 2015 | Fernández-Bargiela, 1987; Andres et al., 2015 |
| Type locality | Near Eilat, Gulf of Aqaba, Red Sea | Red Sea off Sharm El-Sheikh, South Sinai, Egypt | Santa Pola, Spain | Rio de la Plata, Buenos Aires, Argentina | Salt Springs, FL, USA | Camino Las Arenitas, Sinaloa, Mexico | Barra de Chamela, Jalisco, Mexico | Boca de Apiza, Michoacá-n, Mexico | Barra de Navidad, Oaxaca, Mexico | Grand Isle, LA, USA | Concepción, Chile |
| Type host | *Valamugil seheli* | *Crenimugil crenilabis* | *Mugil cephalus* | *Mugil liza* | *Mugil cephalus* | *Mugil curema* | *Mugil curema* | *Mugil curema* | *Mugil curema* | *Mugil cephalus* | *Mugil cephalus* |
| *n* | 7 | 20 | 17 | 17 | 9 | 10 | 7 | 12 | 8 | 16 | 30 |
| BL | 1185–1767 | 1814–2716 | 777–1024 | 501–790 | 354–524 | 524–719 | 195–306 | 576–812 | 439–635 | 477–818 | 580–1110 |
| BW | 348–516 | 530–810 | 201–299 | 131–214 | 124–153 | 133–229 | 107–161 | 141–234 | 170–335 | 159–356 | 210–390 |
| BW/BL (%) | 28* | 28* | 22–36 | 24–29 | 28–35 | 25–35 | 35–72 | 22–35 | 38–59 | 38* | 40–42* |
| FO | 300* | 543–930 | 184–291 | 154–198 | 124–158 | 77–172 | 43–80 | 126–211 | 91–194 | 169* | 147–213* |
| FO/BL (%) | 17–22 | 30–33 | 22–31 | 22–31 | 28–35 | 14–31 | 19–29 | 20–26 | 17–30 | 16–30 | 21–45 |
| HI | 1012* | 1297* | 538* | 279–496 | 157–285 | 320–459 | 87–176 | 349–476 | 254–388 | 341* | 431–545* |
| HI/BL (%) | 69* | 50* | 62* | 56–64 | 44–55 | 61–74 | 44–59 | 58–65 | 50–63 | 59* | 34–39* |
| OSL | 103–123 | 200–245 | 69–93 | 71–81 | 45–67 | 48–61 | 41–55 | 67–93 | 62–91 | 37–72 | 65–125 |
| OSW | 118–167 | 200–245 | 80–107 | 73–100 | 54–72 | 42–68 | 36–55 | 72–95 | 57–92 | 40–93 | 65–125 |
| VSL | 183–230 | 310–439 | 82–109 | 68–97 | 73–84 | 54–95 | 42–65 | 81–121 | 79–101 | 65–121 | 65–115 |
| VSW | 189–222 | 310–439 | 85–115 | 64–97 | 81–87 | 49–89 | 41–68 | 84–115 | 72–110 | 63–126 | 65–115 |
| OSL/VSL | 1:1.6* | 1:1.8* | 1:0.91–1.51 | 1:0.96–1.31 | 1:1.12–1.62 | 1: 1–1.8 | 1:1.02–1.58 | 1: 1.14–1.4 | 1:09–1.5 | 1:1.1* | 1:1–1.2* |
| OSW/VSW | 1:1.4–1.5 | 1:1.55–1.79 | 1:0.87–1.21 | 1:0.75–1.02 | 1:1.17–1.44 | 1:0.92–1.5 | 1:1.13–1.46 | 1:1.16–1.42 | 1:08–1.3 | 1: 0.9–1.6 | 1:1* |
| PL | 30* | 66–93 | 0–35 | 17–33 | 9–28 | 0–20 | 3–6 | 0–15 | 0–12 | 12* | 10–60 |
| PHL | 66–86 | 130–176 | 45–62 | 39–48 | 31–46 | 25–44 | 34–58 | 39–64 | 42–72 | 30–49 | 41–95 |
| PHW | 83–96 | 138–186 | 48–70 | 39–60 | 42–51 | 30–46 | 30–44 | 54–67 | 46–86 | 33–51 | 31–95 |
| OSW/PHW | 1:0.6* | 1:0.75* | – | 1:0.53–0.68 | 1:0.63–0.93 | 1:0.55–0.77 | 1:0.76–0.87 | 1:0.62–0.79 | 1:0.75–0.93 | 1: 0.54* | 1: 0.5–0.7* |
| OEL | 285* | 571–825 | 116–296 | 176–263 | 71–121 | 107–134 | 22–55 | 100–156 | 80–155 | 67–217 | 182* |
| CEND | 600* | 821* | 397–622 | 164–369 | 126–212 | 219–290 | 50–132 | 212–353 | 142–217 | 226* | 312–404* |
| CEND/BL (%) | 37–54 | 31* | 45–61 | 33–50 | 34–45 | 40–48 | 25–43 | 36–48 | 24–37 | 38–55 | 46–47* |
| TL | 218–388 | 125–168 | 75–127 | 59–101 | 31–53 | 102–143 | 36–71 | 81–131 | 55–102 | 79–226 | 67–150 |
| TW | 124–208 | 142–187 | 62–90 | 42–65 | 25–36 | 53–94 | 41–62 | 63–89 | 33–81 | 63–140 | 38–110 |
| TEND | 502* | 436–625 | 395–538 | 176* | 96–186 | 67–232 | 11–48 | 151–277 | 89–177 | 111* | 190–336* |
| | 28–41 | 30* | 45–57 | 19–36 | 27–40 | 12–32 | 5–21 | 25–37 | 14–27 | 10–46 | 29–38* |

**Table 2** (continued)

| | *Forticulcita glabra* (type species) | *F. mugilis* | *F. gibsoni* | *F. platana* | *F. apiensis* | *F. isabelae* n. sp. | *F. minuta* n. sp. | *Ekuarhuni papillatum* n. g. n. sp. | *Overstreetoides pacificus* n. g. n. sp. | *Xiha fastigata* | *Xiha fragilis* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TEND/BL (%) | | | | | | | | | | | |
| ESVL | 390* | 308–435 | 123–197 | 34–63 | 21–44 | 52–82 | 17–31 | 36–73 | 32–58 | 127* | 68–156* |
| ESVW | 37* | 23–28 | 34–62 | 17–27 | 12–24 | 30–48 | –(19) | 32–52 | 24–53 | 35* | 45–75* |
| HSL | 232* | 344–490 | 183–261 | 120–171 | 84–128 | 117–154 | 52–132 | 112–132 | 103–155 | 137* | 149–310 |
| HSW | 67* | 118–160 | 54–96 | 68–98 | 41–55 | 58–95 | 33–60 | 72–108 | 58–112 | 70* | 65–130 |
| HSL/BL (%) | 10* | 16* | 15* | 19–24 | 19–30 | 19–29 | 23–48 | 14–22 | 22–28 | 19* | 20–21* |
| ISVL | 105* | 345* | 53–104 | 32–65 | 26–36 | 40–95 | 21–44 | 22–64 | 37–67 | 60* | 65–100* |
| ISVW | 22* | 35* | 35–77 | 26–64 | 20–34 | 41–69 | 17–30 | 24–49 | 25–72 | 32* | 34–75* |
| PBL | – | – | – | 38–51 | 29–36 | 31–45 | 14–19 | 23–36 | 41–44 | 32* | 36–37 |
| PBW | – | – | – | 36–59 | 24–37 | 32–57 | 16–21 | 22–36 | 44–53 | 28* | 37–50 |
| GPL | – | 95* | 23* | 13–27 | 8–20 | 15–32 | 5–17 | 15–23 | 11–23 | 15* | – |
| OL | 42–147 | 118–170 | 51–137 | 64–86 | 27–42 | 64–75 | 28–46 | 74–99 | 46–77 | 49–93 | 60–127 |
| OW | 44–96 | 118–170 | 47–81 | 33–66 | 22–30 | 38–69 | 15–48 | 37–53 | 33–59 | 40–84 | 36–65 |
| DOT | 0* | 0* | 0* | 3–80 | 7–42 | 0–50 | 0 | 37, 52 | 0–51 | 76* | 0* |
| VL | 77–208 | 93–130 | 53–71 | 40–65 | 28–40 | 43–57 | 24–39 | 42–65 | 51–72 | 42–100 | 42–115 |
| VW | 71–166 | 115–132 | 46–64 | 36–63 | 24–32 | 48–59 | 25–33 | 49–81 | 43–64 | 33–84 | 42–115 |
| EL | 25–34 | 45–54 | 34–44 | 44–52 | 38–49 | 41–52 | 27–49 | 39–61 | 41–59 | 36–56 | 36–60 |
| EW | 14–17 | 30–36 | 18–24 | 20–26 | 14–20 | 19–26 | 16–27 | 21–37 | 21–28 | 18–28 | 19–26 |

*BL* body length, *BW* maximum body width, *FO* forebody, *HI* hindbody, *OSL* oral sucker length, *OSW* oral sucker width, *VSL* ventral sucker length, *VSW* ventral sucker width, *PL* prepharynx length, *PHL* pharynx length, *PHW* pharynx width, *OEL* oesophagus length, *CEND* postcaecal field length, *TL* testis length, *TW* testis width, *TEND* posttesticular field length, *ESVL* external seminal vesicle length, *ESVW* maximum external seminal vesicle width, *HSL* hermaphroditic sac length, *HSW* maximum hermaphroditic sac width, *ISVL* internal seminal vesicle length, *ISVW* maximum internal seminal vesicle width, *PBL* prostatic bulb length, *PBW* prostatic bulb width, *GPL* genital pore length, *OL* ovary length, *OW* ovary width, *DOT* distance of margin posterior of ovary to margin anterior of testis, *VL* vitelline mass length, *VW* vitelline mass width, *EL* egg length, *EW* egg width, *OSL/VSL* sucker length ratio, *OSW/VSW* sucker width ratio, *OSW/PHW* ratio of oral sucker width to pharynx width

*Estimated from the original descriptions

hindbody, elongated-subspherical, 111 long, 76 wide; posttesticular space 177 long, representing 27% of BL. External seminal vesicle spherical, 49 long, 43 wide, just posterior and contiguous with hermaphroditic sac. Hermaphroditic sac thin-walled, slightly bean-shaped, 114 long, 76 wide, representing 17% of BL, containing terminal genitalia, internal seminal vesicle, 51 long, 41 wide, oval to spherical, prostatic bulb 23 long, 33 wide, hermaphroditic duct strongly muscular, wide, eversible, as intromittent organ (Fig. 2b). Genital atrium shallow, genital pore medial, 18 long, anterior to anterior margin of ventral sucker. Ovary spherical to elongate, 90 long, 44 wide, contiguous with testis. Laurer's canal not observed. Vitellarium a single spherical mass, 49 long, 49 wide, dorsal and anterior to testis. Uterus occupying most of hindbody. Eggs in distal portion of uterus, 42–59 long, 21–28 wide, containing develop miracidia having eyespots. Excretory vesicle weakly Y-shaped, extending to approximate level of posterior of hindbody (Fig. 2a).

*Taxonomic summary*

*Type host*: *Mugil curema* Valenciennes, white mullet, Mugilidae. *GenBank accession*: MW287905

*Other host: Mugil cephalus* Linnaeus, *Mugil* sp.

*Site of infection*: intestine.

*Type locality*: Boca de Apiza, Michoacán, México (18° 41′ 5.26″ N, 103° 44′ 12.89″ W).

*Other 19 localities:* Mexico: Topolobampo and Las Arenitas, Sinaloa; La Tovara, Nayarit, Quémaro, Barra de Chamela and Río Cuitzmala, Jalisco; Estero Tecuanillo, Colima; Barra de Nexpa, Michoacán; Playa las Peñitas, Barra de Coyuca, Barra Vieja and Marquelia, Guerrero;

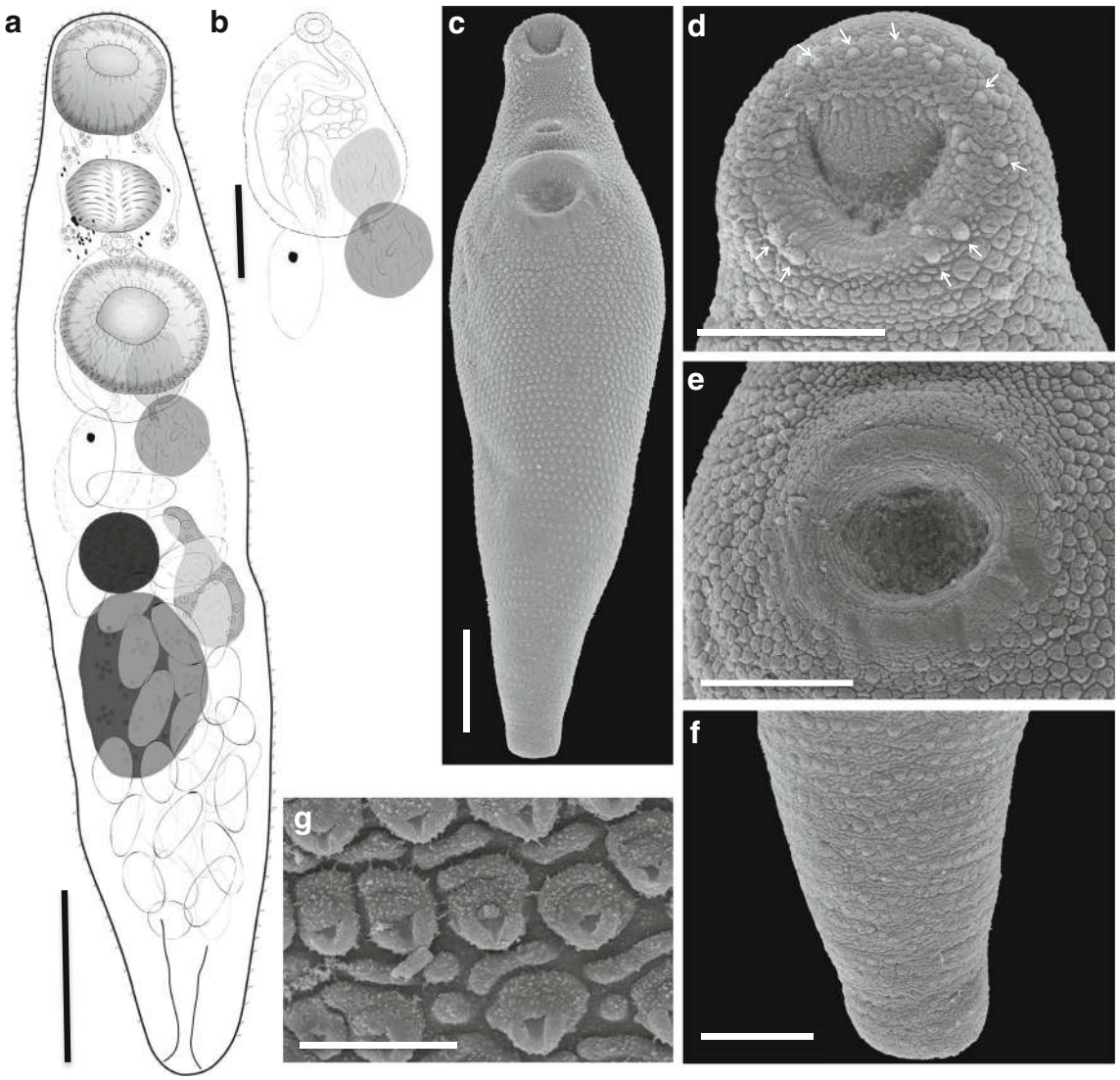


**Fig. 2** *Ekuarhuni papillatum* n. gen. n. sp., from *Mugil curema* (**a**) whole worm, holotype, ventral view. **b** Hermaphroditic sac, ventral view. Scanning electron micrographs of paratype (**c**), whole worm. **d** Oral sucker showing papillae (whit arrows). **e** Ventral sucker. **f** Posterior region. **g** Tegumental spines. Scale bars = 100 μm (**a**); 50 μm (**b**); 100 μm (**c**); 50 μm (**d**–**f**); 10 μm (**g**)

Laguna de Chacahua, Barra de Navidad and Salina Cruz, Oaxaca; Pijijiapán and Puerto Chiapas, Chiapas. Guatemala: Puerto de San José. Costa Rica: El Estero.

*Specimens deposited*: 1 Holotype (CNHE-11429);11 paratypes (CNHE-11430).

*Etymology*: The specific name refers to the presence of papillae on the sucker.

*GenBank* accession: MT957673–MT957740 for LSU; MT957587–MT957620 for ITS2.

*Taxonomic remarks*

*Ekuarhuni papillatum* n. sp. is the type species of *Ekuarhuni* n. gen. This species possesses the main characteristics of Forticulcitinae, i.e. vitellarium being comprised of a single elongate to subspherical mass. This species is characterized by possessing conspicuous glands in the forebody and by the presence of papillae on the oral sucker.

Class Trematoda Rudolphi, 1808

Order Plagiorchiida La Rue, 1957

Suborder Haploporata Pérez-Ponce de León & Hernández-Mena, 2019

Family Haploporidae Nicoll, 1914

Subfamily Forticulcitinae Blasco-Costa, Balbuena, Kostadinova & Olson, 2009a

***Overstreetoides*** gen. n. (Fig. 3a–g)

Diagnosis. Body oval. Tegument spinous. Eyespot pigment dispersed, mostly at pharynx level. Oral sucker subspherical, subterminal. Ventral sucker larger than oral sucker. Forebody approximately half than hindbody. Pharynx well developed. Oesophagus approximately as long as hermaphroditic sac. Caeca saccular, terminating blindly at level of approximate middle of hindbody. Testis spherical to elongate. External seminal vesicle present. Hermaphroditic sac slightly elongate, approximately longer than ventral sucker, approximately twice as long as female duct. Hermaphroditic duct strongly muscular, likely eversible as intromittent organ. Ovary elongate to subspherical, located at middle of body. Vitellarium single mass subspherical located in hindbody. Eggs with developed oculate miracidia. Excretory vesicle strongly muscular, Y-shaped; pore terminal.

*Type species*: *Overstreetoides pacificus*

*Etymology*: The genus is named to Dr. Robin M. Overstreet at the University of Southern Mississippi, USA, in recognition to his studies and contribution of the knowledge of the taxonomy and systematic of haploporids.

*Taxonomic remarks*

*Overstreetoides* n. gen. is placed within Forticulcitinae based on the presence of a vitellarium being comprised of a single elongate to subspherical mass (Blasco-Costa et al. 2009a; Andres et al. 2015). *Overstreetoides* has a body oval and not elongate as the other genera from the subfamily. The new genus has an excretory vesicle muscular.

Class Trematoda Rudolphi, 1808

Order Plagiorchiida La Rue, 1957

Suborder Haploporata Pérez-Ponce de León & Hernández-Mena, 2019

Family Haploporidae Nicoll, 1914

Subfamily Forticulcitinae Blasco-Costa, Balbuena, Kostadinova & Olson, 2009a

Genus *Overstreetoides* n. gen.

***Overstreetoides pacificus*** n. gen. n. sp. (Fig. 3a–g)

Description (based on 8 individuals mature; measurements are given in micrometres (μm)). Body oval, 578 long, widest at midbody, 335 wide, representing 57% of BL. Forebody, 173, representing 30% of BL. Hindbody 314 long, representing 54% of BL. Eyespot pigment dispersed between oral sucker and hermaphroditic sac, mainly at pharynx level (Fig. 3a). Tegumental spines conspicuous, occurring over entire surface of body (Fig. 3c, f, g). Oral sucker subspherical, subterminal, 73 long, 75 wide (Fig. 3d). Ventral sucker subspherical, 93 long, 100 wide, cover completely by spines (Fig. 3e). Ratio of oral sucker to ventral sucker lengths 1:1.2. Ratio of oral sucker to ventral sucker widths 1:1.3. Prepharynx absent. Pharynx large, muscular, subspherical, 56 long, 63 wide. Ratio of oral sucker width to pharynx width 1:0.84. Oesophagus 144 long, slightly bigger than hermaphroditic sac. Intestinal bifurcation typically well posterior to ventral sucker. Caeca sac-like at hindbody, postcaecal space 142 long, representing 24% of BL. Testis single, elongated subspherical, 96 long, 80 wide; posttesticular space 95 long, representing 16% of BL. External seminal vesicle subspherical, 45 long, 48 wide, just posterior and contiguous with hermaphroditic sac (Fig. 3a). Hermaphroditic sac thin-walled, slightly oval, 132 long, 106 wide, representing 22% of BL, containing terminal genitalia, internal seminal vesicle, 63 long, 72 wide, oval to spherical, prostatic bulb 44 long, 53 wide, hermaphroditic duct strongly muscular, wide, eversible, as intromittent organ (Fig. 3b). Genital atrium shallow, genital pore medial, 17 long, anterior to anterior margin of ventral sucker. Ovary spherical to elongate, 64 long, 54 wide, distance between ovary and testis, 51, located at midbody. Laurer's canal not observed. Vitellarium a single subspherical mass, 58 long, 49 wide, dorsal to and contiguous with testis. Uterus occupying most of hindbody. Eggs in distal portion of uterus, 54–55 long, 21–25 wide, containing develop miracidia with eyespots. Excretory vesicle strongly muscular at the end of the body, contiguous to testis, 89 long, 52 wide (39–107 long, 37–93 wide in paratypes) Y-shaped (Fig. 3a).

*Taxonomic summary*

*Type host*: *Mugil curema* Valenciennes white mullet, Mugilidae. *GenBank accession*: MW287906.

*Other host: Mugil cephalus* Linnaeus; *Mugil* sp.

*Site of infection*: intestine.

*Type locality*: Barra de Navidad, Oaxaca, Mexico (15° 48′ 39″ N, 97° 1′ 11″ W).

*Other 9 localities*: Mexico: El Huizache, Sinaloa; La Tovara, Nayarit; Río Cuitzmala, Jalisco; Playa las Peñitas,

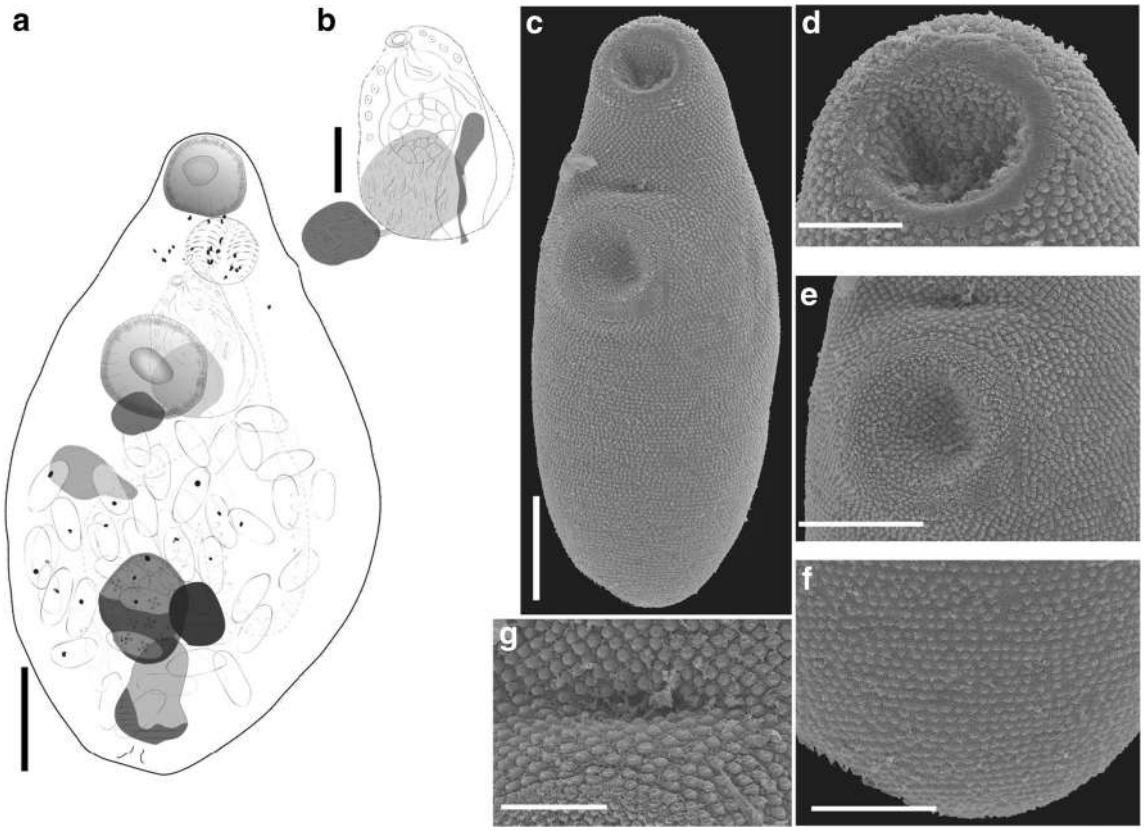


**Fig. 3** *Overstreetoides pacificus* n. gen. n. sp., from *Mugil curema* (**a**) whole worm, holotype, ventral view. **b** Hermaphroditic sac, ventral view. Scanning electron micrographs of paratype (**c**), whole worm. **d** Oral sucker. **e** Ventral sucker. **f** Posterior region. **g** Tegumental spines. Scale bars = 100 μm (**a**); 50 μm (**b**); 100 μm (**c**); 30 μm (**d**); 50 μm (**e**); 40 μm (**f**); 20 μm (**g**)

Barra Vieja and Marquelia, Guerrero; Salina Cruz, Oaxaca; Pijijiapán and Puerto Chiapas, Chiapas.

*Specimens deposited*: 1 holotype (CNHE 11431); 7 paratypes (CNHE 11432).

*Etymology*: The specific epithet refers to the distribution range of the new species in Middle America.

*GenBank accession*: MT957741–MT957773 for LSU; MT957621–MT957634 for ITS2.

*Taxonomic remarks*

*Overstreetoides pacificus* n. sp. is the type species of *Overstreetoides* n. gen. This species possesses the main characteristics of Forticulcitinae, i.e. vitellarium being comprised of a single elongate to subspherical mass. This species is characterized by having an oval body, spines covering complete surface of the body and by having an excretory vesicle very muscular, located at the end of the body.

Class Trematoda Rudolphi, 1808

Order Plagiorchiida La Rue, 1957

Suborder Haploporata Pérez-Ponce de León & Hernández-Mena, 2019

Family Haploporidae Nicoll, 1914

Subfamily Forticulcitinae Blasco-Costa, Balbuena, Kostadinova & Olson, 2009a

Genus *Forticulcita* Overstreet, 1982

***Forticulcita isabelae*** sp. n. (Figs. 4a–b and 5a–e)

Description (based on 10 individuals mature; measurements are given in micrometres (μm)). Body fusiform, 626 long, widest at midbody, 157 wide, representing 25% of BL. Forebody, 160, representing 25% of BL. Hindbody 399 long, representing 63% of BL. Eyespot pigment dispersed between oral sucker and hermaphroditic sac, mostly at pharynx level (Fig. 4a). Spines conspicuous reaching posterior end of the body (Fig. 5a, d, e). Oral sucker subspherical, subterminal, 49 long, 58 wide (Figs. 4a and 5b). Ventral sucker subspherical, 57 long, 58 wide (Figs. 4a and 5c). Ratio of oral sucker to ventral sucker lengths 1:1.16. Ratio of oral sucker to ventral sucker widths 1:1. Prepharynx present, 20 long. Pharynx short, muscular, subspherical, 40 long, 42 wide. Ratio of oral sucker width to pharynx width 1:0.72. Oesophagus 133 long, dorsal to hermaphroditic sac. Intestinal bifurcation opening at posterior of hermaphroditic sac. Caeca sac-like in middle of body, postcaecal space 282 long, representing 45% of BL. Testis single located in middle of hindbody, elongated, 106 long, 53 wide; posttesticular space 167 long, representing 26% of BL. External seminal vesicle elongated, 52 long, 30 wide, dorsal to hermaphroditic sac (Fig. 4a). Hermaphroditic sac thin-walled, oval 140 long, 80 wide, representing 22% of BL, containing terminal

genitalia, internal seminal vesicle, 74 long, 69 wide, spherical, prostatic bulb 45 long, 45 wide, hermaphroditic duct strongly muscular, wide, eversible, as intromittent organ (Fig. 4b). Genital atrium shallow, genital pore medial, 26 long, anterior to anterior margin of ventral sucker. Ovary spherical to elongate, 75 long, 46 wide, located in middle of body, with space between ovary and testis. Laurer's canal not observed. Vitellarium a single spherical mass, 53 long, 57 wide, dorsal to and anterior with testis. Uterus distributed from middle of body to end of the body, with well-developed metraterm entering posterior end of hermaphroditic sac. Eggs in distal portion of uterus, 41–44 long, 21–22 wide, containing develop miracidia with eyespots. Excretory vesicle weakly Y-shaped (Fig. 4a).

*Taxonomic summary*

*Type host*: *Mugil curema* Valenciennes, white mullet, Mugilidae *GenBank accession*: MW287907.

*Other host*: *Mugil cephalus* Linnaeus, *Mugil* sp.

*Site of infection*: intestine.

*Type locality*: Camino Las Arenitas, Sinaloa, Mexico (24° 20′ 42.96″ N, 107° 29′ 23.32″ W).

*Other 9 localities*: Mexico: El Empalme, Sonora; Topolobampo and Cerritos, Sinaloa; Playa Punta Pérula, Jalisco; Barra Vieja and Marquelia, Guerrero; Salina Cruz, Oaxaca. Guatemala: Puerto de San José. Costa Rica: El Estero.

*Specimens deposited*: 1 holotype (CNHE 11433); 9 paratypes (CNHE 11434).

*Etymology*: The species is named in honour Dra. Isabel Blasco-Costa, Université de Genève, Switzerland, in recognition her contribution to the studies in systematic and taxonomy of the haploporids.

*GenBank accession*: MT957774–MT957795 for LSU; MT957635–MT957641 for ITS2.

Class Trematoda Rudolphi, 1808

Order Plagiorchiida La Rue, 1957

Suborder Haploporata Pérez-Ponce de León & Hernández-Mena, 2019

Family Haploporidae Nicoll, 1914

Subfamily Forticulcitinae Blasco-Costa, Balbuena, Kostadinova & Olson, 2009a

Genus *Forticulcita* Overstreet, 1982

***Forticulcita minuta*** sp. n. (Figs. 4c–d and 5f–j)

Description (based on 7 individuals mature; measurements are given in micrometres (μm)). Body slightly pyriform, 305 long, widest at midbody, 107 wide, representing 35% of BL. Forebody, 65, representing 21% of BL. Hindbody 176 long, representing 57% of BL. Eyespot pigment dispersed between oral sucker and hermaphroditic sac (Fig. 4c). Spines conspicuous becoming sparse in posterior of body (Fig. 5f, i, j). Oral sucker spherical, subterminal, 42 long, 48 wide (Fig. 5g). Ventral sucker subspherical, 55 long, 55 wide (Fig. 5h). Ratio of oral sucker to ventral sucker lengths 1:1.3. Ratio of oral sucker to ventral sucker widths 1:1.4. Prepharynx short 6 long. Pharynx large, muscular, subspherical, 43 long, 40 wide. Ratio of oral sucker width to pharynx width 1:0.83. Oesophagus 41 long, dorsal to hermaphroditic sac. Intestinal bifurcation posterior to hermaphroditic sac. Caeca sac-like in middle of body, postcaecal space 132 long, representing 43% of BL. Testis single located at middle of hindbody, subspherical, 71 long, 51 wide; posttesticular space 28 long, representing 9% of BL. External seminal vesicle claviform, 17 long, 19 wide, just posterior and contiguous with hermaphroditic sac (Fig. 4c). Hermaphroditic sac thin-walled, oval, 94 long, 59 wide, representing 30% of BL, containing terminal genitalia, internal seminal vesicle, 44 long, 30 wide, oval to spherical, prostatic bulb 17 long, 19 wide, hermaphroditic duct strongly muscular, wide, eversible, as intromittent organ (Fig. 4d). Genital atrium shallow, genital pore medial, 10 long, anterior to anterior margin or contiguous to ventral sucker. Ovary spherical, 43 long, 30 wide, contiguous with testis. Laurer's canal not observed. Vitellarium a single spherical mass, 39 long, 33 wide, dorsal to and contiguous with testis. Uterus occupying most of hindbody. Eggs in distal portion of uterus, 37–42 long, 16–19 wide, containing develop miracidia having eyespots. Excretory vesicle weakly Y-shaped, extending to testis level (Fig. 4a).

*Taxonomic summary*

*Type host*: *Mugil curema* Valenciennes, white mullet, Mugilidae. *GenBank accession*: MW287908

*Other host: Mugil cephalus* Linnaeus.

*Site of infection*: intestine.

*Type locality*: Barra de Chamela, Jalisco, Mexico (19° 31′ 56.49" N, 105° 4′ 50.98" W).

*Other 10 localities*: Mexico: Topolobampo and El Huizache, Sinaloa; La Tovara, Nayarit; Río Cuitzmala, Jalisco; Estero Tecuanillo, Colima; Playa las Peñitas and Marquelia, Guerrero; Salina Cruz, Oaxaca; Pijijiapán and Puerto Chiapas, Chiapas.

*Specimens deposited*: 1 holotype (CNHE 11435); 6 paratypes (CNHE 11436).

*Etymology*: The specific epithet *minuta* derived from the Latin *diminutus* and refers to the small size of the body.

*GenBank accession*: MT957796–MT957836 for LSU; MT957642–MT957661 for ITS2

*Taxonomic remarks*

With the inclusion of two new species to genus *Forticulcita*, now it contains seven species distributed worldwide associated with mullet fishes. Following Overstreet et al. (2018), we divided the seven congeneric species in two morphotypes. The first contains species with relatively large body size (> 1100 μm long) with numerous eggs relatively small filling most hindbody. The robust morphotype is represented by two species, *Forticulcita glabra* (type species) and *Forticulcita mugilis*. The second morphotype is formed by five species with small body size (< 1100 μm long), and relatively few large eggs present in

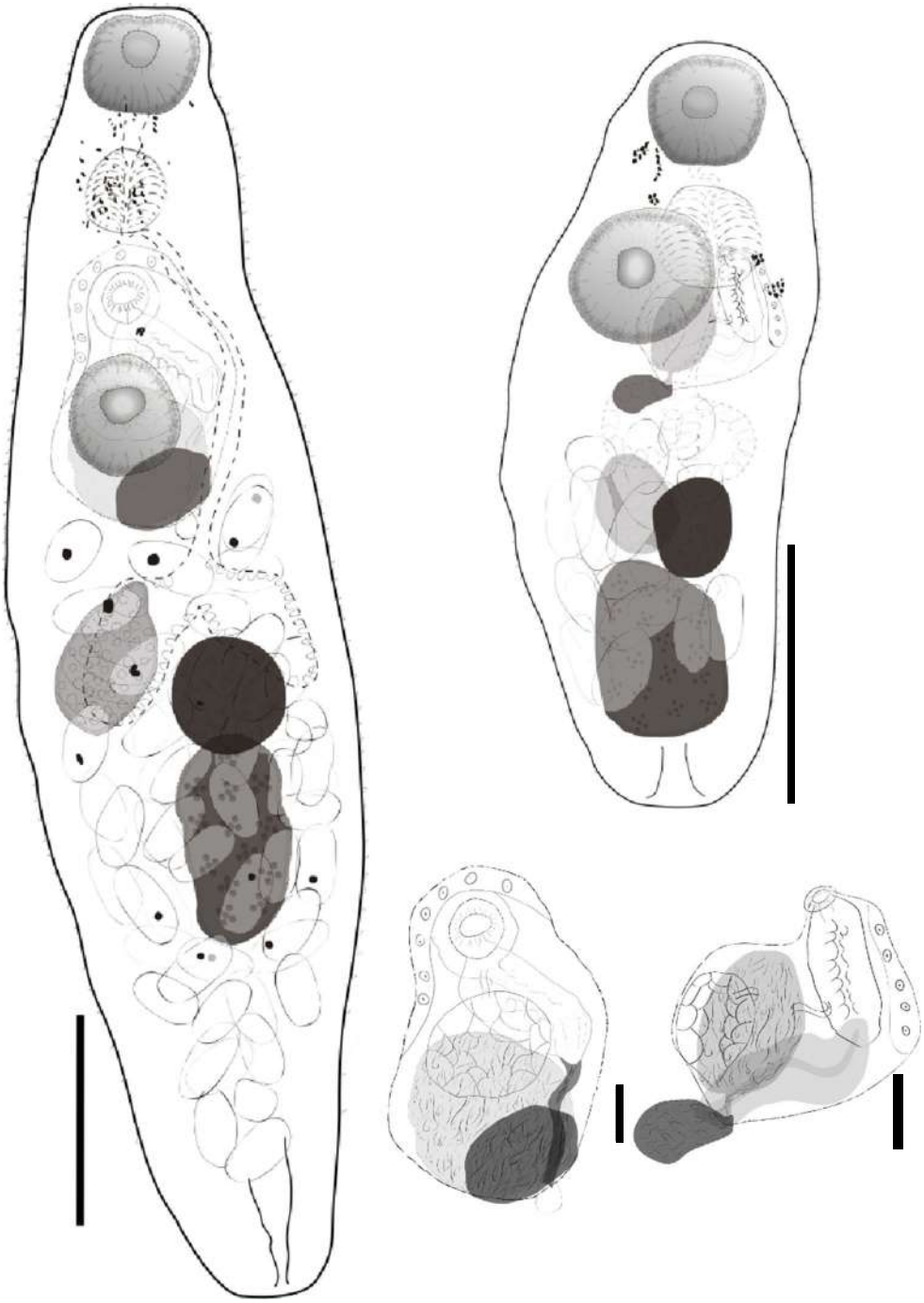

**Fig. 4** **a**, **b** *Forticulcita isabelae* n. sp., from *Mugil curema* (**a**) whole worm, holotype, ventral view. **b** Hermaphroditic sac, ventral view. *Forticulcita minuta* n. sp. from *Mugil curema* (**c**) whole worm, holotype, ventral view. **d** Hermaphroditic sac, ventral view. Scale bars = 100 μm (**a**); 20 μm (**b**, **d**); 100 μm (**c**)

the uterus confined mostly in the hindbody. *Forticulcita minuta* sp. n. can be differentiated from other congeneric species by possessing the smallest body (195–306 μm), with a relatively big pharynx in proportion with the suckers (oral sucker width to pharynx width, 1:0.76–0.87). The other congeneric species described herein as *Forticulcita isabelae* sp. n. also is included in the diminutive morphotype and it is distinguished from *F. minuta* sp. n. in the body size (195–306 μm in *F. minuta* sp. n. vs 524–719 μm in *F. isabelae* sp. n.). *Forticulcita isabelae* sp. n. can be distinguished from *F. apiensis* in the body size (354–524 μm vs 524–719 μm in *F. isabelae* sp. n.) and by having a bigger testis length (31–53 μm vs 102–143 μm in *F. isabelae* sp. n.). *Forticulcita isabelae* sp. n. is distinguished from *F. gibsoni* for being smaller in the body length (777–1024 μm vs 524–719 μm in *F. isabelae* sp. n.) and by having smaller sucker length than *F. gibsoni* (69–93 μm and 82–109 μm vs 48–61 μm and 54–95 μm), oral sucker and ventral sucker length respectively in *F. isabelae* sp. n.). Finally, *F. isabelae* sp. n. can be differentiated from *F. platana*, by having a longer testis length (59–101 μm vs 102–143 μm in

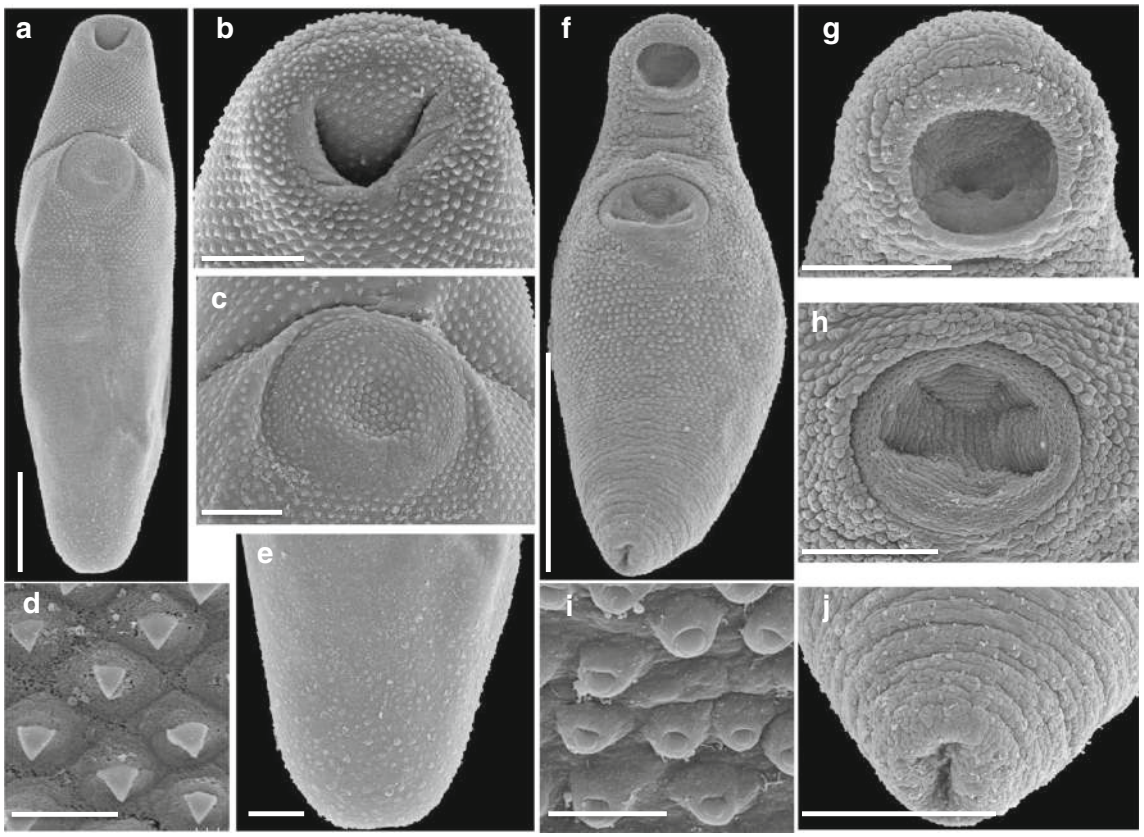


**Fig. 5** **a**–**e** Scanning electron micrographs of paratype of *Forticulcita isabelae* n. sp., from *Mugil curema* (**a**), whole worm. **b** Oral sucker. **c** Ventral sucker. **d** Tegumental spines. **e** Posterior of body. Scanning electron micrographs of the paratype of *Forticulcita minuta* n. sp., from *Mugil curema* (**f**), whole worm. **g** Oral sucker. **h** Ventral sucker. **j** Posterior of body. **i** Tegumental spines. Scale bars = 100 μm (**a**); 30 μm (**b**, **c**); 5 μm (**d**); 30 μm (**e**); 100 μm (**f**); 30 μm (**g**, **h**); 5 μm (**i**); 30 μm (**j**)

*F. isabelae* sp. n.) and by having a smaller oral sucker length (71–81 μm vs 48–61 μm in *F. isabelae* sp. n.) (see Table 2).

*Phylogenetic analysis*

The combined dataset (LSU + ITS2) included 1664 characters and 108 sequences. The phylogenetic analyses inferred with ML and BI showed that Haploporidae is monophyletic and is formed by seven main clades belonging to the recognized seven subfamilies: Haploporinae, Megasoleninae, Waretrematinae, Chalcinotrematinae, Forticulcitinae, Hapladeninae and Pseudohaploporinae with strong support of bootstrap and Bayesian posterior probabilities (see Fig. 6). Particularly, the monophyly of Forticulcitinae was supported with strong support of bootstrap and Bayesian posterior probabilities (100/1). This clade was subdivided into three major subclades. The first branch contained *Xiha fastigata* (KP761088) and it is sister to the other two subclades, which were formed by isolates representing five species of *Forticulcita*: *F. minuta* n. sp., *F. apiensis* (KP761087), *F. isabelae* n. sp., *F. platana* (KP761086) + *F. gibsoni* (FJ211239). The third subclade was formed by sequences representing *Ekuarhuni papillatum* n. gen. n. sp., plus *Overstreetoides pacificus* n. gen. n. sp. This subclade was supported with a moderate bootstrap support value and Bayesian posterior probabilities (70/0.98) (Fig. 6). The genetic divergence estimated with each molecular marker among the four genera of Forticulcitinae ranged from 5.7 to 11.9% with LSU and from 7.3 to 14.6% with ITS2 (see Table 3). The genetic divergence among the five species of *Forticulcita* ranged from 0.8 to 3.4% with LSU and from 1.6 to 6.8% with ITS2. The intraspecific genetic divergence among the 22 isolates of *Forticulcita isabelae* n. sp. ranged from 0 to 0.2% with LSU and with ITS2 all the sequences were identical. The genetic divergence found among the 41 isolates of *Forticulcita minuta* n. sp. with both molecular markers was zero, due that all the sequences were identical. The intraspecific genetic divergence among the 68 isolates of *Ekuarhuni papillatum* n. gen. n. sp. ranged from 0 to 0.4% with LSU and from 0 to 0.5% with ITS2. Finally, the intraspecific genetic divergence among the 33 isolates of *Overstreetoides pacificus* n. gen. n. sp. ranged from 0 to 0.4% with LSU and from 0 to 3.1% with ITS2 (see Table 3).

## Discussion

The phylogenies obtained with the combined (LSU + ITS2) dataset for two nuclear molecular markers unequivocally showed that Haploporidae is monophyletic and agreed with

**Table 3** Pairwise nucleotide sequence comparisons between taxa for the aligned LSU rDNA sequences ($N = 1261$ nt) (below the diagonal) and for ITS2 sequences ($N = 403$ nt) (above the diagonal). Italicized values represent the genetic intraspecific divergence

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *F. gibsoni* | – | 1.6 | 4.7 | 2.6 | 6.2 | 9.4–11.5 | 9.9–10.4 | 13.6 |
| 2 | *F. platana* | 1 | – | 4.1 | 2.1 | 6.2 | 8.3–10.4 | 8.9–9.4 | 14.1 |
| 3 | *F. apiensis* | 2.5 | 2.7 | – | 4.1 | 5.2 | 8.3–10.4 | 8.9–9.4 | 14.1 |
| 4 | *F. isabelae* n. sp. | 0.8–1 | 1.4–1.7 | 2.3–2.5 | *0–0.2*/– | 6.8 | 10.4–12.5 | 10.4–10.9 | 14.6 |
| 5 | *F. minuta* n. sp. | 3.2 | 3.4 | 3.2 | 3.2–3.4 | – | 8.3–10.4 | 7.8–8.3 | 13.6 |
| 6 | *Overstreetoides pacificus* n. g. n. sp. | 8.9–9.4 | 8.9–9.4 | 9.1–9.6 | 8.7–9.4 | 8.1–8.5 | *0–0.4*/*0–3.1* | 7.3–9.9 | 12–14.6 |
| 7 | *Ekuarhuni papillatum* n. g. n. sp. | 9.1–9.6 | 9.1–9.6 | 9.1–9.6 | 9.1–9.8 | 8.3–8.7 | 11.3–11.9 | *0–0.4*/*0–0.5* | 14.1–14.6 |
| 8 | *Xiha fastigata* | 7.2 | 7.4 | 7.4 | 7.4–7.6 | 5.7 | 8.9–9.4 | 10.4–10.6 | – |

previous fundamental phylogenetic studies that contributed significantly to the stable classification of the family (Blasco-Costa et al. 2009a; Andres et al. 2018; Atopkin et al. 2019). Our phylogenetic study added new information to a growing genetic library, which allowed us to explore and evaluate the phylogenetic relationships among the eight recognized subfamilies. The ML and Bayesian analyses recognized seven independent subclades, which agree with the taxonomy of the subfamilies (see Overstreet and Curran 2005; Blasco-Costa et al. 2009a; Andres et al. 2018; Atopkin et al. 2019).

In this study, a combined dataset for two nuclear molecular (LSU + ITS2) markers was built to infer the evolution of the subfamilies within Haploporidae. The haploporids recovered from the Pacific Ocean slopes from three countries, Mexico, Guatemala and Costa Rica, in Middle America were nested within Forticulcitinae (Fig. 6). A detailed morphological study of all these specimens revealed unique morphological traits and synapomorphies shared with other members of Forticulcitinae. This allowed us to propose two new genera, *Ekuarhuni* n. gen. and *Overstreetoides* n. gen., and two new species, *Ekuarhuni papillatum* n. gen. n. sp. and *Overstreetoides pacificus* n. gen. n. sp., as well as two new species of the genus *Forticulcita*, *Forticulcita isabelae* n. sp. and *Forticulcita minuta* n. sp. In addition to morphological and molecular evidence, the genetic divergence found among the genera provided added value. For example, we found high divergence among the genera of Forticulcitinae, ranging from 5.7 to 11.9% for LSU and 7.3 to 14.6% for ITS2. Previous studies involving other genera belonging to haploporid subfamilies, such as Haploporinae, Waretrematinae, Megasoleninae, Chalcinotrematinae and Pseudohaploporinae, have revealed genetic divergence ranging from 10 to 15.3% for LSU and 16.2 to 19.3% for ITS2 (Blasco-Costa et al. 2009a; Andres et al. 2018; Atopkin et al. 2019). These values are similar to or lower than those obtained among the genera of Forticulcitinae. With the addition of *Ekuarhuni* n. gen. and *Overstreetoides* n. gen. to Forticulcitinae, the subfamily now contains four genera that share the unique morphological trait of a single large spherical to subtriangular compact mass of small vitelline follicle at level of or posterior to the gonads.

The phylogenetic relationships within Forticulcitinae were well supported (Fig. 6). *Xiha* is sister to *Ekuarhuni*, *Overstreetoides* and *Forticulcita*, suggesting that all these genera share a common ancestor. Currently, the subfamily contains a total of 11 species recognized, eight of them distributed in the Americas, representing 72% of the diversity suggesting that the subfamily may arose in association with mullets from the Americas, with secondary colonization events to other biogeographical regions.

To date, seven species of *Forticulcita* have been described around the world, primarily in association with mullet fishes. In the Americas, two species have been described, *F. platana* from the lebranche mullet, *M. liza*, in Argentina and *F. apiensis* from the striped mullet, *M. cephalus*, in the USA (Andres et al. 2015); with the addition of the two new species described herein as *F. minuta* n. sp. and *F. isabelae* n. sp., the Americas are now considered to harbour four species. Morphologically, these two new species belong to the diminutive morphotype (< 1100 μm long), together with *F. platana* (501–790 μm), *F. apiensis* (354–524 μm) and *F. gibsoni* (777–1024 μm). In the current study, the five species belonging to the diminutive morphotype were placed in a single clade, and body size could be a synapomorphic character that differentiates this lineage from the robust lineage that includes *F. glabra* (1185–1767 μm) and *F. mugilis* (1814–2716 μm). However, no sequences from the robust morphotype are available to test this hypothesis. Andres et al. (2015) pointed out that these two species classified herein as the robust morphotype might belong to the true *Forticulcita*, while the diminutive morphotype could represent other new genera. Therefore, LSU and ITS2 sequences from robust morphotype species are necessary to understand the evolution of genus *Forticulcita*.

The genetic divergence among the five species of *Forticulcita* ranged from 0.8 to 3.4% for LSU and 1.6 to 6.8% for ITS2, similar to other haploporids. For instance, the genetic divergence found among 4 species of *Saccocoelium* ranged from 0.9 to 4.8% for LSU and from 2.1 to 10.9% for ITS2; between 2 species of *Dicrogaster* ranged 4.6% and 8.7% for LSU and ITS2, respectively; and among 3 species of the

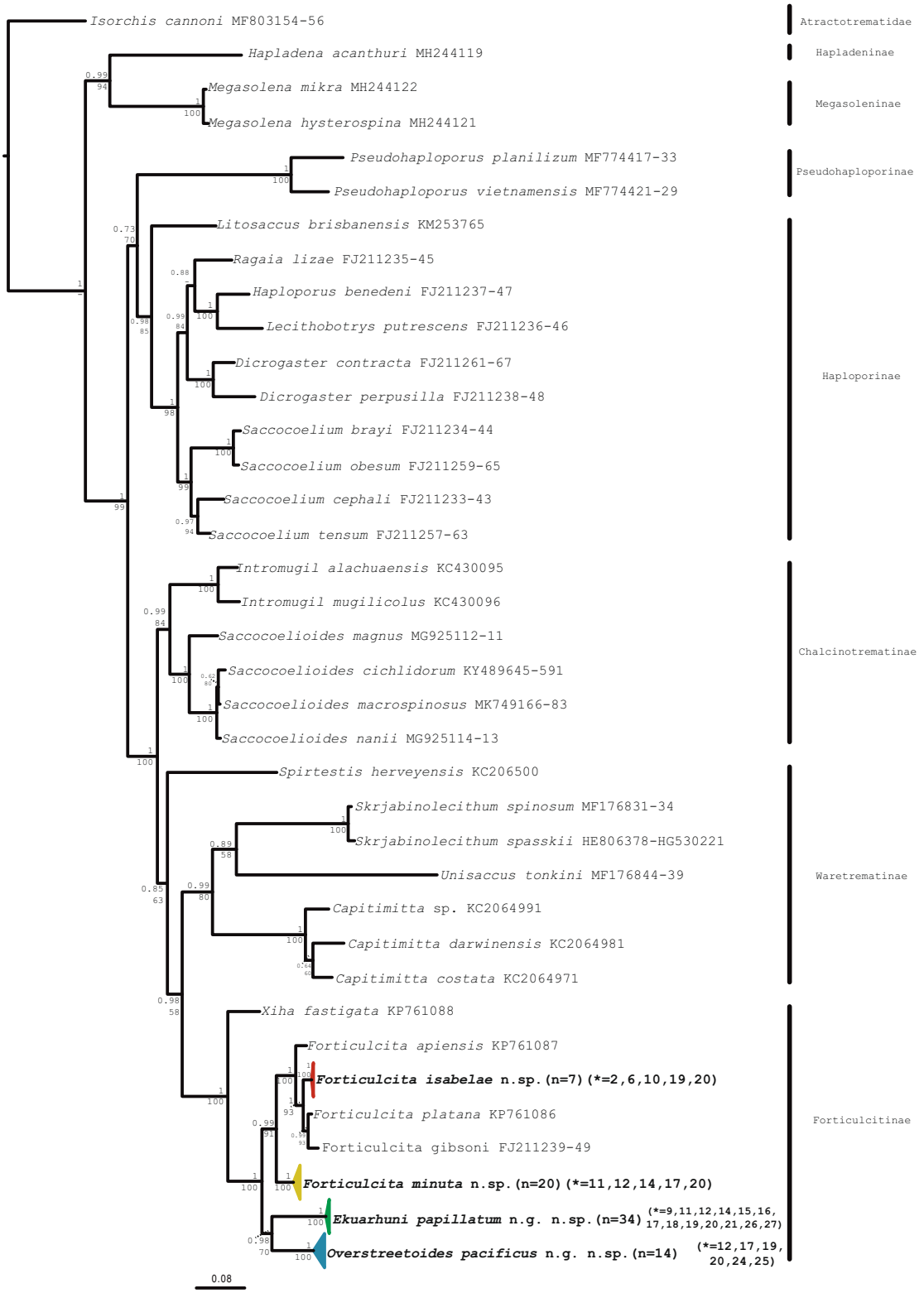


**Fig. 6** Consensus Bayesian inference and maximum likelihood trees inferred with the concatenated (LSU + ITS2) dataset. Numbers near internal nodes show posterior probabilities (BI) and ML bootstrap clade frequencies. *n* = number of specimens sequenced, number of localities correspond to data shown in Table 1, * = type locality

genus *Capitimitta* range from 2.7 to 2.8% for LSU and 7.3 to 11% for ITS2 (Blasco-Costa et al. 2009a; Pulis and Overstreet 2013). Finally, the genetic divergence among five species of *Saccocoelioides* Szidat, 1954 ranged from 1 to 1.6% for LSU and 0.07 to 3.4% for ITS2 (Andrade-Gómez et al. 2019).

The intraspecific genetic divergence found was low in most of the species described herein. For instance, *Overstreetoides pacificus* n. sp. and *Ekuarhuni papillatum* n. sp. ranged from 0 to 0.4% for the LSU and from 0 to 0.2% to *Forticulcita isabelae* n. sp. and it was due that a single sequence from San José Guatemala contains 5 singleton (locality 26; Fig. 1). This intraspecific genetic divergence was similar to found by Atopkin et al. (2015), of 0.5% for LSU with *Skrjabinolecithum spasskii* Belous, 1954, an haloporid from mullets distributed in Far Eastern. The intraspecific genetic divergence of *Overstreetoides pacificus* n. sp. ranged from 0 to 3.1% for ITS2, and it was due that two sequences from Puerto Chiapas, Mexico (locality 25; Fig. 1), showed 11 singleton.

The helminth fauna of the flathead grey mullet and white mullet is relatively well known throughout their distribution in the Americas, and great diversity of parasites has been documented (see Rawson 1976; Paperna and Overstreet 1981; García and Williams 1985; Armas 2006; Muñoz and Olmos 2008; Iannacone and Alvariño 2009; Rosas-Valdez et al. 2012; Andres et al. 2015). Some of the most common parasites recorded to date are haloporids, which are considered to be typical components of the helminth fauna of these fishes (Martin 1973; Andres et al. 2015). Although the complete life cycle of the species of Forticulcitinae is unknown, available evidence from other members of the same family, such as *Pseudohapladena* Yamaguti, 1952, indicates that adult worms live and reproduce sexually in the digestive tracts of mullet fishes that serve as definitive hosts. Eggs are expelled into the environment with the faeces of the host. After the ingestion of the eggs by a snail of the genus *Posticobia* Iredale, 1943, which serves as the first intermediate host, the parasites develop into cercariae. The cercariae emerge and swim to find filamentous algae, where they undergo encystment to develop into metacercariae. Filamentous algae are one of the principal food resources of mullets, and metacercariae are accidentally ingested by definitive hosts (Martin 1973). The intestines of necropsied mullets that contain filamentous algae are commonly infected with species of Forticulcitinae, and the life cycle of all the recorded species can apparently be completed in the open sea, estuaries and lagoons along Pacific Ocean slopes in Middle America. Following dispersal, haloporid cercariae typically undergo encystment on aquatic vegetation, which could explain the wide distribution of Forticulcitinae in Middle America. Andres et al. (2015) noted that the dispersal of a haloporids in aquatic vegetation rafts could explain why *F. gibsoni*, a species distributed in the Red Sea, is sister to *F. platana*, a species distributed in Argentina.

Adults of the four new species *Ekuarhuni papillatum* n. sp., *Overstreetoides pacificus* n. sp., *Forticulcita isabelae* n. sp. and *Forticulcita minuta* n. sp. are frequently found in mullet fishes and are considered to belong to the helminthological core fauna of these fishes from the Pacific Ocean slope in Middle America. The distribution pattern of these haloporids in Middle America has been shaped by a combination of environmental conditions and factors related to the biology of the intermediate and definitive hosts; i.e. the four new species were detected in juvenile mullets from the coasts of Mexico in the northern Pacific to the Pacific coasts of Costa Rica, with different ecological characteristics (prevalence/abundance) (Fig. 1, Table 1). In two localities (Marquelia, Guerrero, and Salina Cruz, Oaxaca), the four described species were found in sympatry, and at least two species were found at each locality. Finally, in two localities, Bacochibampo, Sonora, and Manzanillo, Colima, the mullets were not infected.

In summary, the current study allowed us to characterize and describe the diversity of a group of haloporids that parasitize mullet fishes. Phylogenetic analyses were performed with the combined dataset for two nuclear molecular markers, and the observed genetic divergence together with morphological traits were essential for the description of four new species of Forticulcitinae distributed along the Pacific Ocean coasts in Middle America.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00436-020-06983-y.

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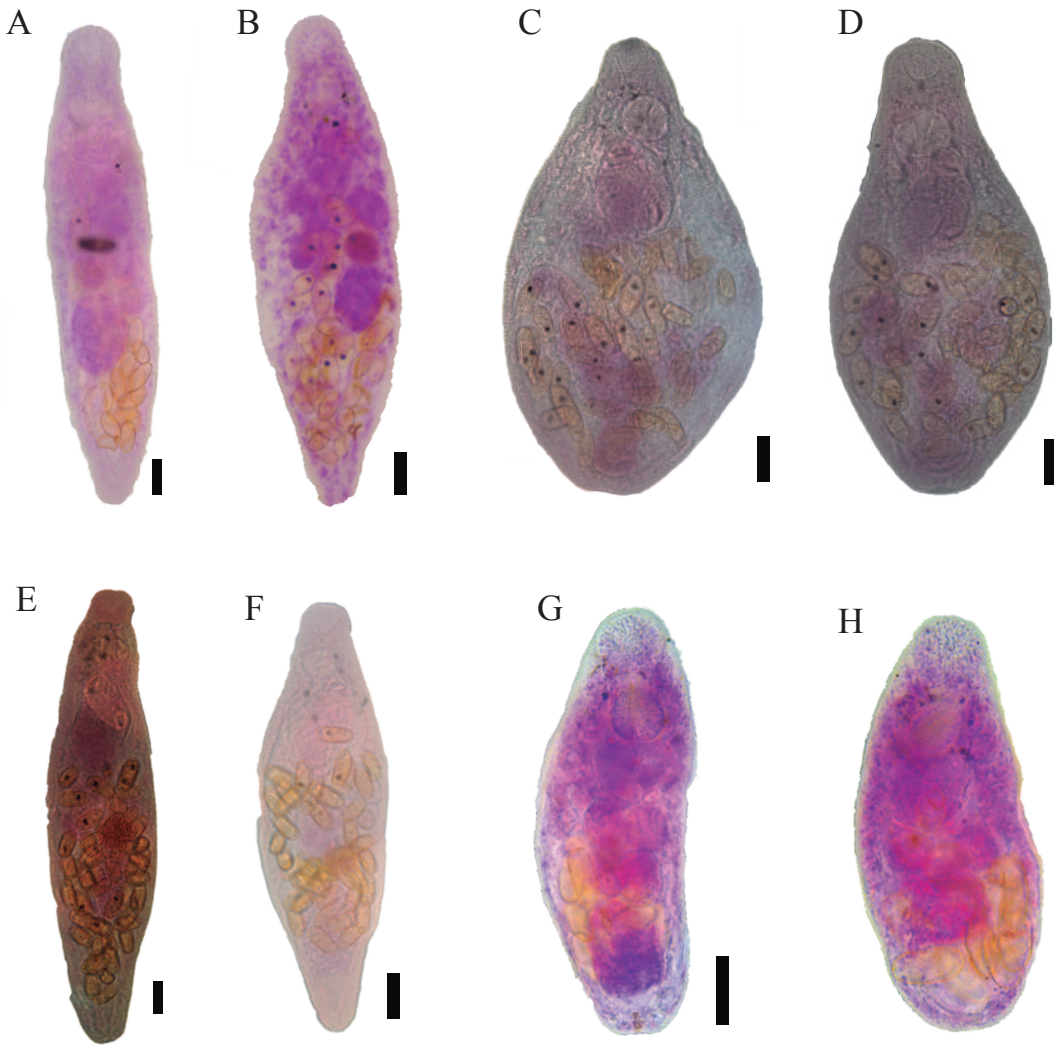
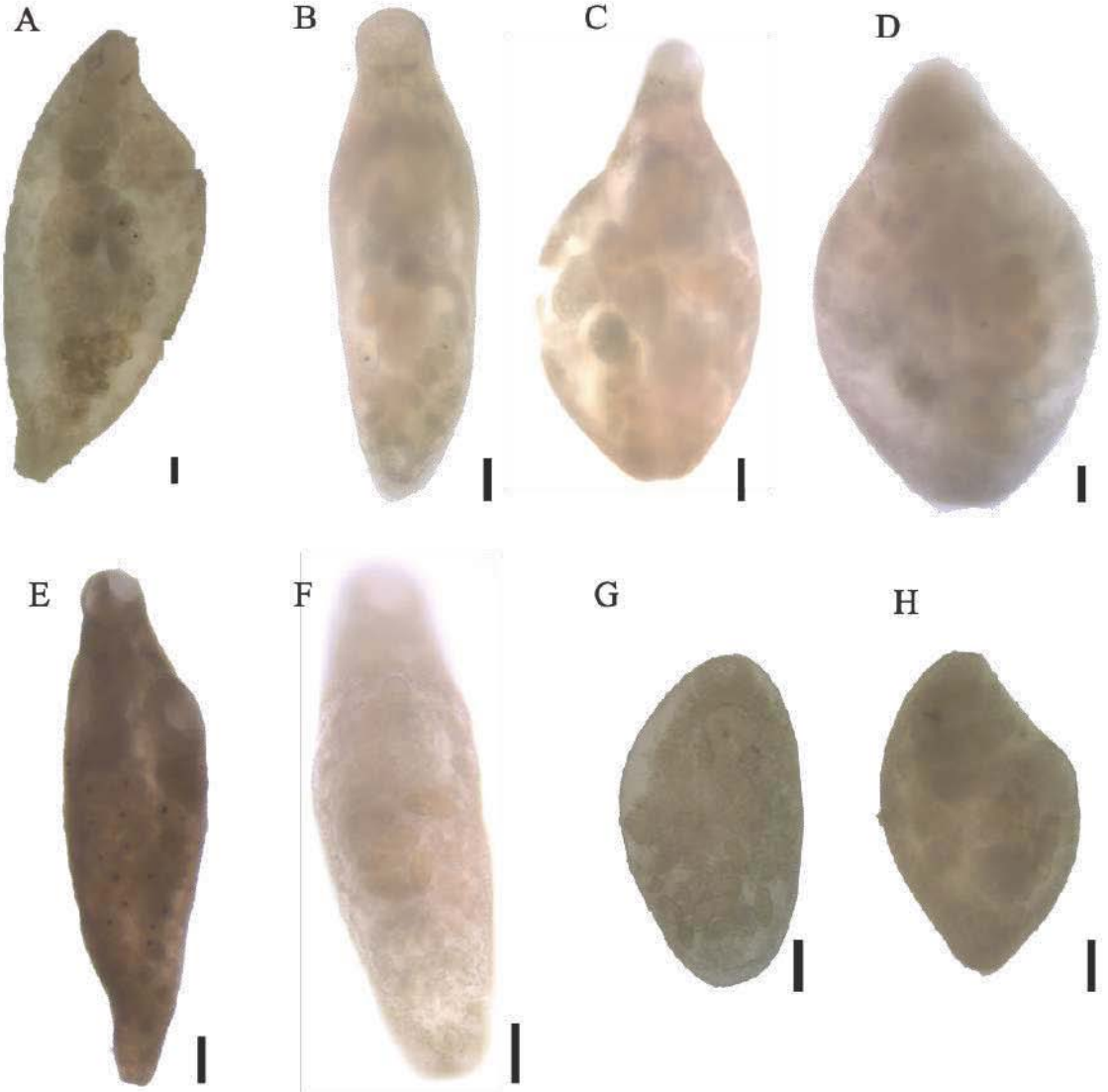



**S1.** Mounted specimens of *Ekuarhuni papillatum* **A)** Holotype (CNHE-11429), **B)** Paratype (CNHE-11430; Specimens collected in Boca de Apiza, Michoacán, Mexico from *Mugil curema*. *Overstreetoides pacificus* **C)** Holotype (CNHE-11431), **D)** Paratype (CNHE-11432); Specimens collected in Barra de Navidad, Oaxaca, Mexico from *Mugil curema*. *Forticulcita isabelae* **E)** Holotype (CNHE-11433), **F)** Paratype (CNHE-11434); Specimens collected in Camino a las Arenitas, Sinaloa, Mexico from *Mugil curema.* *Forticulcita minuta* **G)** Holotype (CNHE-11435), **H)** Paratype (CNHE-11436); Specimens collected in Barra de Chamela, Jalisco, Mexico from *Mugil curema*. Scale bar=50 μm.




**S2.** Photogenophores of *Ekuarhuni papillatum* **A)** Specimen collected in Boca de Apiza, Michoacán, Mexico from *Mugil curema*; **B)** Specimen collected in Peñitas, Guerrero, Mexico from *Mugil* sp.; *Overstreetoides pacificus* **C)** Specimen collected in Barra Vieja, Guerrero, Mexico from *Mugil curema*, **D)** Specimen collected in Peñitas, Guerrero, Mexico from *Mugil* sp.
*Forticulcita isabelae* **E, F)** Specimen collected in Barra Vieja, Guerrero, Mexico from *Mugil curema*. *Forticulcita minuta* **G, H)** Specimens collected in Barra de Chamela, Jalisco, Mexico from *Mugil curema*; GenBank accesion number **A)** MT957699 **B)** MT957712 **C)** MT957753 **D)** MT957751 **E)** MT957787 **F)** MT957788 **G)** MT957807 **H)** MT957806; Scale bar=50 μm.

**III. I. II. Phylogenetic affinities of Forticulcitinae (Haploporidae) parasites of mullet from the Americas, with the description of three new species and notes on the genera and key species. En *Systematic Parasitology.***

**Leopoldo Andrade-Gómez** ✉, Marcelo Tonatiuh González-García, Martín García-Varela

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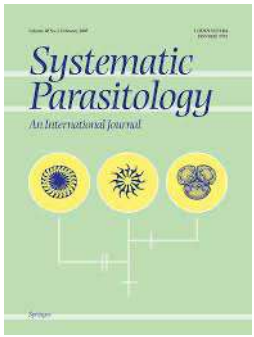

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# Phylogenetic affinities of Forticulcitinae (Haploporidae) parasites of mullet from the Americas, with the description of three new species and notes on the genera and key species

**Leopoldo Andrade-Gómez · M. T. González-García · M. García-Varela**

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**Abstract** Members of Forticulcitinae Blasco-Costa, Balbuena, Kostadinova & Olson, 2009 include endoparasites of mullet fishes distributed worldwide. Adult specimens were collected from the intestines of white mullet (*Mugil curema*) and flathead grey mullet (*Mugil cephalus*) from five localities in the Gulf of Mexico and a single locality in Venezuela. *Photogenophores* were sequenced for two nuclear molecular markers, the large subunit (LSU) and second internal transcribed spacer (ITS2) of nuclear rDNA. The new sequences were aligned with other sequences downloaded from GenBank. The maximum likelihood and Bayesian inferences were deduced using the combined dataset (LSU + ITS2). The phylogenetic analyses revealed four new lineages belonging to Forticulcitinae. Three new species are described in the present study. *Ekuarhuni mexicanus* **n. sp.** can be differentiated from its congeneric species by presenting a longer hermaphroditic sac length (136–180 µm) and a wider testis (91–123 µm). *Forticulcita macropharyngis* **n. sp.** and *Forticulcita venezuelensis* **n. sp.** are the 8th and 9th species described in *Forticulcita*. Both species belong to the diminutive morphotype of *Forticulcita*. *Forticulcita macropharyngis* **n. sp.** can be morphologically distinguished from the other congeneric species by the presence of a massive and muscular pharynx (46–110 µm long, 74–106 µm wide). *Forticulcita venezuelensis* **n. sp.** is the second species of the studied genus recorded in South America and can be differentiated from congeneric species by possessing the largest testis (138–201 µm long, 83–100 µm wide). Finally, the fourth lineage corresponds to *Overstreetoides* Andrade-Gómez & García-Varela, 2021; however, few specimens of this lineage were collected, precluding any description of the species. In addition, a key is proposed for differentiating the genera and species of Forticulcitinae.

**Keywords** Digenea · Americas · *Mugil* · Mugilidae · LSU · ITS2 · Systematics · Phylogeny

L. Andrade-Gómez (✉) · M. T. González-García · M. García-Varela
Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), Avenida Universidad 3000, Ciudad Universitaria, 04510 Mexico City, Mexico
e-mail: l.andrade@ciencias.unam.mx

L. Andrade-Gómez
Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, 04510 Mexico City, Mexico

## Introduction

Forticulcitinae Blasco-Costa et al., 2009a, represents one of the eight recognized subfamilies within Haploporidae Nicoll, 1914 (Atopkin et al., 2019).

This subfamily is composed of small, globally distributed digeneans that at the adult stage, parasitise the intestines of mullet fishes (Mugilidae) (Blasco-Costa et al., 2009a, b; Overstreet, 1982). Currently, the subfamily is morphologically characterized by having a single large spherical to subtriangular compact mass of small vitelline follicles at the level of or posterior to the gonads (Andres et al., 2015; Blasco-Costa et al., 2009a). Based on these features, the subfamily contains 11 species classified into four genera: *Forticulcita* Overstreet, 1982; *Xiha* Andres, Curran, Fayton, Pulis & Overstreet, 2015; *Ekuarhuni* Andrade-Gómez & García-Varela, 2021; and *Overstreetoides* Andrade-Gómez & García-Varela, 2021 (Hassanine, 2007; Blasco-Costa et al., 2009a; Andres et al., 2015; Andrade-Gómez & García-Varela, 2021).

The systematics has been based on its morphological and molecular characteristics since the establishment of the subfamily by Blasco-Costa et al. (2009a). These authors analysed the domains D1–D3 from the large subunit (LSU) and second internal transcribed spacer (ITS2) of the ribosomal DNA of members of Forticulcitinae. Both molecular markers are considered the backbone of phylogenetic studies within Forticulcitinae. Later, other species of *Forticulcita* were described across the world following the previously proposed phylogenetic framework (Andres et al., 2015; Blasco-Costa et al., 2009b). More recently, Andres et al. (2015) erected the genus *Xiha* with two species from the Americas. Finally, Andrade-Gómez and García-Varela (2021) analysed sequences from the LSU and ITS2 of specimens collected from the Pacific coasts of Mexico, Guatemala and Costa Rica, describing two genera, *Ekuarhuni* and *Overstreetoides*.

We evaluated the taxonomy and systematics of the parasitic haploporids associated with flathead grey mullet (*Mugil cephalus* Linnaeus), white mullet (*Mugil curema* Valenciennes) and *Mugil* sp., collected across the coasts of the Gulf of Mexico, Caribbean Sea and Atlantic Ocean. Sequences of the large subunit (LSU) and internal transcribed spacer 2 obtained from nuclear ribosomal DNA, in combination with morphological features, allowed us to describe three new species of Forticulcitinae and recognize an undescribed species of the genus *Overstreetoides*. Additionally, we propose a key for identifying the species and genera of Forticulcitinae.

## Materials and methods

### Sample collection

Between December 2012 and February 2020, a total of 99 individuals of *Mugil cephalus*, *Mugil curema*, and *Mugil* sp. with a standard length of 12–28 cm, were collected in 12 localities in Gulf of Mexico, one in the Caribbean Sea and four localities in Venezuela (Fig. 1; Table 1). Hosts were maintained alive, transported to the laboratory and searched for helminths a few hours after capture; individual fish were euthanized by spinal severance (pithing) following the American Veterinary Medical Association (AVMA, 2013). Forticulcitines were recovered from the intestine of mullets and were fixed in hot distilled water and preserved in 100% ethanol for morphological and molecular studies.

### Morphological analyses

Unflattened specimens preserved in ethanol 100% were stained with Mayer's paracarmine (Merck, Darmstadt, Germany), cleared with methyl salicylate, and mounted on microscope slides in Canada balsam. Mounted specimens were examined under a bright field Leica DM 1000 LED microscope (Leica, Wetzlar, Germany), and drawings were made using a drawing tube attached to the microscope. Measurements were taken using Leica Application Suite microscope software (Leica) and are given in micrometers (μm). Holotypes and Paratypes were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City.

### Amplification and sequencing of DNA

Prior to extraction of the genomic DNA, each unflattened specimen was mounted on microscope slides and some images were taken as reference with bright field Leica DM 1000 LED microscope (Leica, Wetzlar, Germany). Each image was linked with its genomic DNA (Fig. 2), named as *photogenophores* by Andrade-Gómez and García-Varela (2021). Each specimen was removed from the microscope slide and was placed individually in tubes and digested overnight at 56°C. The amplification and sequencing condition of nuclear genes, ITS2 region and D1–D3

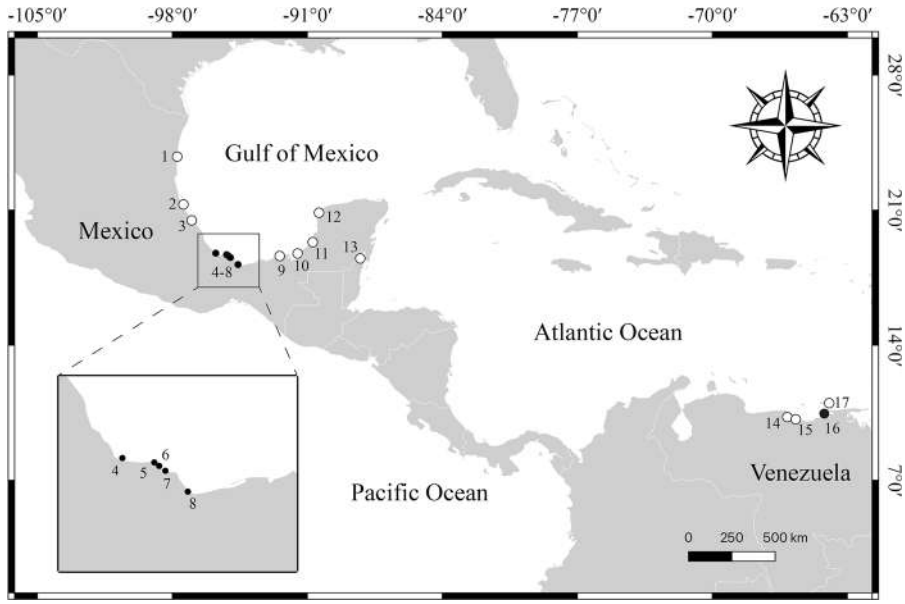


**Fig. 1** Sampling sites (see Table 1) of mugilid fishes from the Americas. In black, are localities where forticulcitines were recovered; in white, infections where negative.

domains of LSU, from our specimens were previously described by Andrade-Gómez and García-Varela (2021).

Alignments and phylogenetic analyses

Sequences obtained in the current research from LSU and ITS2 from rDNA were aligned separately with other select haploporids from different subfamilies downloaded from the GenBank, plus one species from Atroctrematidae that was used as the outgroup (see Table 2). The alignment consisted of 60 sequences with 1,257 nucleotides for the LSU and 50 sequences with 409 nucleotides for the ITS2. The combined alignment contained 50 sequences with 1,666 total positions. Alignments were constructed using the software MUSCLE (Edgar, 2004) with default parameters implemented in SeaView v4. (Gouy et al., 2010) and adjusted manually with the Mesquite program (Maddison & Maddison, 2011). The best model of nucleotide substitution for each data set was estimated with the Akaike Information Criterion (AIC) implemented in jModelTest v0.1.1 (Posada, 2008).

The phylogenetic analyses were performed with the combined database using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. The ML was carried out with the RAxML version 7.0.4 (Silvestro & Michalak, 2011) and Bayesian Inference (BI) analyses were inferred with MrBayes version 3.2.7 (Huelsenbeck & Ronquist, 2001) using the online interface: Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway v3.3 (Miller et al., 2010). The best model for each data set was GTR + I + G (Tavaré, 1986) for the LSU and TVM + I + G (Nguyen & Speed, 1992) for ITS2 implemented in BI method. ML analyses were inferred with the option GTRGAMMAI. To support each node 10,000 bootstrap replicates were run. BI analyses included Markov Chain Monte Carlo (MCMC) searches two simultaneous runs for 10 million generations, sampling every 1,000 generations, a heating parameter value of 0.2 and a "burn-in" of 25%. Tree was drawn using FigTree program v.1.3.1 (Rambaut, 2012). The genetic divergence among taxa were estimated using uncorrected "p" distances with the program MEGA version 6 (Tamura et al., 2013).

## Results

Three new species of the genera *Ekuarhuni* and *Forticulcita* plus one undescribed species belonging to *Overstreetoides* were found parasitizing the intestine of mullets in Gulf of Mexico and Atlantic Ocean

**Table 1** Sampled localities of *Mugil* spp., species of Forticulcitines recovered and GenBank accession number.

| | Locality | Georeference | Collection date | Host | Host Infected/ Host Revised | Species of *Forticulcitinae* | LSU | ITS2 |
|---|---|---|---|---|---|---|---|---|
| 1 | Soto la Marina, Tamaulipas | 23° 46′ 44.6″ N 97° 44′ 49.06″ W | 2016.02.15 | *Mugil curema* | 0/8 | – | | |
| 2 | Tamiahua, Veracruz | 21° 16′ 48.9″ N 97° 26′ 34.6″ W | 2019.12.14 | *Mugil curema* | 0/3 | – | | |
| 3 | Tecolutla, Veracruz | 20° 28′ 15.5″ N 97° 0′ 14.4″ W | 2019.12.13 | *Mugil curema* | 0/10 | – | | |
| 4 | Alvarado, Veracruz | 18° 46′ 47.82″ N 95° 44′ 50.1″ W | 2018.03.12 | *Mugil* sp. | 2/8 | *Ekuaruhuni mexicanus* **n. sp.** | MW796487–494 | MW796524–529 |
| | | | | | | *Overstreetoides* sp. | MW796497 | MW796532 |
| | | | | *Mugil cephalus* | 0/1 | – | | |
| | | | | *Mugil curema* | 1/2 | *Ekuaruhuni mexicanus* **n. sp.** | MW796495–96 | MW796530–31 |
| | | | | | | *Forticulcita macropharyngis* **n. sp.** | MW796500 | MW796535 |
| | | | | | | *Overstreetoides* sp. | MW796498–99 | MW796533–34 |
| 5 | Costa de Oro, Veracruz | 18° 42′ 7.16″ N 95° 11′ 4.7″ W | 2018.03.12 | *Mugil curema* | 1/5 | *Forticulcita macropharyngis* **n. sp.** | MW796501–503 | MW796536–538 |
| 6 | Montepio, Veracruz | 18° 38′ 29.33″ N 95° 5′ 57.2″ W | 2018.03.15 | *Mugil curema* | 3/5 | *Forticulcita macropharyngis* **n. sp.** | MW796504–508 | MW796539–543 |
| 7 | Barra de Sontecomapan, Veracruz | 18° 33′ 20″ N 94° 59′ 21″ W | 2018.03.16 | *Mugil curema* | 2/5 | *Forticulcita macropharyngis* **n. sp.** | MW796509–514 | MW796544–548 |
| 8 | Coatzacoalcos, Veracruz | 18° 11′ 10.9″ N | 2018.12.01 | *Mugil* sp | 2/5 | *Forticulcita macropharyngis* **n. sp.** | MW796515–16 | – |
| | | 94° 35′ 34.6″ W | | *Mugil cephalus* | 4/7 | *Forticulcita macropharyngis* **n. sp.** | MW796517–521 | – |
| 9 | Nuevo Campechito, Campeche | 18° 38′ 55.8″ N 92° 28′ 2.5″ W | 2020.02.19 | *Mugil curema* | 0/7 | – | | |
| 10 | Isla Aguada, Campeche | 18° 45′ 26.6″ N 91° 30′ 54.8″ W | 2020.02.19 | *Mugil curema* | 0/4 | – | | |
| 11 | Champotón, Campeche | 19° 21′ 40.3″ N 90° 43′ 5.37″ W | 2020.02.22 | *Mugil curema* | 0/11 | – | | |

**Table 1** continued

| | Locality | Georeference | Collection date | Host | Host Infected/ Host Revised | Species of *Forticulcitinae* | LSU | ITS2 |
|---|---|---|---|---|---|---|---|---|
| 12 | Celestún, Yucatán | 20° 50′ 53.5″ N 90° 24′ 22″ W | 2020.02.29 | *Mugil curema* | 0/5 | – | | |
| 13 | Chetumal, Quintana Roo | 18° 29′ 29.8″ N 88° 17′ 50.1″ W | 2020.02.24 | *Mugil curema* | 0/2 | – | | |
| 14 | Laguna Tacarigua, Venezuela | 10° 18′ 14.2″ N 65° 52′ 31.4″ W | 2012.01.08 | *Mugil curema* | 0/6 | – | | |
| 15 | Boca de Uchire, Venezuela | 10° 8′ 22.6″ N 65° 25′ 50.4″ W | 2012.01.10 | *Mugil curema* | 0/1 | – | | |
| 16 | Cumaná, Venezuela | 10° 28′ 9.8″ N 64° 11′ 18.7″ W | 2012.01.16 | *Mugil curema* | 1/2 | *Forticulcita venezuelensis* **n. sp.** | MW796522–523 | MW796549–550 |
| 17 | Isla Margarita, Venezuela | 11° 1′ 18.5″ N 63° 56′ 16.7″ W | 2012.01.12 | *Mugil curema* | 0/2 | | | |

slopes. However, only 16 individuals from the 99 *Mugil* spp. were infected with forticulcitines, representing 16% of prevalence (Table 1; Fig. 1).

## Morphological description

**Class Trematoda Rudolphi, 1808**
**Order Plagiorchiida La Rue, 1957**
**Suborder Haploporata Pérez-Ponce de León & Hernández-Mena, 2019**
**Haploporidae Nicoll, 1914**
**Forticulcitinae** Blasco-Costa et al., 2009a
***Ekuarhuni*** Andrade-Gómez & García-Varela, 2021
**Type-species *Ekuarhuni papillatum*** Andrade-Gómez & García-Varela, 2021**, by original designation.**

***Ekuarhuni mexicanus* n. sp.**
*Type host*: *Mugil curema* (Valenciennes), (Mugiliformes: Mugilidae), White mullet.
*Other host: Mugil* sp.
*Type locality*: Alvarado, Veracruz, Mexico (18° 46′ 47.82″ N; 95° 44′ 50.1″ W).
*Type material*: Holotype (CNHE-11582); 9 paratypes (CNHE-11583).
*Site on host*: Intestine.
*GenBank accession*: LSU: MW796487–MW796496; ITS2: MW796524–MW796531.
*ZooBank registration:* To comply with the regulations set out in Article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *Ekuarhuni mexicanus* **n. sp.** is urn:lsid:zoobank.org.act: DC840FD6-4C64-4185-86A3-F852E69B2FB0.
*Etymology*: The new species is named for Mexico, the country where the specimens were collected. The new name is a masculine adjective.

### Description

Based on 10 individuals mature (Fig. 2A, B; Fig. 3). Measurements of holotype are provided in description (μm); measurements of entire type series are included in Table 3. Body slightly fusiform, 836 long, widest at middle of body, 308 wide, representing 37% of body length (BL). Forebody, 213, representing 25% of BL. Hindbody 515 long, representing 62% of BL. Eye-spot pigment scarce dispersed mainly at pharynx level. Tegumental spines becoming scatter in posterior of body (Fig. 3). Oral sucker subspherical, subterminal, 87 long, 99 wide, bearing papillae on the anterior part of the oral sucker. Conspicuous glands dorsal to the oral sucker. Ventral sucker spherical, 125 long, 141 wide. Ratio of oral sucker to ventral sucker lengths 1: 1.44. Ratio of oral sucker to ventral sucker widths 1: 1.42. Prepharynx 21 long. Pharynx muscular, subspherical, 56 long, 73 wide. Ratio of oral sucker width to pharynx width 1: 0.74. Oesophagus 140 long, approximately as long as the hermaphroditic sac. Intestinal bifurcation dorsal to the posterior margin of the ventral sucker. Caeca sac-like at middle of body, postcaecal space 400 long, representing 48% of BL. Testis single located at middle of hindbody or anterior to hindbody, subspherical, 77 long, 94 wide; post-testicular space 342 long, representing 41% of BL. External seminal vesicle subspherical, 59 long, 44 wide, just posterior and contiguous with hermaphroditic sac. Hermaphroditic sac thin-walled, slightly oval, 163 long, 114 wide, representing 19% of BL, containing terminal genitalia, internal seminal vesicle, 79 long, 62 wide, oval to ellipsoidal, prostatic bulb 37 long, 27 wide, hermaphroditic duct muscular, wide, eversible, as intromittent organ. Genital atrium shallow, genital pore medial, 30 long, anterior to anterior margin of ventral sucker.

Ovary spherical, 62 long, 57 wide, located dorsally to ventral sucker. Laurer's canal not observed. Some individuals present a massive seminal receptacle in hindbody. Vitellarium a single spherical mass, 61 long, 62 wide, overlapping anterior portion of testis. Uterus occupying most of hindbody. Eggs in distal portion of uterus, 39–42 long, 20–22 wide, containing well developed miracidia with eyespots. Excretory vesicle weakly Y-shaped.

### Taxonomic Remarks

*Ekuarhuni mexicanus* **n. sp.** is the second species described to the genus, associated with *M. curema* analyzed herein. With the new species described in the current research, Middle America harbors two species of *Ekuarhuni.* The new species possesses the main characteristics of genus *Ekuarhuni*, i.e., conspicuous glands in the forebody, mainly at pharynx level, dorsal to oral sucker (Fig. 2A, B; Fig. 3). The new species can be distinguished from *Ekuarhuni papillatum* by being

**Table 2** Sequences from GenBank used for phylogenetic analysis in the present study.

| Family | Species | Host | Locality | GenBank accession numbres LSU | ITS2 | Reference (s) |
|---|---|---|---|---|---|---|
| Atractotrematidae | *Isorchis cannoni* Huston, Cutmore & Cribb, 2017 | *Siganus lineatus* | Heron Island, Australia | MF803154 | MF803156 | Huston et al. (2018) |
| Haploporidae | | | | | | |
| **Hapladeninae** | *Hapladena acanthuri* Siddiqi & Cable, 1960 | *Acanthurus chirurgus* | Virgin Islands: Christiansted fish market, St. Croix, USA. | MH244119 | MH244119 | Andres et al. (2018) |
| **Megasoleninae** | *Megasolena mikra* Andres et al., 2018 | *Holacanthus ciliaris* | Florida Middle Ground, Gulf of Mexico, USA | MH244122 | MH244122 | Andres et al. (2018) |
| **Pseudohaploporinae** | *Pseudohaploporus planilizum* Atopkin et al., 2019 | *Planiliza subviridis* | Coastal water of Cat Ba Island, Tonkin Bay, northern Vietnam | MF774417 | MF774433 | Atopkin et al. (2019) |
| **Haploporinae** | *Haploporus benedeni* (Stossich, 1887) Looss, 1902 | *Liza ramado* | Santa Pola (sea) Spain | FJ211237 | FJ211247 | Blasco-Costa et al. (2009a) |
| **Chalcinotrematinae** | *Saccocoelioides nanii* Szidat, 1954 | *Prochilodus lineatus* | Los Talas, Buenos Aires Province, Argentina | MG925114 | MG925113 | Curran et al. (2018) |
| **Waretrematinae** | *Capitimitta darwinensis* Pulis & Overstreet, 2013 | *Selenotoca multifasciata* | Northern Territory, Darwin Australia | KC206498 | KC206498 | Pulis and Overstreet (2013) |
| **Forticulcitinae** | *Forticulcita apiensis* Andres, Curran, Fayton, Pulis & Overstreet, 2015 | *Mugil cephalus* | Salt Springs, St. Johns River, Florida, USA | KP761087 | KP761087 | Andres et al. (2015) |
| | *Forticulcita gibsoni* Blasco-Costa et al., 2009b | *Mugil cephalus* | Santa Pola (sea) Spain | FJ211239 | FJ211249 | Blasco-Costa et al. (2009a) |
| | *Forticulcita platana* Andres, Curran, Fayton, Pulis & Overstreet, 2015 | *Mugil liza* | Rio de la Plata, Punta Lara, Argentina | KP761086 | KP761086 | Andres et al. (2015) |
| | *Forticulcita isabelae* Andrade-Gómez & García-Varela, 2021 | *Mugil cephalus* | Cerritos, Sinaloa, México | MT957783 | MT957636 | Andrade-Gómez and García-Varela (2021) |
| | *Forticulcita isabelae* Andrade-Gómez & García-Varela, 2021 | *Mugil* sp. | Perula, Jalisco, México | MT957785 | MT957638 | Andrade-Gómez and García-Varela (2021) |
| | *Forticulcita isabelae* Andrade-Gómez & García-Varela, 2021 | *Mugil curema* | Barra Vieja, Guerrero, México | MT957786 | MT957639 | Andrade-Gómez and García-Varela (2021) |
| | *Forticulcita minuta* Andrade-Gómez & García-Varela, 2021 | *Mugil curema* | Chamela, Jalisco, México | MT957804 | MT957642 | Andrade-Gómez and García-Varela (2021) |
| | *Forticulcita minuta* Andrade-Gómez & García-Varela, 2021 | *Mugil* sp. | Tecuanillo, Colima, México | MT957809 | MT957647 | Andrade-Gómez and García-Varela (2021) |
| | *Forticulcita minuta* Andrade-Gómez & García-Varela, 2021 | *Mugil* sp. | Penitas, Guerrero, México | MT957810 | MT957648 | Andrade-Gómez and García-Varela (2021) |

**Table 2** continued

| Family | Species | Host | Locality | GenBank accession numbres LSU | ITS2 | Reference (s) |
|---|---|---|---|---|---|---|
| | *Overstreetoides pacificus* Andrade-Gómez & García-Varela, 2021 | *Mugil* sp. | Cuitzmala, Jalisco, México | MT957745 | MT957621 | Andrade-Gómez and García-Varela (2021) |
| | *Overstreetoides pacificus* Andrade-Gómez & García-Varela, 2021 | *Mugil* sp. | Penitas, Guerrero, México | MT957748 | MT957624 | Andrade-Gómez and García-Varela (2021) |
| | *Overstreetoides pacificus* Andrade-Gómez & García-Varela, 2021 | *Mugil curema* | Barra Vieja, Guerrero, México | MT957754 | MT957630 | Andrade-Gómez and García-Varela (2021) |
| | *Ekuarhuni papillatum* Andrade-Gómez & García-Varela, 2021 | *Mugil curema* | Chamela, Jalisco, México | MT957690 | MT957590 | Andrade-Gómez and García-Varela (2021) |
| | *Ekuarhuni papillatum* Andrade-Gómez & García-Varela, 2021 | *Mugil curema* | Boca de Apiza, Michoacán, México | MT957700 | MT957599 | Andrade-Gómez and García-Varela (2021) |
| | *Ekuarhuni papillatum* Andrade-Gómez & García-Varela, 2021 | *Mugil curema* | Estero, Costa Rica | MT957738 | MT957620 | Andrade-Gómez and García-Varela (2021) |
| | *Xiha fastigata* (Thatcher & Sparks, 1958) | *Mugil cephalus* | Grand Isle, Louisiana and Davis Bayou, Mississippi USA | KP761088 | KP761088 | Andres et al. (2015) |

**Table 3** Comparative metrical data for *Ekuarhuni* spp. and the diminutive morphotype species of *Forticulcita*. *=Estimated from the published drawing. BL body length, BW maximum body width, FO forebody, HI hindbody, OSL oral sucker length, OSW oral sucker width, VSL ventral sucker length, VSW ventral sucker width, PL prepharynx length, PHL pharynx length, PHW pharynx width, OEL oesophagus length, CEND postcaecal field length, TL testis length, TW testis width, TEND posttesticular field length, ESVL external seminal vesicle length, ESVW maximum external seminal vesicle width, HSL hermaphroditic sac length, HSW maximum hermaphroditic sac width, ISVL internal seminal vesicle length, ISVW maximum internal seminal vesicle width, PBL prostatic bulb length, PBW prostatic bulb width, GPL genital pore length, OL ovary length, OW ovary width, DOT distance of margin posterior of ovary to margin anterior of testis, VL vitelline mass length, VW vitelline mass width, EL egg length, EW egg width, OSL/VSL sucker length ratio, OSW/VSW sucker width ratio, OSW/PHW ratio of oral sucker width to pharynx width.

| | *Ekuarhuni papillatum* | ***Ekuarhuni mexicanus* n. sp.** | *F. gibsoni* | *F. platana* | *F. apiensis* | *F. isabelae* | *F. minuta* | ***F. macropharyngis* n. sp.** | ***F. venezuelensis* n. sp.** |
|---|---|---|---|---|---|---|---|---|---|
| Reference | Andrade-Gómez & García-Varela, 2021 | This study | Blasco-Costa et al., 2009b | Andres et al., 2015 | Andres et al., 2015 | Andrade-Gómez & García-Varela, 2021 | Andrade-Gómez & García-Varela, 2021 | This study | This study |
| Type-Locality | Boca de Apiza, Michoacán, Mexico | Alvarado Veracruz, Mexico | Santa Pola, Spain | Rio de la Plata, Buenos Aires, Argentina | Salt Springs, Florida, USA | Camino Las Arenitas, Sinaloa, Mexico | Barra de Chamela, Jalisco, Mexico | Costa de Oro, Veracruz, Mexico | Laguna Tacarigua, Venezuela |
| Type-Host | *Mugil curema* | *Mugil curema* | *Mugil cephalus* | *Mugil liza* | *Mugil cephalus* | *Mugil curema* | *Mugil curema* | *Mugil curema* | *Mugil cephalus* |
| n | 12 | 10 | 17 | 17 | 9 | 10 | 7 | 8 | 10 |
| BL | 576–812 | 677–931 | 777–1, 024 | 501–790 | 354–524 | 524–719 | 195–306 | 491–768 | 583–746 |
| BW | 141–234 | 229–376 | 201–299 | 131–214 | 124–153 | 133–229 | 107–161 | 241–375 | 230–281 |
| BW/BL (%) | 22–35 | 25–49 | 22–36 | 24–29 | 28–35 | 25–35 | 35–72 | 36–55 | 31–43 |
| FO | 126–211 | 158–266 | 184–291 | 154–198 | 124–158 | 77–172 | 43–80 | 131–230 | 130–167 |
| FO/BL (%) | 20–26 | 22–31 | 22–31 | 22–31 | 28–35 | 14–31 | 19–29 | 24–31 | 18–23 |
| HI | 349–476 | 402–555 | 538* | 279–496 | 157–285 | 320–459 | 87–176 | 295–489 | 389–542 |
| HI/BL (%) | 58–65 | 53–65 | 62* | 56–64 | 44–55 | 61–74 | 44–59 | 53–65 | 66–73 |
| OSL | 67–93 | 60–96 | 69–93 | 71–81 | 45–67 | 48–61 | 41–55 | 49–84 | 59–81 |
| OSW | 72–95 | 72–108 | 80–107 | 73–100 | 54–72 | 42–68 | 36–55 | 73–83 | 78–90 |
| VSL | 81–121 | 85–149 | 82–109 | 68–97 | 73–84 | 54–95 | 42–65 | 78–116 | 64–83 |
| VSW | 84–115 | 94–154 | 85–115 | 64–97 | 81–87 | 49–89 | 41–68 | 96–126 | 75–90 |
| OSL/VSL | 1:1.14–1.4 | 1:1.16–1.92 | 1:0.91–1.51 | 1:0.96–1.31 | 1:1.12–1.62 | 1:1–1.8 | 1:1.02–1.58 | 1:1.13–1.86 | 1:0.94–1.27 |
| OSW/VSW | 1:1.16–1.42 | 1:1.13–1.74 | 1:0.87–1.21 | 1:0.75–1.02 | 1:1.17–1.44 | 1:0.92–1.5 | 1:1.13–1.46 | 1:1.16–1.58 | 1:0.89–1.09 |

**Table 3** continued

| | *Ekuarhuni papillatum* | ***Ekuarhuni mexicanus* n. sp.** | *F. gibsoni* | *F. platana* | *F. apiensis* | *F. isabelae* | *F. minuta* | ***F. macropharyngis* n. sp.** | ***F. venezuelensis* n. sp.** |
|---|---|---|---|---|---|---|---|---|---|
| PL | 0–15 | 11–25 | 0–35 | 17–33 | 9–28 | 0–20 | 3–6 | 6–23 | 9–16 |
| PHL | 39–64 | 38–64 | 45–62 | 39–48 | 31–46 | 25–44 | 34–58 | 46–110 | 46–61 |
| PHW | 54–67 | 52–76 | 48–70 | 39–60 | 42–51 | 30–46 | 30–44 | 74–106 | 61–73 |
| OSW/PHW | 1:0.62–0.79 | 1:0.63–0.81 | – | 1:0.53–0.68 | 1:0.63–0.93 | 1:0.55–0.77 | 1:0.76–0.87 | 1:0.93–1.34 | 1:0.75–0.88 |
| OEL | 100–156 | 117–159 | 116–296 | 176–263 | 71–121 | 107–134 | 22–55 | 77–130 | 119–151 |
| CEND | 212–353 | 328–483 | 397–622 | 164–369 | 126–212 | 219–290 | 50–132 | 105–449 | 257–362 |
| CEND/BL (%) | 36–48 | 45–55 | 45–61 | 33–50 | 34–45 | 40–48 | 25–43 | 19–58 | 44–49 |
| TL | 81–131 | 76–150 | 75–127 | 59–101 | 31–53 | 102–143 | 36–71 | 44–90 | 138–201 |
| TW | 63–89 | 91–123 | 62–90 | 42–65 | 25–36 | 53–94 | 41–62 | 44–88 | 83–100 |
| TEND | 151–277 | 206–350 | 395–538 | 176* | 96–186 | 67–232 | 11–48 | 127–304 | 135–266 |
| TEND/BL (%) | 25–37 | 29–45 | 45–57 | 19–36 | 27–40 | 12–32 | 5–21 | 26–40 | 22–36 |
| ESVL | 36–73 | 36–77 | 123–197 | 34–63 | 21–44 | 52–82 | 17–31 | 40–69 | 28–79 |
| ESVW | 32–52 | 28–53 | 34–62 | 17–27 | 12–24 | 30–48 | – (19) | 31–61 | 32–60 |
| HSL | 112–132 | 136–180 | 183–261 | 120–171 | 84–128 | 117–154 | 52–132 | 162–199 | 128–147 |
| HSW | 72–108 | 101–141 | 54–96 | 68–98 | 41–55 | 58–95 | 33–60 | 101–140 | 100–141 |
| HSL/BL (%) | 14–22 | 16–25 | 15* | 19–24 | 19–30 | 19–29 | 23–48 | 21–35 | 18–22 |
| ISVL | 22–64 | 63–88 | 53–104 | 32–65 | 26–36 | 40–95 | 21–44 | 50–93 | 50–83 |
| ISVW | 24–49 | 44–67 | 35–77 | 26–64 | 20–34 | 41–69 | 17–30 | 51–85 | 53–82 |
| PBL | 23–36 | 23–45 | – | 38–51 | 29–36 | 31–45 | 14–19 | 44–60 | 34–48 |
| PBW | 22–36 | 21–40 | – | 36–59 | 24–37 | 32–57 | 16–21 | 32–71 | 34–53 |
| GPL | 15–23 | 13–30 | 23* | 13–27 | 8–20 | 15–32 | 5–17 | 15–25 | 10–23 |
| OL | 74–99 | 58–85 | 51–137 | 64–86 | 27–42 | 64–75 | 28–46 | 49–89 | 60–75 |
| OW | 37–53 | 53–81 | 47–81 | 33–66 | 22–30 | 38–69 | 15–48 | 42–78 | 58–68 |
| DOT | 37, 52 | 0–95 | 0* | 3–80 | 7–42 | 0–50 | 0 | 14, 52 | 0–65 |
| VL | 42–65 | 54–70 | 53–71 | 40–65 | 28–40 | 43–57 | 24–39 | 46–67 | 63–75 |
| VW | 49–81 | 62–76 | 46–64 | 36–63 | 24–32 | 48–59 | 25–33 | 39–57 | 57–72 |
| EL | 39–61 | 32–46 | 34–44 | 44–52 | 38–49 | 41–52 | 27–49 | 30–53 | 40–48 |
| EW | 21–37 | 19–24 | 18–24 | 20–26 | 14–20 | 19–26 | 16–27 | 18–37 | 18–22 |

bigger; i.e., the body width is bigger than *E. papillatum* (141–234 µm *vs* 229–376 µm in *Ekuarhuni mexicanus* **n. sp.**); the sucker ratios are slightly bigger (1: 1.14–1.4 length; 1:1.16–1.42 width *vs* 1: 1.16–1.92 length; 1: 1.13–1.74 width, in *Ekuarhuni mexicanus* **n. sp.**); testis width is bigger (63–89 µm *vs* 91–123 µm in *Ekuarhuni mexicanus* **n. sp.**); hermaphroditic sac is bigger (112–132 µm length; 72–108 µm width *vs* 136–180 µm length; 101–141 µm width, respectively in *Ekuarhuni mexicanus* **n. sp.**); internal seminal vesicle is bigger (22–64 µm length; 24–49 µm width *vs* 63–88 µm length; 44–67 µm width, respectively in *Ekuarhuni mexicanus* **n. sp.**). Finally, the new species can be distinguished from *Ekuarhuni papillatum* by its geographical distribution, i.e., *Ekuarhuni papillatum* was recorded in the Pacific slopes of Middle America, meanwhile *Ekuarhuni mexicanus* **n. sp.** was found in Gulf of Mexico.

**Class Trematoda Rudolphi, 1808**
**Order Plagiorchiida La Rue, 1957**
**Suborder Haploporata Pérez-Ponce de León & Hernández-Mena, 2019**
**Haploporidae Nicoll, 1914**
**Forticulcitinae** Blasco-Costa et al., 2009a
***Forticulcita*** Overstreet, 1982
**Type-species *Forticulcita glabra*** Overstreet, 1982**, by original designation.**
***Forticulcita macropharyngis* n. sp.**
*Type host*: *Mugil curema* (Valenciennes), (Mugiliformes: Mugilidae), White mullet.
*Other host*: *Mugil cephalus* (L.).
*Type locality*: Costa de Oro, Veracruz, Mexico (18° 42′ 7.16″ N; 95° 11′ 4.7″ W).
*Other 4 localities*: Coatzacoalcos, Montepio, Alvarado, Sontecomapan in Veracruz, Mexico.
*Type material*: Holotype (CNHE-11584); 7 paratypes (CNHE-11585).
*Site on host*: Intestine.
*GenBank accession*: LSU: MW796500–MW796521; ITS2: MW796535–MW796548.
*ZooBank registration:* To comply with the regulations set out in Article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *Forticulcita macropharyngis* **n. sp.** is urn:lsid:- zoobank.org.act: 847A44FF-5924-461F-86D3-E893A841B6BA

*Etymology*: The new species is named for its massive pharynx that readily distinguishes from other congeneric species of *Forticulcita*. The new name is treated as a feminine adjective.

Description

Based on 8 individuals mature (Fig. 2C–D; Fig. 4A–B). Measurements of holotype are provided in description (µm); measurements of entire type series are included in Table 3. Body slightly pyriform, 592 long, widest at midbody, 266 wide, representing 45% of BL. Forebody, 169, representing 29% of BL. Hindbody 343 long, representing 58% of BL. Eye-spot pigment scarce dispersed mostly at pharynx level. Spines conspicuous reaching posterior end of the body. Oral sucker subspherical, subterminal, 70 long, 80 wide. Ventral sucker subspherical, 115 long, 126 wide. Ratio of oral sucker to ventral sucker lengths 1: 1.64. Ratio of oral sucker to ventral sucker widths 1: 1.58. Prepharynx present, 13 long. Pharynx massive, muscular, subspherical, 94 long, 94 wide. Ratio of oral sucker width to pharynx width 1: 1.18. Oesophagus 95 long, dorsal to ventral sucker. Intestinal bifurcation posterior to posterior margin of ventral sucker. Caeca sac-like in hindbody, postcaecal space 180 long, representing 30% of BL. Testis single located at posterior to middle of body, subspherical, 65 long, 59 wide; post-testicular space 211 long, representing 36% of BL. External seminal vesicle slightly elongated, 40 long, 31 wide, dorsal to hermaphroditic sac.

Hermaphroditic sac thin-walled, oval to elongated 168 long, 135 wide, representing 28% of BL, containing terminal genitalia, internal seminal vesicle, 93 long, 85 wide, spherical, conspicuous prostatic bulb 60 long, 71 wide, hermaphroditic duct strongly muscular, eversible, as intromittent organ. Genital atrium shallow, genital pore medial, 18 long, anterior to ventral sucker.

Ovary transversally elongate, 49 long, 78 wide, located in middle of body, anterior and contiguous to testis. Laurer's canal not observed. Vitellarium a single subspherical mass, 54 long, 57 wide, contiguous to testis and intercaecal. Uterus filling mostly the middle of body, not reaching the end of the body, with well-developed metraterm entering posterior end of hermaphroditic sac. Eggs in distal portion of uterus, 50–53 long, 25–26 wide, containing well developed

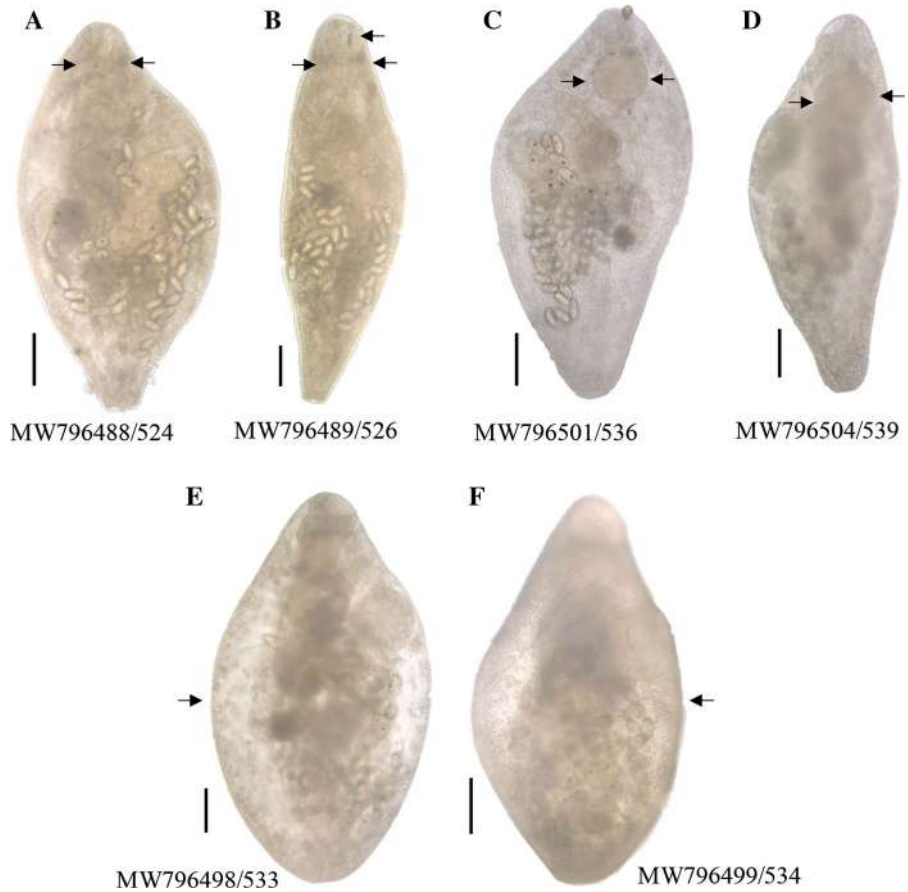


**Fig. 2** *Photogenophores* of **(A, B)** *Ekuarhuni mexicanus* **n. sp.** Specimens collected from *Mugil* sp. in Alvarado, Veracruz, Mexico. *Forticulcita macropharyngis* **n. sp.**, from *Mugil curema* (**C**) specimen collected from Costa de Oro, Veracruz, Mexico; (**D**) specimen collected from Montepio, Veracruz, Mexico. (**E**, **F**) *Overstreetoides* sp. Specimens collected from *Mugil curema* in Alvarado, Veracruz, Mexico. GenBank accession numbers are indicated. Arrows indicated diagnostic characters. Scale bars= 100 μm.

miracidia with eyespots. Excretory vesicle weakly Y-shaped.

**Class Trematoda Rudolphi, 1808**
**Order Plagiorchiida La Rue, 1957**
**Suborder Haploporata Pérez-Ponce de León & Hernández-Mena, 2019**
**Haploporidae Nicoll, 1914**
**Forticulcitinae** Blasco-Costa et al., 2009a
***Forticulcita*** Overstreet, 1982
**Type-species *Forticulcita glabra*** Overstreet, 1982**, by original designation.**

***Forticulcita venezuelensis* n. sp.**
*Type host*: *Mugil curema* (Valenciennes), (Mugiliformes: Mugilidae), White mullet.
*Type locality*: Cumaná, Venezuela (10° 28′ 9.8″ N; 64° 11′ 18.7″ W).
*Type material*: Holotype (CNHE-11586); 9 paratypes (CNHE-11587).
*Site on host*: Intestine.
*GenBank accession*: LSU: MW796522–MW796523; ITS2: MW796549–MW796550.

*ZooBank registration:* To comply with the regulations set out in Article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *Forticulcita venezuelensis* **n. sp.** is urn:lsid:- zoobank.org.act: 37877376-FA47-4AC5-A143-B3629FF80D88

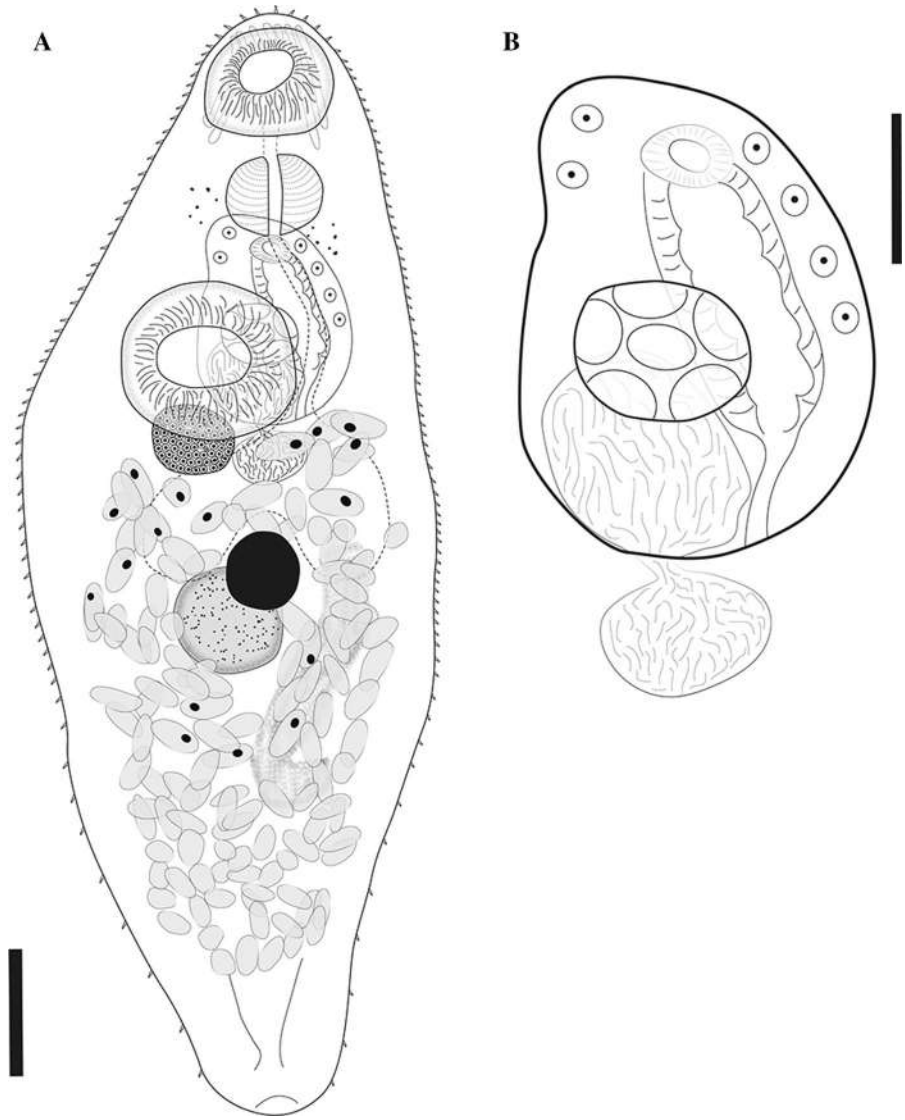


**Fig. 3** *Ekuarhuni mexicanus* **n. sp.**, from *Mugil curema* (**A**) whole worm, holotype, ventral view; (**B**) Hermaphroditic sac, ventral view. Scale bars = 100 μm (A); 50 μm (B)

*Etymology*: The new species is named *venezuelensis* (Latinised feminine adjectival name) refers to Venezuela country where the specimens were collected.

Description

Based on 10 individuals mature (Fig. 4C–D). Measurements of holotype are provided in description (μm); measurements of entire type series are included in Table 3. Body slightly fusiform, 713 long, widest at the first third of the body, 276 wide, representing 39% of BL. Forebody, 167, representing 23% of BL. Hindbody 473 long, representing 66% of BL. Eye-spot pigment scarce dispersed at pharynx level. Spines conspicuous, becoming sparse in posterior of body. Oral sucker spherical, subterminal, 81 long, 85 wide. Ventral sucker subspherical, 76 long, 81 wide. Ratio of oral sucker to ventral sucker lengths 1: 0.94. Ratio of oral sucker to ventral sucker widths 1: 0.95. Prepharynx short 13 long. Pharynx, muscular, subspherical, 56 long, 73 wide. Ratio of oral sucker width to pharynx width 1: 0.85. Oesophagus 119 long, dorsal to ventral sucker. Intestinal bifurcation posterior to hermaphroditic sac. Caeca sac-like in middle of body,

terminating at first third of testis, postcaecal space 335 long, representing 47% of BL. Testis single located posterior to middle of body, elongated, 201 long, 98 wide; post-testicular space 172 long, representing 24% of BL. External seminal vesicle spherical, 36 long, 40 wide, just posterior and contiguous with hermaphroditic sac.

Hermaphroditic sac thin-walled terminating posterior to ventral sucker, subspherical, 147 long, 141 wide, representing 21 % of BL, containing terminal genitalia, internal seminal vesicle, 73 long, 66 wide, oval to spherical, prostatic bulb 37 long, 39 wide, hermaphroditic duct strongly muscular, eversible, as intromittent organ. Genital atrium shallow, genital pore medial, 13 long, anterior to ventral sucker.

Ovary spherical, 73 long, 68 wide, anterior to testis. Laurer's canal not observed. Vitellarium a single subspherical mass, 74 long, 66 wide, located intercaecal. Uterus distributed between ventral sucker level and postesticular zone, not reaching the end of body. Eggs in distal portion of uterus, 40–50 long, 18–23 wide, containing well developed miracidia with eyespots. Excretory vesicle weakly Y-shaped.

Taxonomic Remarks

To date, *Forticulcita* represents the most specious genus within Forticulcitinae with 9 described species, parasitizing mullets worldwide. Andrade-Gómez and García-Varela (2021) divided the genus *Forticulcita* into two morphotypes. The diminutive morphotype consisted of five species (*F. gibsoni* Blasco-Costa et al., 2009b; *F. apiensis* Andres, Curran, Fayton, Pulis & Overstreet, 2015; *F. platana* Andres, Curran, Fayton, Pulis & Overstreet, 2015; *F. isabelae* Andrade-Gómez & García-Varela, 2021; and *F. minuta* Andrade-Gómez & García-Varela, 2021) that were characterized by having a small body size (<1,100 μm long), and relatively few large eggs present in the uterus confined mostly in the hindbody. The two new species described herein belong to this group because *F. macropharyngis* **n. sp.** and *F. venezuelensis* **n. sp.** present a relatively small body size (<1,100 μm long) with relatively few large eggs present in the uterus. With the inclusion of the two new species, the America harbor 6 species; 2 in South America (*F. venezuelensis* **n. sp.** and *F. platana*), one

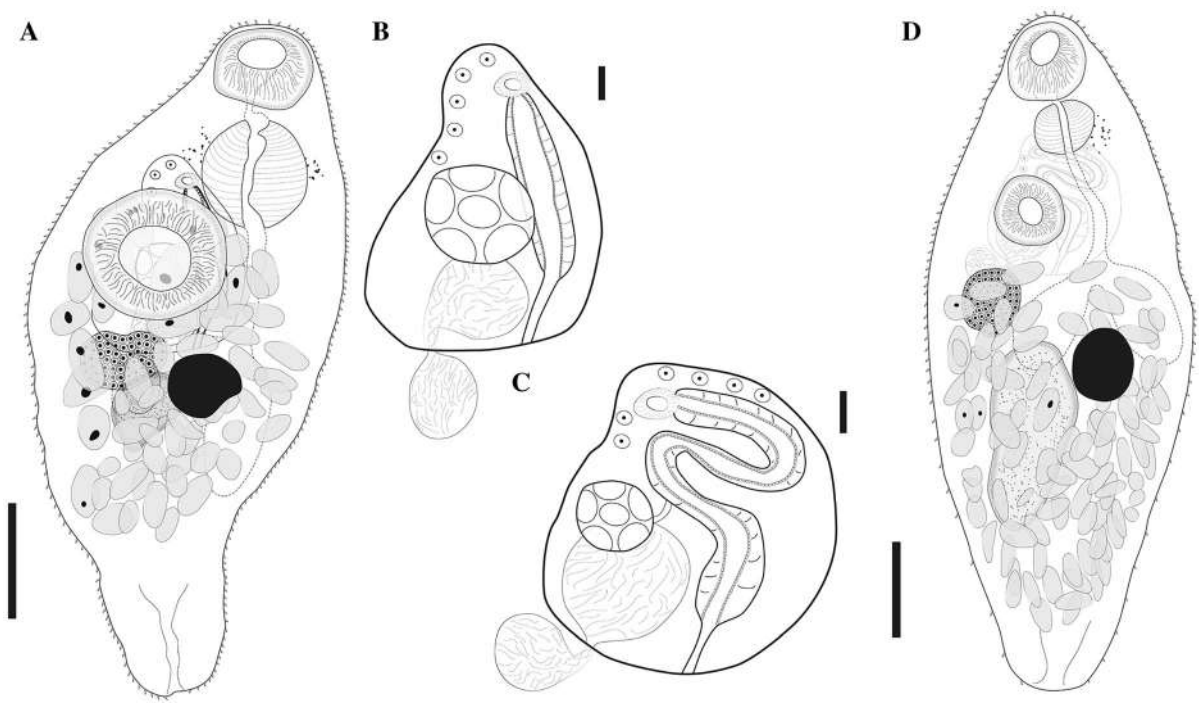


**Fig. 4** **(A–B)** *Forticulcita macropharyngis* **n. sp.**, from *Mugil curema* (**A**) whole worm, holotype, ventral view; (**B**) Hermaphroditic sac, ventral view; **(C–D)** *Forticulcita venezuelensis* **n. sp.**, from *Mugil curema* (**C**) Hermaphroditic sac, ventral view (**D**) whole worm, holotype, ventral view. Scale bars = 100 μm (A); 20 μm (B–C); 100 μm (D).

in Middle America (*F. macropharyngis* **n. sp.**), and one in North America (*F. apiensis*) from the Atlantic slopes; and 2 in Middle America (*F. isabelae*, and *F. minuta*) from the Pacific slopes.

*Forticulcita macropharyngis* **n. sp.**, can be differentiated from the other species of diminutive morphotype by possessing the biggest pharynx (46–110 µm long, 74–106 µm wide) (Fig. 2C–D; Fig. 4A–B) with the biggest ratio of oral sucker width to pharynx width (1:0.93–1.34; see Table 3). Likewise, the other congeneric species described herein as *Forticulcita venezuelensis* **n. sp.** can be differentiated from the other species of diminutive morphotype by possessing the biggest testis (138–201 µm long, 83–100 µm wide; see Table 3, Fig. 4C–D).

**Class Trematoda Rudolphi, 1808**
**Order Plagiorchiida La Rue, 1957**
**Suborder Haploporata Pérez-Ponce de León & Hernández-Mena, 2019**
**Haploporidae Nicoll, 1914**
**Forticulcitinae** Blasco-Costa et al., 2009a
***Overstreetoides*** Andrade-Gómez & García-Varela, 2021

**Type-species *Overstreetoides pacificus*** Andrade-Gómez & García-Varela, 2021
***Overstreetoides* sp.**
*Hosts*: *Mugil curema* (Valenciennes), White mullet; *Mugil* sp. (Mugiliformes: Mugilidae).
*Locality*: Alvarado, Veracruz, Mexico (18° 46′ 47.82″ N; 95° 44′ 50.1″ W).
*Site in host*: Intestine.
*Material: Photogenophore* and sequences.
*GenBank accession*: LSU: MW796497–MW796499; ITS2: MW796532–MW796534.

Remarks

Three mature individuals were sequenced from *Mugil* sp. and *Mugil curema* collected from Alvarado, Veracruz, México. The sequences obtained match 97% similar with *Overstreetoides pacificus*, suggesting that those individuals represent an undescribed species. The abundance of *Overstreetoides* sp. was the lowest, only three individuals collected. Therefore, the lack of specimens precludes the description of the species. However, the *photogenophores* of *Overstreetoides* sp. (Fig. 2E, F) showed a body oval like genus *Overstreetoides* as reported by Andrade-Gómez and García-Varela (2021).

Phylogenetic analysis

The combined data set (LSU + ITS2) include 50 sequences aligned to 1,666 positions. The phylogenetic analyses inferred with ML and BI showed that the subfamily Forticulcitinae was well supported (1/100) with *X. fastigata* as sister to a clade of *Ekuarhuni* spp. + (*Overstreetoides* + *Forticulcita*) (Fig. 5). The 27 sequences obtained in the present study were nested in Forticulcitinae. For instance, eight new sequences of *Ekuarhuni mexicanus* **n. sp.**, were sister group to *Ekuarhuni papillatum* receiving strong support (1/100). Likewise, three new sequences of *Overstreetoides* sp. were sister to *Overstreetoides pacificus* receiving strong support (1/100). Apparently, these new sequences represent a new species of the genus *Overstreetoides*. On the other hand, the genus *Overstreetoides* was sister group to *Forticulcita* spp., however this near relationship received moderate support (0.69/76). Finally, *Forticulcita* was divided into two well-supported clades (1/94). The first was formed by *F. minuta* and the two new sequences of *F. venezuelensis* **n. sp.** receiving strong support (0.99/98), and the second clade was formed by the 14 new sequences of *F. macropharyngis* **n. sp.** as sister to *F. apiensis*, *F. gibsoni*, and *F. platana* + *F. isabelae* with strong support (1/98). In addition, the three new species described herein plus the undescribed species of *Overstreetoides* received strong support, i.e., *Ekuarhuni mexicanus* **n. sp.** (0.99/100); *Forticulcita venezuelensis* **n. sp.**, *F. macropharyngis* **n. sp.**, and *Overstreetoides* sp. (1/100) validating the independent lineages status (Fig. 5).

The intraspecific genetic divergence among the 14 isolates of *Forticulcita macropharyngis* **n. sp.** ranged from 0 to 0.08% ± 0.008 (0–1 nt) with LSU and with ITS2 all the sequences were identical. In contrast there was no intraspecific variation observed in the two sequences of *Forticulcita venezuelensis* **n. sp.**, or the three sequences of *Overstreetoides* sp. Meanwhile, the intraspecific genetic divergence among the 8 isolates of *Ekuarhuni mexicanus* **n. sp.** was zero with LSU and from 0 to 0.53% ± 0–0.003 (0–2 nt) with ITS2 (see Table 4).

The genetic divergence estimated among the four genera of Forticulcitinae ranged from 3.64 to 6.08 % ±

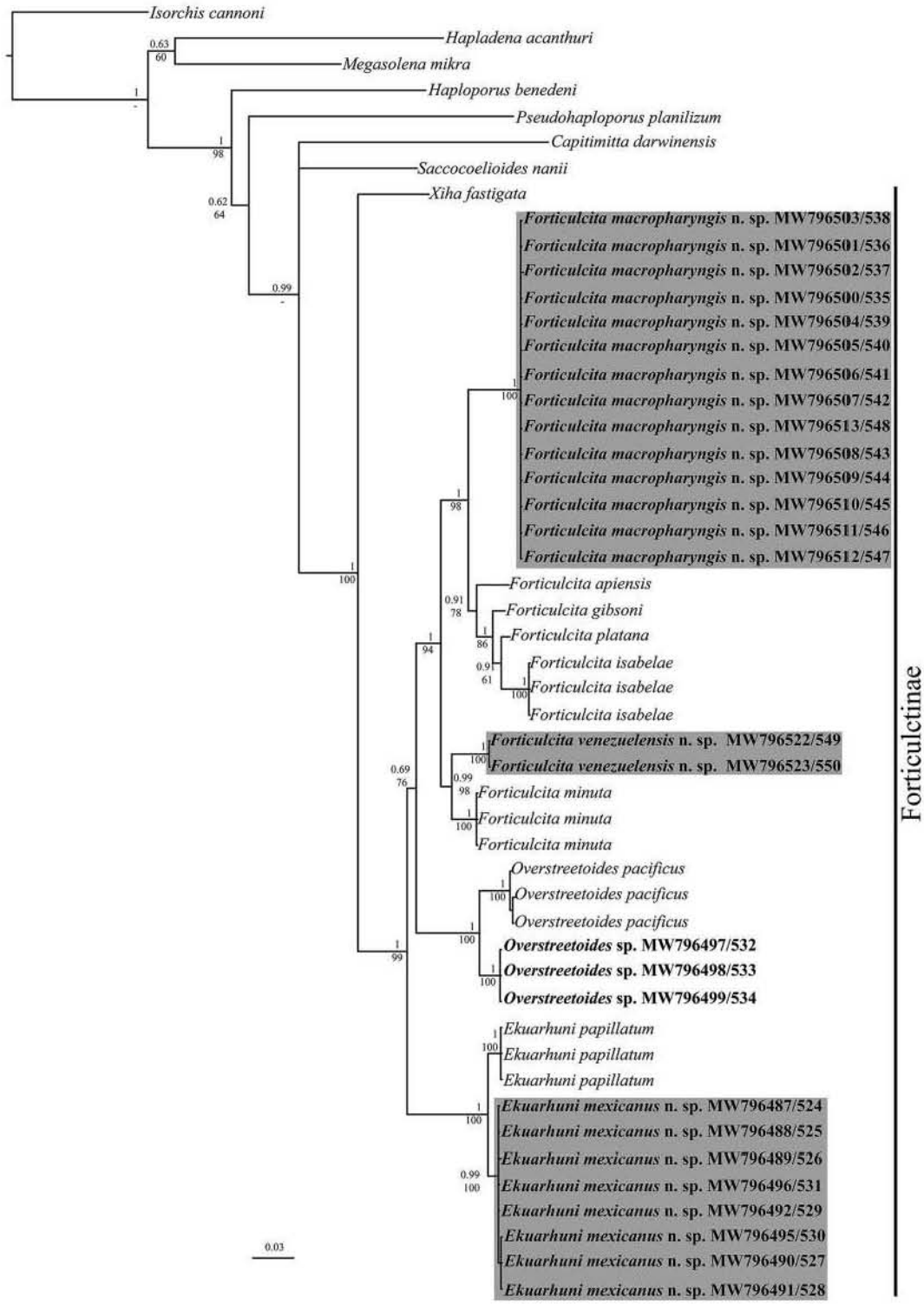


**Fig. 5** Consensus Bayesian inference and Maximum likelihood trees inferred with the concatenated (LSU + ITS2) dataset. Numbers near internal nodes show posterior probabilities (BI) and ML bootstrap clade frequencies. In bold are sequences generated in this study; in gray are the new species described herein.

**Table 4** Pairwise nucleotide sequence comparisons between taxa for the aligned LSU rDNA sequences ($N = 1257$ nt) (below the diagonal) and for ITS2 sequences ($N = 409$ nt) (above the diagonal). In bold is represented the genetic intraspecific divergence.

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *F. gibsoni* | – | 1.13 | 5.41 | 3.10 | 6.03 | 5.07 | 6.9 | 7.12–7.41 | 7.41 | 8.36 | 7.8–8.36 | 11.36 |
| 2 | *F. platana* | 0.42 | – | 4.51 | 2.36 | 5.08 | 3.67 | 6.15 | 5.57–5.84 | 5.84 | 6.7 | 6.18–6.7 | 10.32 |
| 3 | *F. apiensis* | 1.34 | 1.37 | – | 3.71 | 5.08 | 4.51 | 6.15 | 6.7–6.97 | 7.24 | 7.77 | 7.51–7.77 | 10.96 |
| 4 | *F. isabelae* | 0.84 | 1.05 | 1.54 | – | 5.08 | 4.72 | 6.95 | 7.16–7.43 | 7.43 | 7.77 | 6.99–7.51 | 9.79 |
| 5 | *F. minuta* | 2.18 | 2.27 | 2.11 | 2.19 | – | 4.55 | 5.07 | 6.43–6.7 | 7.71 | 8.02 | 7.75–8.02 | 9.87 |
| 6 | *F. macropharyngis* **n. sp.** | 2.52–2.6 | 2.51–2.59 | 2.27–2.35 | 2.75–2.83 | 3.48–3.57 | **0–0.08/**– | 5.88 | 5.57–5.84 | 6.63 | 6.43 | 5.65–6.17 | 9.79 |
| 7 | *F. venezuelensis* **n. sp.** | 2.35 | 2.19 | 2.59 | 2.67 | 1.46 | 3.48–3.57 | – | 8.31 | 7.77–8.04 | 9.09 | 9.09–9.36 | 11.73 |
| 8 | *Overstreetoides pacificus* | 4.87–4.95 | 4.78–4.86 | 4.78–4.86 | 4.62–4.70 | 4.21–4.29 | 5.51–5.67 | 4.37–4.45 | **0–0.08/ 0–0.26** | 2.62–2.89 | 6.99–7.26 | 7.26–7.8 | 10.5–10.76 |
| 9 | *Overstreetoides* sp. | 4.61 | 4.53 | 4.29 | 4.13 | 3.64 | 5.27–5.35 | 4.21 | 1.7–1.78 | – | 7.26 | 7.53–7.8 | 10.24 |
| 10 | *Ekuarhuni papillatum* | 5.21 | 5.27 | 5.19 | 5.35 | 4.29 | 5.43–5.52 | 4.38 | 6–6.08 | 5.67 | – | 1.34–1.6 | 11.23 |
| 11 | *Ekuarhuni mexicanus* **n. sp.** | 5.04 | 5.11 | 5.02 | 5.19 | 4.29 | 4.95–5.03 | 4.38 | 5.83–5.92 | 5.67 | 0.65 | **–/0–0.53** | 10.72–11.23 |
| 12 | *Xiha fastigata* | 5.12 | 5.10 | 5.02 | 5.43 | 4.05 | 5.67–5.75 | 4.53 | 5.02–5.10 | 5.26 | 5.59 | 5.51 | – |

0.005–0.006 with LSU and from 5.57 to 11.73 % ± 0.011–0.017 with ITS2 (see Table 4). The genetic divergence between the two species of *Ekuarhuni* was 0.65 and 1.34% ± 0.002 and 0.006 in LSU and ITS2, respectively. The genetic divergence between the two lineages of *Overstreetoides* was 1.7 and 2.89% ± 0.003 and 0.008 in LSU and ITS2, respectively. Finally, the genetic divergence among the seven diminutive morphotype species of *Forticulcita* ranged from 0.42 to 3.57% ± 0.001–0.005 with LSU and from 1.13 to 6.95% ± 0.005–0.012 with ITS2 (Table 4).

**Key to the species of the Forticulcitinae** Blasco-Costa et al., 2009a.

1a. Oval body with muscular excretory vesicle… ……..... ***Overstreetoides***

**Andrade-Gómez & García-Varela,** 2021

***Overstreetoides pacificus* Andrade-Gómez & García-Varela**, 2021

Remarks: *O. pacificus* is the only species described, however, a second lineage was recognized in this study. In order to describe it, new material is needed to be collected.

1b. Body elongate to fusiform ……..... (2)

2a. Hermaphroditic sac armed with spines. …… ……..……***Xiha* Andres, Curran, Fayton, Pulis & Overstreet, 2015**

2a, 1. Distributed in Atlantic coasts of North America.…….. ……***Xiha fastigata* (Thatcher & Sparks, 1958)**

2a, 2. Distributed in Pacific coasts of South America ……***Xiha fragilis* (Fernández-Bargiela, 1987)**

Remarks: Lack of material of *Xiha fragilis* (Fernández-Bargiela, 1987) precludes the correct diagnosis of the species. Andres et al. (2015) pointed out that more specimens are needed to corroborate the species status.

2b. Hermaphroditic sac unarmed of spines…… …… ..... (3)

3a. Conspicuous glands in the forebody, at pharynx level, crossing dorsally to oral sucker…… ……..…… ***Ekuarhuni* Andrade-Gómez & García-Varela,** 2021

3a, 1. Hermaphroditic sac length < 135 µm; and testis width < 90 µm …… ……..…… ***Ekuarhuni papillatum* Andrade-Gómez & García-Varela,** 2021

3a, 2. Hermaphroditic sac length > 135 µm; and testis width > 90 µm …… …… ***Ekuarhuni mexicanus* n. sp.**

3b. Lack of conspicuous glands in the forebody…... ***Forticulcita* Overstreet,** 1982

3b, 1. Body size > 1100 µm length with numerous eggs relatively small filling most hindbody (robust morphotype).……... ……..…..…. ..... (4)

3b, 2. Body size < 1100 µm length relatively few large eggs present in the uterus confined mostly in the hindbody (diminutive morphotype)…… ……..…..... (5)

4a. Body size < 1800 µm length; and forebody proportion < 25% .....…… …… ***Forticulcita glabra* Overstreet,** 1982

4b. Body size > 1800 µm length; and forebody proportion > 25% …… ….. …… ***Forticulcita mugilis* Hassanine, 2007**

5a. Post-testicular space > 45% of body length and distributed in Mediterranean Sea …… ***Forticulcita gibsoni* Blasco-Costa et al.**, 2009b

5b. Post-testicular space < 45% of body length and distributed in coasts of America …… ……..…. (6)

6a. Small body < 350 µm length with a short oesophagus < 60 µm …… …… ***Forticulcita minuta* Andrade-Gómez & García-Varela**, 2021

6b. Body > 350 µm length with a relatively long oesophagus > 60 µm …….…… (7)

7a. Small ovary (< 45 µm length; < 30 µm width) ……...… ***Forticulcita apiensis* Andres, Curran, Fayton, Pulis & Overstreet, 2015**

7b. Ovary large (> 45 µm length; > 30 µm width) ……..……..…… (8)

8a. The width of the body (>230 µm), pharynx (> 60 µm) and hermaphroditic sac (> 100 µm) are big ……..……..……(9)

8b. The width of the body (<230 µm), pharynx (< 60 µm), and hermaphroditic sac (< 100 µm) are relatively thin …….... (10)

9a. Very muscular pharynx, with a big ratio of oral sucker width to pharynx width (> 0.9) and a small testis (< 130 µm length) …..... ……..***Forticulcita macropharyngis* n. sp.**

9b. Simple pharynx, with a ratio of oral sucker width to pharynx width (< 0.9) and a big

testis (> 130 µm length) …… ……..…… ***Forticulcita venezuelensis* n. sp.**

10a. Oral sucker big (> 70 µm, in length and width) and with a hermaphroditic duct lined with gland cells. ……..…… ……***Forticulcita platana* Andres, Curran, Fayton, Pulis & Overstreet, 2015**

10b. Oral sucker small (<70 µm, in length and width) and with a hermaphroditic duct with no gland cells. ……..…***Forticulcita isabelae* Andrade-Gómez & García-Varela,** 2021

Remarks: *Forticulcita glabra* Overstreet, 1982 is the type species described from Mediterranean Sea (Overstreet, 1982). No sequences of the robust morphotype are available. Andres et al. (2015) mentioned that these two species could represent a true *Forticulcita.* We based the key on the original descriptions.

## Discussion

Members of Forticulcitinae are considered to be typical components of the helminth fauna of mugilid fishes, distributed mostly in the coastal systems of the Americas (Choudhury et al., 2017). This subfamily was described by Blasco-Costa et al. (2009a) based on the morphological and molecular data of three species; a few years later, the diversity increased significantly (Andrade-Gómez & García-Varela, 2021; Andres et al., 2015). With the inclusion of the three new species described herein, the subfamily now contains 14 species.

The current study represents the continuation of our effort to uncover the diversity of Forticulcitinae species that inhabit mugilid fishes over a wide geographic range. The phylogenetic analyses inferred with the combined dataset (LSU + ITS2) examining two nuclear molecular markers recovered four new lineages of Forticulcitinae species from the Gulf of Mexico and Atlantic Ocean slopes. A detailed morphological study of our specimens allowed us to describe three of the four lineages found, i.e., *Ekuarhuni mexicanus* **n. sp.**, *Forticulcita macropharyngis* **n. sp.**, and *F. venezuelensis* **n. sp.** The genetic divergences found among the species were similar to those reported by Andres et al. (2015) and Andrade-Gómez and García-Varela (2021); the latter found the highest divergence values among the genera of Forticulcitinae (5.7–11.9% for LSU and 7.3–14.6% for ITS2); however, with the inclusion of new lineages, the values obtained herein were lower. For instance, the genetic divergence between *Ekuarhuni mexicanus* **n. sp.** and *Ekuarhuni papillatum* was 0.65 and 1.34–1.6% for the markers LSU and ITS2, respectively. These values were very similar to those reported by Andres et al. (2015) between *F. gibsoni* and *F. platana*; the authors obtained values of 0.4 and 1.5% for LSU and ITS2, respectively. Likewise, the genetic divergence among *F. venezuelensis* **n. sp.**, and the other *Forticulcita* spp. ranged between 1.46 and 3.57 and between 3.67 and 5.88% for LSU and ITS2, respectively. For *F. macropharyngis* **n. sp.**, the interspecific genetic divergence found was 2.27–3.57 and 5.07–6.9% for LSU and ITS2, respectively. Moreover, the undescribed species of *Overstreetoides* and *O. pacificus* ranged from 1.7–1.78 and 2.62–2.89% for LSU and ITS2, respectively. These values were consistent with those previously reported and upheld the validity of the three new species, as well as the independent lineage of *Overstreetoides*.

The phylogenetic analysis presented by Andrade-Gómez and García-Varela (2021) estimated that Waretrematinae Srivastava, 1937 was the sister group to Forticulcitinae. In contrast, the present analysis (Fig. 5) did not resolve the placement of this subfamily relative, as observed by Andres et al. (2015). *Saccocoelioides nanii* Szidat, 1954, representing Chalcinotrematinae Overstreet & Curran, 2005, and *Capitimitta darwinensis* Pulis & Overstreet, 2013 representing Waretrematinae, formed a polytomy. This could be explained by the fact that many haploporid species were absent in the current analysis.

The phylogenetic analysis inferred with the combined (LSU + ITS2) dataset placed *Xiha fastigata* as sister to the remaining species of the subfamily, and this result agreed with previous phylogenetic studies suggesting that Forticulcitinae might have a New World origin (Andrade-Gómez & García-Varela, 2021; Andres et al., 2015). With the inclusion of the new species, the phylogenetic tree yielded that *Ekuarhuni* formed a clade that is sister to another subclade with moderate support (0.69/76) formed by *Overstreetoides* and *Forticulcita* (see Fig. 5). Likewise, the phylogenetic trees revealed that the species from Middle America yield a biogeographical pattern. For instance, species distributed along the shoreline coast of the Pacific Ocean, are sister taxa with species distributed on the Gulf of Mexico and Atlantic Ocean

slopes i.e., *Forticulcita minuta* nested with *Forticulcita venezuelensis* **n. sp.**; *Ekuarhuni papillatum* nested with *Ekuarhuni mexicanus* **n. sp.**; and *Overstreetoides pacificus* nested with *Overstreetoides* sp. The same pattern of distribution in sister species of helminths associated with freshwater and marine mullets from Middle America has been documented recently in digeneas of the genus *Pseudoparacreptotrema* Pérez-Ponce de León, Pinacho-Pinacho, Mendoza-Garfias, Choudhury & García-Varela, 2016 and acanthocephalans of the genus *Floridosentis* Ward, 1953 (see Pérez-Ponce de León et al., 2020; Rosas-Valdez et al., 2020). Our results suggested that the complete closure of the Isthmus of Panama, which occurred at approximately 5.5–3.1 Mya (Coates & Obando, 1996), was the principal barrier that prevented migration and consequent gene flow among the populations, as observed in different lineages of Metazoa (Lessios, 2008). This vicariant event might have favoured the diversification of the Forticulcitinae species in the Americas, with a secondary colonization event occurring in Mediterranean Sea, likely by rafting (Andrade-Gómez & García-Varela, 2021; Andres et al., 2015).

In the Gulf of Mexico, the species *Ekuarhuni mexicanus* **n. sp.**, *Forticulcita macropharyngis* **n. sp.**, and *Overstreetoides* sp. were found in both studied species of mullet in five of the 13 sampled localities (4–8 localities, Fig. 1). The abundance of each new species was different in each of the five localities (Bush et al., 1997), with *Forticulcita macropharyngis* **n. sp.**, being the most abundant (0.78), followed by *Ekuarhuni mexicanus* **n. sp.** (0.52) and *Overstreetoides* sp. (0.07). The five positive localities with confirmed infections are part of two of the largest freshwater systems exiting into the Gulf of Mexico (the Papaloapan River and Coatzacoalcos River); near of the Gulf of Mexico, these freshwater systems form coastal lagoons (González-Ramírez & Parés-Sierra, 2019). It is likely that these brackish systems are fundamental to completing the life cycle of the Forticulcitinae species. Although the complete life cycles of the *Forticulcita*, *Ekuarhuni* and *Overstreetoides* species are unknown, available evidence from other members of the subfamily, such as *Xiha fragilis* (Fernández-Bargiela, 1987) (recorded as *Dicrogaster fastigatus* by Lado et al., 2013), reported that adult worms live and reproduce sexually after 20 days in the digestive tracts of mullets that serve as definitive hosts. Eggs containing miracidium with eye spots hatch within the uteruses of trematodes, suggesting that free miracidium penetrates the intermediate host, the snail *Heleobia conexa* Gaillard (Cochliopidae: Rissooidea), and the miracidium develops into a redia with several cercariae. The cercariae are released and encysted with the help of a structure called "caudal filament" that allows the cercariae to stick to aquatic vegetation. Juvenile mullets that inhabit estuarine nursery areas (Whitfield et al., 2012) feed on aquatic vegetation to complete their life cycles. Overstreet and Curran (2005) pointed out that snails belonging to the superfamily Rissooidae are the likely the first intermediate host of haploporids. In the Gulf of Mexico, the genus *Heleobia* Stimpson has not been recorded; however, 15 species belonging to Cochliopidae Tryon have been reported (Czaja et al., 2020). These snails might contain members of Forticulcitinae at the larvae stage, and further studies focusing on these snails are needed to understand the life cycle and distribution of Forticulcitinae species in the Americas.

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**Declarations**

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

**Ethical approval** Specimens in Mexico were collected under the Cartilla Nacional de Colector Científico (FAUT 0202) issued by the Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT), to MGV. All applicable international, and institutional guidelines for the use and care of animals were followed.

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## IV. DISCUSIÓN GENERAL.

En la presente tesis se realizó un estudio helmintológico de dos especies de mugílidos, *Mugil curema* y *Mugil cephalus* en 42 localidades de México; además se incluyeron muestras de Costa Rica y Venezuela. Se colectaron digéneos adultos de las familias Haplosplanchnidae, Monorchiidae, y Haploporidae. En este proyecto nos enfocamos específicamente en describir la composición taxonómica de los haplopóridos asociados a dos especies de mugílidos. Overstreet y Curran (2005) mencionaron que los miembros de la familia Haploporidae presentan un tegumento extremadamente delicado, y al ser de tamaño pequeño son degradados rápidamente una vez que el huésped muere. Esto resultó en un desafío metodológico para la colecta de los especímenes, no obstante, esto se resolvió manteniendo a los peces vivos hasta su estudio helmintológico. Asimismo, otro desafío metodológico fue la presencia de infecciones mezcladas de haplopóridos, es decir, en algunas localidades encontramos hasta tres linajes de haplopóridos. Por lo tanto, para la fijación de los ejemplares seguimos la metodología propuesta por Overstreet y Curran (2005), quienes recomendaron emplear agua destilada a punto de ebullición para sacrificar a los organismos y posteriormente fijarlos en etanol absoluto. Este método permite revisar las características morfológicas de los individuos y procesarlos para la extracción del DNA genómico. Además, este método permitió obtener fotografías de los ejemplares (*fotogenóforos*) (Andrade-Gómez y García-Varela 2021) los cuales son análogos a los *hologenóforos* (Astrin et al. 2013). Con esta metodología pudimos vincular la morfología de cada organismo procesado con sus respectivos datos moleculares.

## IV. I. Composición taxonómica de Chalcinotrematinae en *Mugil* spp. de México

La subfamilia Chalcinotrematinae Overstreet y Curran 2005, está conformada por haplopóridos parásitos principalmente de peces dulceacuícolas distribuidos en América (Overstreet y Curran 2005). Se caracterizan morfológicamente por presentar folículos vitelinos irregulares en forma y tamaño dispuestos alrededor de las gónadas, así como en la parte posterior del cuerpo. Esta subfamilia la integran seis géneros, *Chalcinotrema* Texeira de Freitas 1947, *Paralecithobotrys* Freitas 1948*, Megacoelium* Szidat 1954*, Unicoelium* Thatcher y Dossman 1975, *Intromugil* Overstreet y Curran 2005 y *Saccocoelioides* Szidat 1954 (Curran et al. 2018). Los primeros cuatro géneros parasitan peces dulceacuícolas, mientras que las cinco especies reportadas del género *Intromugil* están asociadas a peces del género *Mugil* en Brasil, Venezuela y Estados Unidos. Por otro lado, el género *Saccocoelioides* tiene algunos miembros que son capaces de parasitar a mugílidos, así como a otras 11 familias de peces dulceacuícolas. En el presente estudio registramos miembros del género *Saccocoelioides.*

Antes del presente trabajo, dos registros del género *Saccocoelioides* habían sido reportados en México asociados a *Mugil* spp. El primero corresponde con *S. overstreeti* Fernández-Bargiela, 1987 en *Mugil cephalus* de una localidad en Jalisco y el segundo con *S. beauforti* (Hunter y Thomas, 1961) Overstreet, 1971 en *Mugil curema* de Tabasco (Cabañas-Carranza, 2001; López-Jiménez, 2001). En el presente estudio se analizaron dos especies de mugílidos, *Mugil cephalus* y *Mugil curema* en 13 localidades del Golfo de México y Océano Pacífico en México. En el primer artículo derivado de la tesis, se describió *Saccocoelioides macrospinosus* de *Poecilia catemaconis* Miller (Poeciliidae) y de *Mugil curema* de Catemaco y Alvarado, Veracruz. Esta es la especie número 24 descrita del género, todas distribuidas en América, siendo el género más diverso de la familia. Asimismo, *S. macrospinosus* es la cuarta especie descrita del género en México,

después de *Saccocoelioides chauhani* Lamothe-Argumedo-1974*, Saccocoelioides lamothei* y *Saccocoelioides olmecae* Andrade-Gómez, Pinacho-Pinacho, Hernández-Orts, Sereno-Uribe y García-Varela, 2017 los cuales son parásitos de peces charácidos y eleótridos, respectivamente. Por otra parte, en este artículo registramos por primera vez a *Saccocoelioides orosiensis* Curran, Pulis, Andres y Overstreet, 2018 en *Mugil curema* en Montepio, Veracruz. Esta es la cuarta especie del género *Saccocoelioides* asociada a peces del género *Mugil.* Las otras especies de *Saccocoelioides* que se han registrado en estos mugílidos son, *Saccocoelioides overstreeti, S. beauforti*, y *S. macrospinosus* en Chile, Estados Unidos y México, respectivamente (Overstreet y Curran 2005; Curran et al. 2018).

*Saccocoelioides orosiensis* es una especie descrita en *Poecilia gillii* (Kner) en Costa Rica, y ha sido ampliamente registrada en localidades de Nicaragua, Honduras y México asociada a peces de las familias Poeciliidae y Cichlidae (Curran et al. 2018). En el presente trabajo se encontró por primera vez en peces de la familia Mugilidae. Por lo tanto, *S. orosiensis* posee un amplio espectro hospedatorio ya que es capaz de parasitar tres familias de peces no relacionadas. Cabe destacar que la mayoría de estos registros son en vertientes del Atlántico y Golfo de México (Andrade-Gómez et al. 2017; Curran et al. 2018).

En el segundo artículo derivado de la tesis, se realizó un estudio integrativo evaluando características morfológicas y moleculares, particularmente el gen de la subunidad mayor del DNA ribosomal (28S) y dos genes mitocondriales, el citocromo oxidasa subunidad 1 (*cox1*) y la deshidrogenasa subunidad 1 (*nad1*) de especímenes identificados como *Saccocoelioides* sp. en 29 localidades de las costas del Océano Pacífico en México, Guatemala, El Salvador, Honduras, Nicaragua y Costa Rica de cinco familias de huéspedes, Mugilidae, Eleotridae, Gobiidae, Poeciliidae y Profundulidae. Los

análisis mostraron que todos los individuos colectados corresponden a una sola especie, *Saccocoelioides lamothei*. Esta especie fue descrita del eleótrido *Dormitator latifrons* (Richardson) en Guerrero, México. La nueva evidencia sugiere que esta especie se encuentra ampliamente distribuida en vertientes del Océano Pacífico, y es capaz de parasitar cinco familias de peces. La evidencia generada sugiere que esta especie presenta una plasticidad fenotípica inducida por los huéspedes. Particularmente, los especímenes de *S. lamothei* colectados de peces del género *Mugil* se obtuvieron en 12 de las 29 localidades, en México y Guatemala. Los datos sugieren que este haplopórido utiliza a los peces del género *Mugil* como un puente ecológico permitiendo dispersar y parasitar a especies de peces de agua dulce y de esta forma dispersarse. Esto se observa en la red de haplotipos (Publicación 2; Fig. 5), el haplotipo CB se encuentra ampliamente distribuido, y los individuos asociados a este haplotipo fueron colectados en mugílidos y eleótridos (Publicación 2; Fig. 4), lo que sugiere que estos peces juegan un papel clave en la dispersión del parásito.

Con respecto a los registros previos de *Saccocoelioides overstreeti* y *S. beauforti* en Tabasco y Jalisco, los resultados encontrados en la presente tesis no soportan la validez de estos. *Saccocoelioides overstreeti* es una especie que se describió en *Mugil cephalus* de Chile (Bargiela 1987). Sin embargo, faltan estudios moleculares para corroborar su estatus, así como su distribución. Mientras que *Saccocoelioides beauforti* fue descrita en *Mugil cephalus* en aguas costeras del sureste de Estados Unidos, y recientemente analizada por Curran et al. (2018). Con base en nuestros resultados, no encontramos evidencia que *Saccocoelioides beauforti* se encuentre distribuida en México. Además, no existen ejemplares depositados (*voucher*) en la CNHE que nos permita corroborar la identificación de las especies previamente registradas. En este sentido, es indispensable que, en futuros estudios helmintológicos, se depositen ejemplares en colecciones

nacionales con el propósito de contar con ejemplares disponibles para corroborar y comparar las identidades taxonómicas. Por lo tanto, los datos obtenidos en la presente tesis sugieren que, en México las especies de *Saccocoelioides* asociadas a *Mugil* spp. son, *S. lamothei* en vertientes del Océano Pacífico; *Saccocoelioides macrospinosus* y *S. orosiensis* en costas del Golfo de México.

**IV. II. Composición taxonómica de Forticulcitinae en *Mugil* spp. de México**

La subfamilia Forticulcitinae Blasco-Costa, Balbuena, Kostadinova y Olson, 2009, está conformada por haplopóridos parásitos de mugílidos distribuidos globalmente. Los miembros de esta subfamilia se caracterizan morfológicamente por presentar un solo folículo vitelino circular en la parte media del cuerpo (Blasco-Costa et al. 2009). Previo a este estudio, la subfamilia la integraban cinco especies de *Forticulcita* Overstreet, 1982 y dos especies de *Xiha* Andres, Curran, Fayton, Pulis y Overstreet, 2015 (Andres et al. 2015). En México se habían reportado tres registros de esta subfamilia, el primero corresponde con *Xiha fastigata* asociado a *Mugil cephalus* en el estado de Jalisco; y los otros dos corresponden con Forticulcitinae gen. sp. asociado a *Mugil curema* en los estados de Jalisco y Tabasco (Cabañas-Carranza 2001; Lira-Guerrero 1997).

En el tercer artículo derivado de la tesis, se analizaron 204 individuos de *Mugil* spp. en 27 localidades de la vertiente de Océano Pacífico de México, Guatemala y Costa Rica. En este estudio se analizaron características morfológicas internas, así como la ultraestructura de la superficie corporal utilizando microscopía electrónica de barrido; además de caracteres moleculares tales como los genes ribosomales (28S e ITS2) de los haplopóridos encontrados. Los resultados mostraron que existe una mayor diversidad de haplopóridos de la subfamilia Forticulcitinae con cuatro nuevas especies. Los *fotogenóforos* nos ayudaron a describir dos géneros nuevos de Forticulcitinae debido a

que pudimos vincular la morfología del parásito con la secuencia de DNA. Los géneros nuevos son, *Ekuarhuni* Andrade-Gómez y García-Varela, 2021 para *Ekuarhuni papillatum* Andrade-Gómez y García-Varela, 2021 y *Overstreetoides* Andrade-Gómez y García-Varela, 2021 para *Overstreetoides pacificus* Andrade-Gómez y García-Varela, 2021. Los dos géneros nuevos descritos presentan características diagnósticas de la subfamilia como la presencia de un solo folículo vitelino en la parte media del cuerpo. Sin embargo, estos especímenes presentaban características únicas que no se asemejaban con los géneros previos. Por ejemplo, *Ekuarhuni* presenta glándulas ubicadas dorsalmente a la ventosa oral, mientras que *Overstreetoides* presenta un cuerpo redondo y una vesícula excretora muscular. Además, la evidencia molecular apoya la asignación de dos nuevos géneros para la ciencia. Paralelamente en este trabajo se describieron otras dos especies del género *Forticulcita, F. isabelae* Andrade-Gómez y García-Varela, 2021 y *F. minuta* Andrade-Gómez y García-Varela, 2021.

En el cuarto artículo derivado de la tesis, se analizaron 99 individuos de *Mugil* spp. en 17 localidades con vertientes al Golfo México, así como en cuatro localidades del Océano Atlántico de Venezuela. En este artículo se describen tres nuevas especies de Forticulcitinae. La primera de ellas corresponde al género recientemente asignado, *Ekuarhuni* para la especie *E. mexicanus* Andrade-Gómez, González-García y García-Varela, 2021, y dos especies de *Forticulcita, F. macropharyngis* Andrade-Gómez, González-García y García-Varela, 2021, y *F. venezuelensis* Andrade-Gómez, González-García y García-Varela, 2021. Además, se reconoció un linaje del género *Overstreetoides* sp. que no se logró describir por falta de material. Cabe destacar que un aporte importante de este manuscrito fue la generación de una clave taxonómica de las 14 especies que componen a la subfamilia Forticulcitinae.

Los resultados generados en este proyecto revelaron una gran diversidad de especies de Forticulcitinae asociados a *Mugil* spp. en ambas costas de México. La nueva evidencia sugiere que en México las especies *Ekuarhuni papillatum, Overstreetoides pacificus, F. isabelae,* y *F. minuta* se distribuyen en vertientes del Océano Pacífico. Mientras que las especies *Ekuarhuni mexicanus, Forticulcita macropharyngis,* y *Overstreetoides* sp. se encuentran distribuidas en costas del Golfo de México, la mayoría en localidades de Veracruz. Además, con las nuevas especies descritas, la subfamilia Forticulcitinae incrementó de forma sustancial los miembros que la integran. De contar con siete especies, ahora la familia contiene 14 especies.

Estas 14 especies que integran a la subfamilia Forticulcitinae fueron descritas de cinco especies de mugílidos, que corresponden con los géneros *Crenimugil* y *Mugil*. Por ejemplo, *Forticulcita glabra* Overstreet, 1982 y *F. mugilis* Hassanine, 2007 fueron descritas de *Crenimugil seheli* (Forsskål) y *C. crenilabis* (Forsskål) en el Mar Rojo, respectivamente. *Forticulcita platana* fue descrita de *Mugil liza* en Argentina. *Forticulcita gibsoni, F. apiensis, Xiha fastigata,* y *X. fragilis* fueron descritas de *Mugil cephalus* en el Mar Mediterráneo, en Florida y Luisiana en el Golfo de México, y en Concepción, Chile, respectivamente. *Forticulcita isabelae, F. minuta, Ekuarhuni papillatum, Overstreetoides pacificus, Forticulcita macropharyngis,* y *Ekuarhuni mexicanus y F. venezuelensis,* fueron descritas de *Mugil curema* en México y Venezuela, las primero cuatro especies en costas con vertientes al Pacífico mexicano, las otras dos en costas con vertientes al Golfo de México, y la última en Venezuela. Como se observa, 12 de las 14 especies de forticulcitinos han sido descritas en mugílidos del género *Mugil.* La especie *Mugil curema* es la que mayor número alberga con siete especies descritas, seguido de *Mugil cephalus* con cuatro especies, y finalmente *Mugil liza* con solamente una. De las 12 especies asociadas a mugílidos del género *Mugil,* 11 de ellas fueron

descritas en América: tres en Suramérica (*Forticulcita venezuelensis, F. platana* y *Xiha fragilis*), seis en Centroamérica (*Forticulcita isabelae, F. minuta, F. macropharyngis, Ekuarhuni papillatum, E. mexicanus* y *Overstreetoides pacificus*), y dos en Norteamérica (*Forticulcita apiensis* y *Xiha fastigata*). Solamente, *Forticulcita gibsoni* asociada a *Mugil cephalus* fue descrita en el Mar Mediterráneo. Con excepción de *Xiha fragilis*, los análisis filogenéticos reportados en la presente tesis muestran las 11 especies mencionadas (Publicación 4, Fig. 5). En contraste, no existen datos moleculares para corroborar el estatus de las dos especies del morfotipo robusto de *Forticulcita* (*Forticulcita glabra* y *F. mugilis)*, las cuales fueron descritas de mugílidos del género *Crenimugil* en el Mar Rojo.

Con base en el árbol filogénetico (Publicación 4, Fig. 5), *Xiha fastigata* se muestra como grupo hermano del resto de las especies de forticulcitinos. El género *Ekuarhuni* es hermano del grupo conformado por *Forticulcita* y *Overstreetoides*. Como se mencionó previamente, *Forticulcita gibsoni* es la única especie distribuida en Europa que se incluyó en los análisis, debido a que es la única que tiene datos moleculares. Nuestros análisis (Publicación 4, Fig. 5) mostraron que la especie *Forticulcita gibsoni* es hermano de las especies *F. platana* y *F. isabelae,* estas especies tienen distribución en Argentina y México, respectivamente. Andres et al. (2015) obtuvieron datos similares a los de la presente tesis. Ellos indicaron que una posible explicación para que *Forticulcita gibsoni* se encuentre distribuida en Europa es mediante “balsas de vegetación acuática” (Thiel y Haye 2006), estas pudieron haber transportado al primer huésped intermediario; o bien las metacercarias de los haplopóridos que suelen enquistarse en la vegetación (Lado et al. 2013; Andres et al. 2015). Sin embargo, es necesario colectar y practicar un estudio helmintológico a mugílidos de las costas del occidente de África para corroborar esta

hipótesis. Es decir, si los forticulcitinos de América pueden ser transportados mediante "balsas de vegetación acuática" y encontrarse en esta región del mundo.

Hasta el momento, las 14 especies de forticulcitinos han sido descritas en cinco especies de mugílidos, dos corresponden al género *Crenimugil* y tres de *Mugil*. En la filogenia de la familia Mugilidae, la cual está conformada por 4 subfamilias, se muestra que *Crenimugil* y *Mugil* corresponden a subfamilias diferentes (ver Xia et al. 2016). El género *Mugil* corresponde con la subfamilia Mugilinae y *Crenimugil* con Rhinomugilinae, este último género posee una distribución principalmente en costas de Europa, África y Asia (Durand y Borsa 2015; Xia et al. 2016). La filogenia de los mugilídos muestra que la subfamilia Mugilinae, es hermano del grupo formado por las subfamilias Rhinomugilinae y Cheloninae (Xia et al. 2016). Esta evidencia aunada a los datos que nosotros obtuvimos sugiere que las especies del género *Mugil* (Mugilinae) posiblemente originaron a los parásitos de la subfamilia Forticulcitinae. Posteriormente, un segundo evento de colonización o especiación ocurrió en los peces del género *Crenimugil* (Rhinomugilinae). Esta hipótesis coincide con los datos que nosotros obtuvimos, debido a que el género *Xiha,* el cual es parásito de mugílidos del género *Mugil,* es grupo hermano del resto de los forticulcitinos (Publicación 4, Fig. 5). Sin embargo, para corroborar esta hipótesis, son indispensables datos moleculares de las especies de morfotipo robusto de *Forticulcita*, debido a que estas son las que están asociadas a mugilídos del género *Crenimugil.* Además, con estos datos, se podrán resolver dos preguntas de sumo interés dentro de la subfamilia. La primera ayudará a comprender el patrón de radiación dentro de la subfamilia; y, por otra parte, se podrá corroborar el estatus del género *Forticulcita*. En este sentido, se ha sugerido que las dos especies del morfotipo robusto corresponden con una "verdadera" *Forticulcita* (Andres et al. 2015). Si las dos especies del morfotipo robusto resultan parafiléticas, se requerirá un re-arreglo

taxonómico. Es decir, las cinco especies del morfotipo diminuto de *Forticulcita*, incluyendo las que se describen en la presente tesis podrían corresponder a otro género aún sin describir.

En la presente tesis se identificaron a los huéspedes, *Mugil cephalus* y *M. curema*, ambas especies corresponden a dos complejos de especies, las cuales tienen una distribución global (Durand y Borsa 2015). Dentro del complejo de especie de *M. cephalus* se han reconocido 13 linajes, de los cuales dos linajes están reportados en México, uno en las costas del Pacífico mexicano; y el otro linaje en vertientes del Golfo de México; y éstos se encuentran estrechamente relacionados (Durand y Borsa 2015; Neves et al. 2020). Mientras que el complejo de especies de *M. curema*, se han reconocido tres linajes, de los cuales dos están reportados en México con el mismo patrón que *M. cephalus,* es decir, uno en las costas del Pacífico mexicano; y el otro linaje en vertientes al Golfo de México; y también se encuentran estrechamente relacionados (Durand y Borsa 2015; Neves et al. 2020). Esta evidencia coincide con nuestros resultados debido a que encontramos linajes hermanos de forticulcitinos en ambas costas de México. Es decir, *Forticulcita minuta, F. isabelae, Ekuarhuni papillatum* y *Overstreetoides pacificus* están asociados a los linajes de los complejos de especie, tanto de *Mugil cephalus* y *M. curema* distribuidos en las vertientes del Pacifico mexicano; estos parásitos son grupo hermano respectivamente con *Forticulcita macropharyngis*, *Ekuarhuni mexicanum*, y *Overstreetoides* sp., los cuales son parásitos de los linajes de los complejos de especie, tanto de *M. cephalus* y *M. curema* distribuidos en el Golfo de México. Esto coincide con lo reportado por Rosas-Valdez et al. (2020), donde encontraron el mismo patrón en acantocéfalos del género *Floridosentis* Ward 1953 asociados a *Mugil curema* y *M. cephalus* en costas de México, Guatemala, El Salvador, Costa Rica y Venezuela. Estos

autores reportaron hasta cinco linajes del género *Floridosentis* asociados a ambas especies de mugilídos.

Aunado a lo anterior, nuestros resultados se asemejan a lo reportado en monogéneos del género *Ligophorus* Euzet y Suriano, 1977; donde se han descrito más de 50 especies y muchas de éstas en el Mar Mediterráneo parásitos de mugilídos (Blasco-Costa et al. 2012). Se ha observado que muchas de las especies de *Ligophorus* se encuentran co-existiendo, por ejemplo, en *Mugil liza* de Brasil se describieron cuatro especies de *Ligophorus*; los cuales fueron los primeros registros de este género para el país (Abdallah et al. 2009). Este sistema es similar al nuestro, es decir, varias especies de parásitos estrechamente relacionadas asociadas a una especie de mugílido. Blasco-Costa et al. (2012) señalaron que la duplicación dentro del huésped, así como el cambio de huésped pudieron haber contribuido a la diversificación de *Ligophorus*. En este sentido, la gran diversidad de forticulcitinos observada sugiere que los modos de especiación de éstos pudieron haber ocurrido de forma similar a *Ligophorus.* Para reconstruir un escenario de diversificación para los representantes de los forticulcitinos y para verificar los posibles eventos de co-especiación, se podrían enfocar estudios en análisis cofilogenéticos de los forticulcitinos y sus huéspedes.

Por otra parte, los resultados encontrados en la presente tesis no soportan la validez del registro previo de *Xiha fastigata* en *Mugil cephalus* de Jalisco (Cabañas-Carranza 2001). El género *Xiha* se caracteriza morfológicamente por presentar espinas en el ducto hermafrodita. Los ejemplares que nosotros colectamos no presentaban esta característica. Las secuencias generadas de los diferentes especímenes del presente trabajo no se agruparon con *Xiha fastigata*, lo que sugiere que esta especie no se encuentra distribuida en México. Por lo tanto, los datos obtenidos en la presente tesis sugieren que estos registros deberían ser considerados como Forticulcitinae gen. sp., ya que pueden

corresponder a las especies de los géneros *Ekuarhuni, Overstreetoides* o *Forticulcita*. Mientras que los registros de Forticulcitinae gen. sp. en *Mugil curema* de Jalisco y Tabasco de Lira-Guerrero (1997) y López-Jiménez (1999) no se lograron identificar a nivel de especie debido a la ausencia de vouchers depositados en una colección para corroborar su identidad taxonómica.

En la presente tesis se sustenta la relación ecológica y evolutiva de los haplopóridos con los mugílidos. La familia Haploporidae está estrechamente relacionada con los mugílidos debido a que cinco de las ocho subfamilias de haplóporidos parasitan a estos peces. Los registros previos sugieren que más del 50% de especies de haplopóridos parasitan mugilídos (Overstreet y Curran 2005), lo que muestra una estrecha asociación parásito- huésped.

En México, la diversidad de este grupo de parásitos era desconocida debido al tamaño y las características inherentes de la familia. Sin embargo, con la nueva información generada en la presente tesis, ahora conocemos un poco más sobre la diversidad de haplopóridos en *Mugil* spp. Consideramos, que futuras investigaciones deben estar dirigidas en conocer el ciclo de vida de estos parásitos. Los primeros estudios podrían estar enfocados en las cinco localidades positivas con infecciones en el Golfo de México. Estas localidades forman parte de dos de los sistemas de agua dulce más grandes que vierten al Golfo de México, el río Papaloapan y el río Coatzacoalcos (González-Ramírez y Parés-Sierra 2019). Estos sistemas de agua dulce forman lagunas costeras los cuales son probablemente indispensables para completar el ciclo de vida de las especies de Forticulcitinae. Aunado a lo anterior, Overstreet y Curran (2005) señalaron que los caracoles pertenecientes a la superfamilia Rissooidae son probablemente el primer huésped intermediario de los haplopóridos. En México, se han reportado 15 especies de la familia Cochliopidae Tryon (Rissooidae) (Czaja et al. 2020). Aparentemente estos

caracoles pueden contener los estadios larvales de los miembros de Forticulcitinae. Por lo tanto, es indispensable concentrar los estudios en estos caracoles debido a que los primeros estadios larvales se alojan dentro de éstos. Esto permitirá comprender el ciclo de vida, así como la distribución de las especies de Forticulcitinae en México y América. De manera general, se detectó una diversidad de haplopóridos asociados a los dos complejos de especie tanto, *Mugil curema* y *Mugil cephalus*, sugiriendo que las poblaciones de los mugílidos del Golfo de México y del Pacifico poseen sus propios parásitos.

**IV. III. Perspectiva de la asociación parásito-huésped**

En la presente tesis se realizó un estudio integrativo incorporando diferentes fuentes de evidencia como datos morfológicos, moleculares, geográficos, y ecológicos con el objetivo de describir la diversidad de haplopóridos en mugilídos (Cuadro 3). Como se mencionó previamente, las dos subfamilias de haplopóridos encontradas fueron Chalcinotrematinae y Forticulcitinae.

Las especies analizadas en la presente tesis de la subfamilia Chalcinotrematinae corresponden con el género *Saccocoelioides*. De las 24 especies registradas hasta el momento, 17 de ellas están asociadas a peces estrictamente dulceacuícolas, mientras que las otras siete especies se han registrado también en peces salobre. Dos de las tres especies analizadas de *Saccocoelioides* en la presente tesis son de ambientes tanto dulceacuícolas como salobres, solamente *Saccocoelioides tkachi* es de peces estrictamente dulceacuícolas (Cuadro 3). Los datos publicados en la presente tesis sugieren que al menos *S. lamothei* ha sido transportado mediante mugílidos, los cuales al ser peces eurihalinos y euritermos pueden moverse a través de ambientes salobres hacia dulceacuícolas. Esto generaría que los parásitos puedan colonizar nuevos huéspedes. A

su vez, podría explicar porqué esta subfamilia de haplopóridos es la única que se encuentra distribuida en peces dulceacuícolas. Por otro lado, las especies analizadas de la subfamilia Forticulcitinae corresponden con 3 géneros, *Overstreetoides, Ekuarhuni,* y *Forticulcita* (Cuadro 3). En la presente tesis se analizaron siete de las 14 especies de esta subfamilia, sin embargo, todos los forticulcitinos han sido registrados en mugílidos, es decir, peces salobres.

En parásitos existen diferentes hipótesis sobre la forma en cómo divergen éstos. Una de ellas, es la regla de Fahrenholz, la cual indica que la filogenia del parásito debe ser un espejo de la filogenia del huésped, es decir, una co-especiación estricta (Eichler 1948). En el presente trabajo no se realizó un estudio filogenético sobre los huéspedes, no obstante, filogenias publicadas sobre ellos existen. En la filogenia presentada por Betancur et al. (2017) se observa que los mugilídos son grupo hermano de la familia Embiotocidae (Peces marinos). Por otro lado, peces estrictamente dulceacuícolas como *Astyanax aeneus*, que alberga a *S. tkachi,* no están estrechamente relacionadas con los mugilídos. Este mismo patrón, lo observamos con los otros huéspedes analizados en la presente tesis, como eleotridos, gobiidos, poeciliidos, y profundulidos, es decir, que no están estrechamente relacionadas con los mugilídos. Esto apoya que los miembros de la subfamilia Chalcinotrematinae que parasitan principalmente peces dulceacuícolas, han sido transportados por los mugilídos y que mediante transferencia horizontal han podido colonizar nuevos huéspedes y que en ellos han divergido especies de esta subfamilia. Esto se puede asumir porque no existe una concordancia filogenética entre los huéspedes y parásitos, es decir, una co-especiación estricta. Este tipo de evidencia es la que usualmente se ha reportado, es decir, una transferencia horizontal de parásitos (Bell et al. 2021).

En contraste, los miembros de la subfamilia Forticulcitinae parasitan exclusivamente a mugilídos, la filogenia dentro de los mugílidos (Xia et al. 2016) aunado a los datos de forticulcitinos generados hasta el momento sugiere que podría existir una concordancia filogenética entre mugilídos y forticulcitinos. Sin embargo, aun quedan varias especies de lisas distribuidas globalmente que no han sido objeto de estudio helmintológico. Una vez que se obtengan más datos generados sobre los forticulcitinos en este grupo de peces se podría poner a prueba la hipótesis de co-especiación entre ellos.

Cuadro 3. Especies analizadas bajo un enfoque integrativo durante el proyecto doctoral. * especies nuevas descritas.

| Especie de haplopórido | Huésped | Ambiente | Datos geográficos | Datos moleculares | Datos morfológicos |
|---|---|---|---|---|---|
| *Saccocoelioides tkachi* Curran, Pulis, Andres y Overstreet, 2018 | *Astyanax aeneus*<br>*A. fasciatus* | Dulceacuícola | En ríos de Costa Rica y Nicaragua | DNA ribosomal LSU, ITS2<br><br>DNA mitocondrial *cox1* | Glándulas vitelógenas irregulares en forma y tamaño. Tamaño del cuerpo menor a 1.7 mm. Forma alargada del cuerpo. La forma del ovario es subesférico. Distribución de las glándulas vitelógenas en la parte media y posterior del cuerpo. |
| *S. lamothei* Violante-Gónzalez y Aguirre-Macedo, 2008 | *Dormitator latifrons*<br>*Mugil cephalus*<br>*M. curema*<br>*Dajaus monticola*<br>*Poeciliopsis gracilis*<br>*Poecilia sphenops*<br>*P. gillii*<br>*P. mexicana*<br>*Sicydium multipunctatum*<br>*S. salvini*<br>*Awaous banana*<br>*Profundulus* sp. | Dulceacuícola y salobre | En costas y ríos con vertientes al Océano Pacífico en México, Guatemala, El Salvador, Honduras, Nicaragua y Costa Rica | DNA ribosomal LSU<br><br>DNA mitocondrial *cox1*, *nad1* | Glándulas vitelógenas irregulares en forma y tamaño distribuidas principalmente en la parte posterior del cuerpo. Los datos encontrados sugieren que el largo y ancho del cuerpo varían conforme a la familia de peces que parasitan. |

| | | | | | |
|---|---|---|---|---|---|
| *S. macrospinosus** | *Poecilia catemaconis*<br>*Mugil curema* | Dulceacuícola y salobre | En lago de Catemaco y costas de Veracruz | DNA ribosomal LSU, ITS2<br><br>DNA mitocondrial *cox1* | Glándulas vitelógenas irregulares en forma y tamaño. Se puede distinguir por la forma y tamaño del cuerpo 440–850 µm de largo y 120–245 µm de ancho. También se puede distinguir por el largo de la ventosa oral, así como el radio de las ventosas. |
| *Overstreetoides pacificus** | *Mugil curema*<br>*M. cephalus* | Salobre | En costas de México con vertientes al Océano Pacífico | DNA ribosomal LSU, ITS2 | Una sola glándula vitelógena esférica a mitad del cuerpo. Cuerpo ovalado con vesícula excretora muscularizada. |
| *Ekuarhuni papillatum** | *Mugil curema*<br>*M. cephalus* | Salobre | En costas de México, Guatemala y Costa Rica con vertientes al Océano Pacífico | DNA ribosomal LSU, ITS2 | Una sola glándula vitelógena esférica a mitad del cuerpo. Cuerpo elongado con glándulas conspicuas en la parte anterior del cuerpo con el saco hermafrodita y testículo pequeños. |
| *E. mexicanus** | *Mugil curema* | Salobre | En costas de Veracruz | DNA ribosomal LSU, ITS2 | Una sola glándula vitelógena esférica a mitad del cuerpo. Cuerpo elongado con glándulas conspicuas en la parte anterior del cuerpo con el saco hermafrodita y testículo grandes. |

| | | | | | |
|---|---|---|---|---|---|
| *Forticulcita minuta** | *Mugil curema*<br>*M. cephalus* | Salobre | En costas de México con vertientes al Océano Pacífico | DNA ribosomal LSU, ITS2 | Una sola glándula vitelógena esférica a mitad del cuerpo. Cuerpo elongado con el tamaño más pequeño. |
| *F. isabelae** | *Mugil curema*<br>*M. cephalus* | Salobre | En costas de México, Guatemala y Costa Rica con vertientes al Océano Pacífico | DNA ribosomal LSU, ITS2 | Una sola glándula vitelógena esférica a mitad del cuerpo. Cuerpo elongado con ovario grande, pero con un ancho del cuerpo, faringe, relativamente pequeños y con un saco hermafrodita sin glándulas. |
| *F. macropharyngis** | *Mugil curema*<br>*M. cephalus* | Salobre | En costas de Veracruz | DNA ribosomal LSU, ITS2 | Una sola glándula vitelógena esférica a mitad del cuerpo. Cuerpo elongado con ovario y ancho del cuerpo grandes y con una faringe masivamente muscular. |
| *F. venezuelensis** | *Mugil curema* | Salobre | En costas de Venezuela | DNA ribosomal LSU, ITS2 | Una sola glándula vitelógena esférica a mitad del cuerpo. Cuerpo elongado con ovario y ancho del cuerpo grandes y con un testículo testículo muy grande. |

## V. CONCLUSIONES GENERALES.

1. Los datos observados sugieren que existen infecciones mezcladas de especies de Forticulcitinae con diferentes valores de prevalencia y abundancia en ambas costas de México asociados a mugílidos.
2. La diversidad de haplopóridos es mayor de lo que se consideraba en México, particularmente en mugílidos. De conocer tres especies registradas en mugílidos, ahora se cuentan con nueve especies registradas en muglídos de México.
3. Se reconocieron siete nuevas especies de la subfamilia Forticulcitinae incrementando la diversidad de la subfamilia a 14 especies.
4. Se describieron dos géneros nuevos y siete nuevas especies de haplopóridos asociados a *Mugil curema* y *Mugil cephalus* en costas de México, y una especie más de Venezuela asociada a *Mugil curema*. Estas especies son, *Overstreetoides pacificus, Ekuarhuni papillatum, E. mexicanus, Forticulcita minuta, F. isabelae, F. macropharyngis, F. venezuelensis* y *Saccocoelioides macrospinosus.*
5. Las nuevas especies se describieron empleando un enfoque integrativo. Se utilizaron caracteres morfológicos, ultraestructurales en combinación con caracteres moleculares y datos geográficos.
6. Los marcadores moleculares ribosomales (28S e ITS2) mostraron información para esclarecer las relaciones filogenéticas dentro de la familia Haploporidae, así como para delimitar a las especies. Los genes mitocondriales (*cox1* y *nad1*) funcionan para evaluar las poblaciones de las especies en la familia Haploporidae.
7. Se detectó la presencia de un linaje de *Overstreetoides* sp., en Alvarado, Veracruz del Golfo de México que no pudo describirse por falta de ejemplares.
8. El género *Saccocoelioides* es el grupo más diverso de haplopóridos con 24 especies descritas en América asociadas a 12 familias de peces, Mugilidae,

Poeciliidae, Characidae, Cichlidae, Eleotridae, Gobiidae, Prochilodontidae, Anostomidae, Curimatidae, Loricariidae, Goodeidae y Profundulidae.

9. Se registró por primera vez la presencia de *Saccocoelioides orosiensis* en *Mugil curema* (Mugilidae) de Montepio, Veracruz, siendo la tercera familia de peces que se reporta de este haplopórido. La mayoría de los registros de esta especie son en vertientes del Atlántico y Golfo de México en Costa Rica, Nicaragua, Honduras y México asociados peces de la familia Poeciliidae y Cichlidae.
10. *Saccocoelioides lamothei* es la especie que parasita al mayor número de familias de peces, Mugilidae, Eleotridae, Poeciliidae, Gobiidae, y Profundulidae en vertientes al Océano Pacífico en cinco países, México, Guatemala, Honduras, Nicaragua y Costa Rica.
11. Las redes de haplotipo sugieren que los mugílidos funcionan como un puente ecológico debido que pueden dispersar a algunos parásitos a diferentes huéspedes que habitan ambientes dulceacuícolas, salobre y marinos. Asimismo, la información recopilada sugiere que estos peces han ayudado a la diversificación de los haplopóridos.

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## VII. Apéndice.

Artículos publicados como becario de doctorado por CONACYT (CVU. 640068) y financiados por el Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-IN207219) que no forman parte de la tesis. Sin embargo, fueron realizados de forma paralela y colaborativa durante este período.

### VII. I. Exploring the genetic diversity of *Tylodelphys* (Diesing, 1850) metacercariae in the cranial and body cavities of Mexican freshwater fishes using nuclear and mitochondrial DNA sequences, with the description of a new species. En *Parasitology Research.*

Ana Lucia Sereno-Uribe, **Leopoldo Andrade-Gómez**, Gerardo Pérez-Ponce de León, Martín García-Varela

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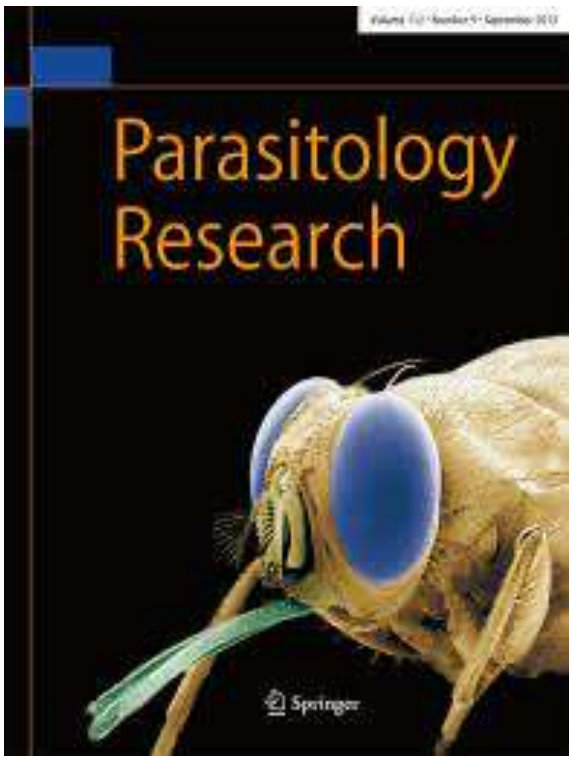

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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

# Exploring the genetic diversity of *Tylodelphys* (Diesing, 1850) metacercariae in the cranial and body cavities of Mexican freshwater fishes using nuclear and mitochondrial DNA sequences, with the description of a new species

Ana L. Sereno-Uribe[1] · Leopoldo Andrade-Gómez[1,2] · Gerardo Pérez Ponce de León[1] · Martín García-Varela[1]

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**Abstract**
Members of the genus *Tylodelphys* Diesing, 1850 are endoparasites of fish-eating birds, particularly ciconiids, anhingids, and podicipedids across the globe. Metacercariae of *Tylodelphys* spp. were collected from the cranial and body cavities of freshwater fishes in central and northern Mexico; adults were recovered from the intestine of two species of freshwater diving birds of the family Podicipedidae, commonly known as grebes, in two locations of central Mexico. Specimens were sequenced for two molecular markers, the internal transcribed spacers (ITS1 and ITS2) plus 5.8S gene of the nuclear ribosomal DNA and of the cytochrome *c* oxidase subunit 1 from mitochondrial DNA. The genetic divergence among the 25 samples (16 metacercariae and 9 adults) and between the newly sequenced specimens and those deposited in the GenBank were estimated. Maximum likelihood and Bayesian inference analyses inferred with each data set revealed the existence of five genetic lineages. Eight metacercariae analyzed in this study were nested in two divergent lineages previously recognized as *Tylodelphys* sp. 5 and *Tylodelphys* sp. 6 (sensu Locke et al., *Int J Parasitol*, 45:841–855, 2015). Five adult specimens recovered from the intestine of the least grebe (*Tachybaptus dominicus* Linnaeus, 1766) in Tecocomulco Lake, Hidalgo State, nested in a single clade with other sequences identified previously as *Tylodelphys aztecae*, expanding its distribution range in other areas of central Mexico. The isolates of the metacercariae found in the cranial cavity of the shortfin silverside, *Chirostoma humboldtianum* Valenciennes, 1835 from Zacapu Lake in central Mexico formed a monophyletic lineage and were recognized as an undescribed species of *Tylodelphys.* The lack of adult specimens of this lineage in our samples prevented a formal description. However, the metacercariae collected in the cranial cavity of the silverside, *Chirostoma jordani* Woolman, 1894 and the adult specimens recovered from the intestine of the western grebe, *Aechmophorus occidentalis* (Lawrence, 1858) from Cuitzeo Lake formed a monophyletic clade, allowing us to link both stages of the life cycle and to describe this as a new species, *Tylodelphys kuerepus* n. sp. The new species represents the eighth species of the genus described in the Americas and the fourth in the Nearctic region. We briefly discuss the ecological associations between the metacercariae and their second intermediate hosts in relation to the genetic diversity patterns uncovered in our study.

**Keywords** Digenea · *Tylodelphys* · Central Mexico · Species description · Cox 1 · ITS

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✉ Martín García-Varela
garciav@unam.mx

[1] Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad Universitaria, Avenida Universidad 3000, 04510 Mexico, Distrito Federal, Mexico

[2] Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, Avenida Universidad 3000, 04510 Mexico, Distrito Federal, Mexico

## Introduction

Adults of the genus *Tylodelphys* Diesing, 1850 are endoparasites of fish-eating birds, particularly ciconiids, anhingids, and podicipedids across the globe (King and Van As 1997; Drago and Lunaschi 2008; Chibwana et al. 2015; Blasco-Costa et al. 2017). As in other diplostomids, members of the genus *Tylodelphys* exhibit a complex life cycle involving a freshwater snail (lymnaeids or planorbids) as the first intermediate host. The metacercaria is found unencysted parasitizing

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**VII. II. Assessing the taxonomic validity of *Austrodiplostomum* spp. (Digenea: Diplostomidae) through nuclear and mitochondrial data. En *Journal of Parasitology*.**

Ana Lucia Sereno-Uribe, **Leopoldo Andrade-Gómez**, Margarita Ostrowski de Núñez, Gerardo Pérez-Ponce de León, Martín García-Varela

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# ASSESSING THE TAXONOMIC VALIDITY OF *AUSTRODIPLOSTOMUM* SPP. (DIGENEA: DIPLOSTOMIDAE) THROUGH NUCLEAR AND MITOCHONDRIAL DATA

**Ana Lucia Sereno-Uribe[1], Leopoldo Andrade Gómez[1,2], Margarita Ostrowski de Núñez[3], Gerardo Pérez-Ponce de León[1], and Martín García-Varela[1]**

[1] Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), Avenida Universidad 3000, Ciudad Universitaria, CP 04510, Distrito Federal, México.

[2] Universidad Nacional Autónoma de México (UNAM), Posgrado en Ciencias Biológicas, Avenida Universidad 3000, Ciudad Universitaria, CP 04510, Distrito Federal, México.

[3] Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 1428 Buenos Aires, Argentina.

Correspondence should be sent to Martín García-Varela at: *garciav@ib.unam.mx*

**KEY WORDS**

*Austrodiplostomum*
*Austrodiplostomum compactum*
*Austrodiplostomum mordax*
*ITS1*
*ITS2*
*5.8S*
*LSU*
*COI*
Phylogenetic Analyses
South America
Middle America
North America
*Nannopterum brasilianus*
*Nannopterum auritus*

**ABSTRACT**

Adults of the genus *Austrodiplostomum* are parasites in cormorants of the New World, whereas metacercariae are parasites from eye globe and brain of freshwater and brackish water fishes. In this study, specimens of *Austrodiplostomum mordax* from South America (type-species) were analyzed together with other specimens of *Austrodiplostomum* spp. collected from several locations across Middle America and North America. Partial DNA sequences of the mitochondrial gene cytochrome *c* oxidase subunit I (*COI*), the internal transcribed spacers (*ITS1*, *ITS2*, and *5.8S* gene), and the D2–D3, domains of the large subunit (*LSU*) of nuclear ribosomal DNA, were generated for both developmental stages and compared with available sequences of *Austrodiplostomum* spp. Phylogenetic analyses inferred with each molecular marker using maximum likelihood and Bayesian inference revealed the existence of 4 lineages representing 2 described species, *A. mordax* and *Austrodiplostomum compactum* (syn. *Austrodiplostomum ostrowskiae*) and 2 undescribed species of *Austrodiplostomum* recognized in previous studies. The *COI* haplotype network inferred with 172 sequences detected 28 haplotypes divided into 4 clusters, separating each other by 33 and 40 substitutions and with a genetic divergence ranging from 9 to 12%. The largest group included specimens identified as *A. compactum* plus those identified as *A. ostrowskiae*, supporting the synonymy of both species. As a result, we conclude that *A. compactum* is widely distributed across the Americas, in locations of the United States, Mexico, El Salvador, Honduras, Costa Rica, Venezuela, Peru, and Brazil. The other 2 undescribed species of the genus *Austrodiplostomum* were previously recorded in the United States and now are reported in Mexico. These 2 species cannot be described because adult forms have not been found in their definitive hosts. Finally, the species *A. mordax* has been found only in some lakes from Argentina, and it was validated in this study through molecular analyses.

Species of the genus *Austrodiplostomum* Szidat and Nani, 1951 (Digenea: Diplostomidae) use fish-eating birds of the genus *Nannopterum* Brisson as definitive hosts (Ostrowski de Núñez, 1970, 1977, 1982, 2017; Dronen, 2009; Drago et al., 2011; O'Hear et al., 2014; García-Varela et al., 2016), whereas the metacercariae are found parasitizing the eyes in the vitreous liquid and brain of >32 species of freshwater and brackish fishes from 13 families, such as Cichlidae, Heptapteridae, Characidae, Eleotridae, Ictaluridae, Ariidae, Poeciliidae, Clupeidae, Gerreidae, Catostomidae, Sciaenidae, Atherinopsidae, and Acentrorhynchidae across the Americas (Ramos et al., 2013; García-Varela et al., 2016; Rosser et al., 2016a, 2016b; Ostrowski de Núñez, 2017). Planorbid snails (*Biomphalaria glabrata* Say, *Biomphalaria straminea* Dunker, *Biomphalaria prona* Martens, and *Biomphalaria havanensis* Pfeiffer) are used as the first intermediate hosts of these species of the genus *Austrodiplostomum* (Ostrowski de Núñez, 1982; Pinto and Melo, 2013; Rosser et al., 2016a).

Currently, *Austrodiplostomum* contains 2 valid species, i.e., *Austrodiplostomum mordax* Szidat and Nani, 1951 (type-species), and *Austrodiplostomum compactum* (Lutz, 1928) Dubois, 1970 (Ostrowski de Núñez, 2017). However, the taxonomic status of both species has been controversial. In the first taxonomic revision of the genus, Dubois (1970) considered *A. mordax* as a synonym of *A. compactum* and placed it within subgenus

**VII. III. Morphological and molecular evidence reveals a new species of *Lyperosomum* Looss, 1899 (Digenea: Dicrocoeliidae) from *Melanerpes aurifrons* (Wagler, 1829) from northern Mexico. En *Journal of Helminthology.***

Marcelo Tonatiuh González-García, Mirza Patricia Ortega-Olivares, **Leopoldo Andrade-Gómez**, Martín García-Varela

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*Journal of Helminthology*

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# Morphological and molecular evidence reveals a new species of *Lyperosomum* Looss, 1899 (Digenea: Dicrocoeliidae) from *Melanerpes aurifrons* (Wagler, 1829) from northern Mexico

M.T. González-García[1], M.P. Ortega-Olivares[1], L. Andrade-Gómez[1,2] and M. García-Varela[1]

[1]Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, Ciudad de México C.P. 04510, México and [2]Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, C. P. 04510, Distrito Federal, México

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Morphology; taxonomy; *Lyperosomum*; LSU; *cox 1*; molecular phylogeny; Mexico

**Author for correspondence:**
M. García-Varela, E-mail: garciav@ib.unam.mx

**Abstract**

A new species of the genus *Lyperosomum* Looss, 1899, from the intestine of the golden-fronted woodpecker (*Melanerpes aurifrons*) from northern Mexico is described. *Lyperosomum cuauhxinqui* sp. n. is morphologically distinguished from other congeneric species from the Americas by a higher oral/ventral sucker ratio and its body length and width. The sequences of domains D1–D3 of the large subunit (LSU) of nuclear ribosomal DNA and cytochrome *c* oxidase subunit 1 (*cox 1*) from the mitochondrial DNA of the new species were obtained and compared with available sequences from GenBank. The genetic divergence estimated between the new species and other congeneric species ranged from 2 to 6% and 13.4 to 17.3% for LSU and *cox 1*, respectively. Phylogenetic analyses based on the two (LSU and *cox 1*) molecular markers consistently showed that *L. cuauhxinqui* sp. n. was nested within the genus *Lyperosomum*, with strong bootstrap support (100%) and Bayesian posterior probabilities (1.0). In particular, the LSU tree indicated that the sequence of the new species is closely related to sequences from *Zonorchis alveyi*, *Zonorchis delectans* and *Zonorchis* sp. from Central America, suggesting that these sequences should be transferred to the genus *Lyperosomum*. The new species represents the first record from Mexico and the fifth species identified in the Americas. Our study also revealed that the taxonomy of the genus *Lyperosomum* should be re-examined by combining molecular, morphological and ecological characteristics.

## Introduction

Dicrocoeliidae Looss, 1899 is a family of digenean parasites from the bile ducts, gallbladder and intestines of birds and, rarely, mammals distributed around the world, including approximately 400 species classified into 46 genera (Hildebrand *et al*., 2016, 2019; Tkach *et al*., 2018). The genus *Lyperosomum* Looss, 1899 is among the most diverse genera in this family, with approximately 33 recognized species, mostly parasitizing passerine birds (Hildebrand *et al*., 2019). The species of *Lyperosomum* are characterized by the following traits: oral sucker smaller than the ventral sucker, testes positioned closely to the ventral sucker, ovary posterior and distant from the posterior testis, genital pore located anterior to the intestinal bifurcation and vitellarium forming two relatively long lateral bands of follicles, beginning at the level of the testes and not reaching the caecal ends (Pojmańska, 2008). Based on these morphological traits, the history of the taxonomy and species composition of the genus *Lyperosomum* has been complex and unstable due to the phenotypic plasticity of some diagnostic characteristics that define the species. Recently, Hildebrand *et al*. (2019) conducted one of the most extensive studies of the genus *Lyperosomum*, combining morphological and molecular data. Their analyses also included species representing the genera *Skrjabinus* Bhalerao, 1936 and *Zonorchis* Travassos, 1944 from Dicrocoeliidae. These authors found that the species of *Lyperosomum* analysed were paraphyletic because some species of *Zonorchis* were nested in the genus *Lyperosomum*.

In the Americas, four species of the genus *Lyperosomum* have been recorded. *Lyperosomum intermedium* Denton & Kinsella, 1972 was described from the pancreas of rice rats, *Oryzomys palustris* Harlan, 1837, from Georgia and Florida in the US (Denton & Kinsella, 1972). *Lyperosomum petiolatum* (Railliet, 1900) Hildebrand, Pyrka, Sitko, Jeżewski, Zaleśny, Tkach & Laskowski, 2019 was isolated from the gall bladder of blue jays, *Cyanocitta cristata* (Linnaeus, 1758), from Texas, Mississippi and Nebraska in the USA (Denton & Byrd,

**VII. IV. Morphological and molecular data reveal a new species of *Lueheia* (Acanthocephala: Plagiorhynchidae) from *Turdus migratorius* (Turdidae) in central Mexico and its phylogenetic implications within the family. En *Parasitology Research.***

Martín García-Varela, **Leopoldo Andrade-Gómez**, Jorge López-Caballero, Berenit Mendoza-Garfias, Alejandro Oceguera-Figueroa, Rosario Mata-López

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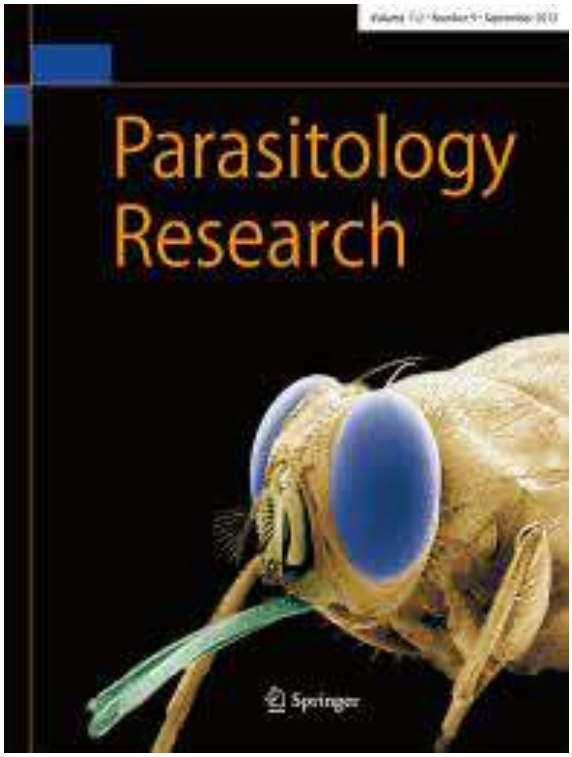

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FISH PARASITOLOGY - ORIGINAL PAPER

# Morphological and molecular data reveal a new species of *Lueheia* (Acanthocephala: Plagiorhynchidae) from *Turdus migratorius* (Turdidae) in central Mexico and its phylogenetic implications within the family

Martín García-Varela[1] · Leopoldo Andrade-Gómez[1,2] · Jorge López-Caballero[3] · Berenit Mendoza-Garfias[1] · Alejandro Oceguera-Figueroa[1] · Rosario Mata-López[3]

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## Abstract

Members of the genus *Lueheia* Travassos, 1919, are endoparasites of birds, particularly passerines, throughout the Americas. Adults of *Lueheia* sp., (Plagiorhynchidae Golvan, 1960; Porrorchinae Golvan, 1956) were recovered from the intestine of the American robin (*Turdus migratorius phillipsi* Bangs) in Mexico City, and two other species of acanthocephalans identified as *Porrorchis nickoli,* (Plagiorhynchidae: Porrorchinae) Salgado-Maldonado and Cruz-Reyes, 2002 and *Centrorhynchus microcephalus* (Bravo-Hollis, 1947) Golvan, 1956 (Centrorhynchidae Van Cleave, 1916), were recovered from the Virginia opossum (*Didelphis virginiana* Allen) and groove-billed ani (*Crotophaga sulcirostris* Swainson), respectively in southeastern Mexico. Specimens of three species were sequenced at two molecular markers, the small subunit (SSU) and large subunit (LSU) of the nuclear rDNA and compared with other sequences available in GenBank. Maximum likelihood and Bayesian inference analyses of the combined (LSU + SSU) dataset and each individual dataset revealed that the specimens of *Lueheia* sp. formed an independent lineage, which is recognized herein as a new species, *Lueheia aztecae* n. sp., representing the fifth species of the genus in the Americas, and the second in the Nearctic region. The new species can be morphologically distinguished from the other five species in the genus by having a cylindrical proboscis, armed with 24–26 longitudinal rows with 9–10 hooks each. Phylogenetic inference performed with the combined dataset consisting of two genes (LSU + SSU) revealed that *Lueheia aztecae* n. sp. and *P. nickoli* belonging to subfamily Porrorchinae, formed two independent lineages, indicating that the subfamily is paraphyletic. *Porrorchis nickoli* and *C. microcephalus* formed a clade with other species of the genus *Centrorhynchus*, suggesting that *P. nickoli* should be transferred to genus *Centrorhynchus*, to form *C. nickoli* n. comb. In addition, we briefly discuss the ecological associations between the members of the families Plagiorhynchidae and Centrorhynchidae.

**Keywords** Acanthocephala · *Lueheia* · Central Mexico · Species description · Molecular markers · Phylogeny

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✉ Martín García-Varela
garciav@ib.unam.mx

[1] Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), Avenida Universidad 3000, Ciudad Universitaria, 04510 México City, México

[2] Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, 04510 México City, Mexico

[3] Departamento de Biología Evolutiva, Facultad de Ciencias, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, 04510 México City, Mexico

## Introduction

Members of Plagiorhynchidae Golvan, 1960 are acanthocephalans that use birds, mammals, and rarely reptiles as definitive hosts and are distributed worldwide (Smales 2002; Amin 2013). Currently, the family includes three subfamilies: Porrorchinae Golvan, 1956, with six genera; Sphaerechinorhynchinae Golvan, 1956, represented by a single genus; and Plagiorhynchinae Meyer, 1931, with two genera (see Amin 2013). Golvan (1956) reviewed the taxonomy of Plagiorhynchidae and recognized the subfamily Porrorchinae with five genera. Currently, the subfamily includes approximately 37 species, classified into six genera:

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Martín García-Varela, **Leopoldo Andrade-Gómez**

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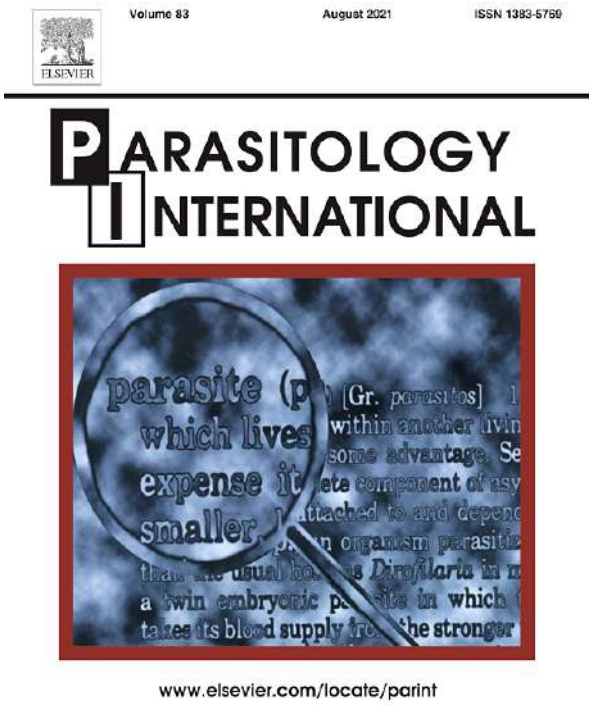

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# First steps to understand the systematics of Echinorhynchidae Cobbold, 1876 (Acanthocephala), inferred through nuclear gene sequences

Martín García-Varela [a,*], Leopoldo Andrade-Gómez [a,b]

[a] Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, Ciudad de México C.P. 04510, Mexico
[b] Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, Ciudad de México, C.P. 04510, Mexico

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ABSTRACT

Acanthocephalans of the order Echinorhynchida are one of the most diverse groups in their phylum, with approximately 470 species classified into 11 families that largely consist of parasites of freshwater, brackish and marine fishes and, sporadically, reptiles and amphibians distributed worldwide. Previous phylogenies inferred with molecular data have supported the paraphyly or polyphyly of some families, suggesting that most of them have been diagnosed based on unique combinations of characters, rather than shared derivative features. We expand the taxonomic sampling of several genera such as *Acanthocephalus*, *Echinorhynchus* and *Pseudoacanthocephalus* of Echinorhynchidae from diverse biogeographical zones in the Americas, Europe and Asia with the aim of testing the monophyly of the family by using two molecular markers. Sequences from small (SSU) and large (LSU) subunits of ribosomal DNA were obtained for six species representing the genera *Acanthocephalus* and *Echinorhynchus* from the Neotropical, Nearctic, Palearctic and Oriental regions. These sequences were aligned with other sequences available in the GenBank dataset from Echinorhynchidae. Phylogenetic trees inferred with the combined (SSU + LSU) and the individual data sets consistently placed the genera *Acanthocephalus, Pseudoacanthocephalus* and *Echinorhynchus* into three independent lineages. Two families, Paracanthocephalidae Golvan, 1960, and Pseudoacanthocephalidae Petrochenko, 1956, were resurrected to accommodate the genera *Acanthocephalus* and *Pseudoacanthocephalus*, respectively. The species of the genus *Acanthocephalus* from the Nearctic, Palearctic and Oriental biogeographic regions formed a clade that was well supported. However, *Acanthocephalus amini* from the Neotropical region was nested inside Arhythmacanthidae. Therefore, the genus *Calakmulrhynchus* was created to accommodate *A. amini* and resolve the paraphyly of *Acanthocephalus*. Finally, the diagnoses of the families Echinorhynchidae and Arhythmacanthidae were amended. The molecular phylogenies should be used as a taxonomic framework to find shared derived characters (synapomorphies) and build a more robust classification scheme that reflects the evolutionary history of the acanthocephalans.

## 1. Introduction

Members of the class Palaeacanthocephala Meyer, 1931, are the most diverse group within the phylum Acanthocephala, with approximately 845 species classified into three orders, namely, Heteramorphida Amin and Ha, 2008; Polymorphida Petrochenko, 1956; and Echinorhynchida Southwell and Macfie, 1925, with one, three and 11 families, respectively [1]. Palaeacanthocephalans have diverse life-cycles, including malacostracans (crustaceans) as intermediate hosts and vertebrates (fishes, amphibians, reptiles, birds and mammals) as definitive hosts [2,3]. In some cases, some species of fish, amphibians and reptiles act as paratenic hosts (transport) to facilitate transmission to the appropriate definitive hosts [2,3]. Occasionally, some species of palaeacanthocephalans can alter the behaviour or coloration of their intermediate hosts and increase their susceptibility to predation [3].

The earliest molecular phylogenetic analyses in acanthocephalans were based exclusively on the small subunit (SSU) of ribosomal DNA [4–7]. More recently, the large subunit (LSU) of ribosomal DNA was added as another molecular marker to infer the evolution of the acanthocephalans [8–11]. The phylogenies inferred with each molecular marker or the combination of both have supported the monophyly of Palaeacanthocephala, and both molecular markers are considered the

* Corresponding author.
*E-mail address:* garciav@ib.unam.mx (M. García-Varela).

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