UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE BIOLOGÍA

SISTEMÁTICA

INFERENCIA FILOGENÉTICA DE LA SUBCLASE DIGENEA (PLATYHELMINTHES) UTILIZANDO MARCADORES MOLECULARES Y LAS IMPLICACIONES EN SU SISTEMÁTICA

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS BIOLÓGICAS

PRESENTA:

DAVID IVÁN GUADALUPE HERNÁNDEZ MENA

TUTOR PRINCIPAL: DR. GERARDO PÉREZ PONCE DE LEÓN. INSTITUTO DE BIOLOGÍA, UNAM

COMITÉ TUTOR: DR. JOSÉ MARTÍN GARCÍA VARELA. INSTITUTO DE BIOLOGÍA, UNAM
DR. ROGELIO AGUILAR AGUILAR. FACULTAD DE CIENCIAS, UNAM

CD. MX. MAYO, 2018

UNAM POSGRADO
Ciencias Biológicas

COORDINACIÓN

Lic. 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 del Subcomité por Campo de Conocimiento de Biología Experimental y Biomedicina del Posgrado en Ciencias Biológicas, celebrada el día 12 de marzo de 2018, se aprobó el siguiente jurado para el examen de grado de **DOCTOR EN CIENCIAS** del alumno **HERNÁNDEZ MENA DAVID IVÁN GUADALUPE** con número de cuenta **405087505** con la tesis titulada: **"Inferencia filogenética de la subclase Digenea (Platyhelminthes) utilizando marcadores moleculares y las implicaciones en su sistemática",** realizada bajo la dirección del **DR. GERARDO PÉREZ PONCE DE LEÓN:**

Presidente:	DR. JUAN JOSÉ MORRONE LUPI
Vocal:	DR. ALEJANDRO FRANCISCO OCEGUERA FIGUEROA
Secretario:	DR. JOSÉ MARTÍN GARCÍA VARELA
Suplente:	DRA. CLAUDIA PATRICIA ORNELAS GARCÍA
Suplente	DRA. MARÍA DEL CARMEN GUZMÁN CORNEJO

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

A T E N T A M E N T E
"POR MI RAZA HABLARA EL ESPIRITU"
Cd. Universitaria, Cd. Mx., a 18 de mayo de 2018.

POSGRADO EN CIENCIAS BIOLÓGICAS
COORDINACIÓN

DR. ADOLFO GERARDO NAVARRO SIGÜENZA
COORDINADOR DEL PROGRAMA

c.c.p. Expediente del (la) interesado (a).

AGRADECIMIENTOS

Al Posgrado en Ciencias Biológicas de la Universidad Nacional Autónoma de México por el apoyo logístico durante los cuatro años que duro el programa de doctorado.

Al Consejo Nacional de Ciencia y Tecnología (CONACYT) por la beca otorgada durante mis estudios de doctorado.

Al Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-UNAM IN204514 y PAPIIT-UNAM IN202617) por el financiamiento al presente proyecto.

Al Dr. Gerardo Pérez Ponce de León (tutor principal) por su interés y entusiasmo en el proyecto, por sus valiosas enseñanzas en el ámbito académico y personal, y por su apoyo económico y moral durante todo mi proceso de formación académica en el Instituto de Biología. Muchas gracias.

A mi Comité Tutoral, el Dr. Martín García Varela y el Dr. Rogelio Aguilar Aguilar por todo su apoyo y valiosos comentarios para el mejoramiento del presente proyecto durante su desarrollo.

AGRADECIMIENTOS A TÍTULO PERSONAL

A los miembros del jurado Dr. Juan José Morrone, Dr. Alejandro Oceguera, Dr. Martín García, Dra. Patricia Ornelas y Dra. Carmen Guzmán por el tiempo que se tomaron para la revisión del documento de tesis, en donde sus aportaciones fueron muy valiosas para el mejoramiento del mismo.

Al Dr. Armando Rodríguez Reyes y a Rocío González Acosta por facilitar el proceso de los tramites para obtener el grado.

A todos los que a continuación voy a mencionar les quiero agradecer por su apoyo incondicional en los distintos aspectos de mi formación académica, por su ayuda en el trabajo de campo, pero sobretodo por su invaluable amistad a lo largo de los cuatro años que duro el doctorado, ya que gracias a ustedes, esta ha sido una de las mejores experiencias en mi vida.

A Luis García Prieto por sus consejos en el ámbito personal, su invaluable amistad y ser un guía espiritual en el aspecto académico.

A Berenit Mendoza Garfías por el apoyo técnico en el área de microscopía, por su amistad y por su apoyo en lo académico.

A los doctores Jairo Arroyave, Ulises Rosas, Alejandro Oceguera, David Ortiz, Ricardo Paredes e Ismael Hinojosa, por ser parte del gran y tan interesante grupo de los Vengativos, y por la gran cantidad de alcohol, tacos y demás marrullerías. Gracias por su don de gente amigos.

A Víctor Hugo Reinoso, Hyla y Melissa Plasman por su amistad.

A Carlos Palmero y Jesús Orts por todos los consejos en lo académico, pero sobretodo por las innumerables cervezas que nos hemos tomado juntos, gracias amigos.

A compañeros y amigos de la Colección Nacional de Helmintos Brenda Atziri, Ofelia, Jonathan, Ángeles de Charli, Josué, Gisela, Ricardo, Nayeli, Nanci, Gerardo, Yanet, Leti hija y Leti Barragán, por compartir tantos eventos en el laboratorio, salidas al campo y sobretodo buenas fiestas.

A Carlos Pinacho, la Morra, Polo, Eduardo y Alejandra del C-104, por su amistad.

A mis amigos del laboratorio Ana Santacruz, Brenda Solórzano, Rosario Briosio y Jorge López por su amistad y por soportarme.

A Laura y Alexa por ayudarme en el laboratorio con los especímenes de mi proyecto.

A Uriel, Sara, Rosario Mata y a Lassaro Guevara por permitirme colaborar con ellos.

A Christina Lynggaard por ser una gran colaboradora y porque literalmente me salvaste la vida, muchas gracias.

A Flavia por las sugerencias para la elaboración del proyecto y por su valiosa amistad.

A Alma Islas y a Omar Lagunas por invitarme a colaborar en sus proyectos de posgrado y por su amistad.

A Valeria Salinas por la donación de parásitos de murciélagos, por su amistad y apoyo en situaciones complicadas.

A Andrea Rebollo por su amistad y por el apoyo durante el trabajo de campo.

A Omar Domínguez, Eloísa Torres y Rodolfo Pérez por su apoyo en campo, enseñanzas académicas y por compartir unas buenas cervezas.

A Pati Ornelas y Carlos Pedraza por su apoyo durante las colectas de campo, por sus enseñanzas académicas y por su amistad.

A Erick Moreno, Edain y Jorge Kimba, por su ayuda en el campo y por su amistad.

A Oscar Maraver por su valiosa amistad durante tantos años.

A Mariana García por haber compartido todos esos años, que en definitiva no han sido fáciles, pero que definitivamente se acercan tiempos mejores. Gracias por haberme soportado. Te quiero.

A la familia Mena Vera, por enseñarle la humildad que existe en el trabajo, y por el amor que se puede tener por la misma tierra en la que se camina, se trabaja y se vive. Muchas gracias, los quiero.

A mis padres Félix y Gabriela por todo el apoyo incondicional durante la toma de mis decisiones, de momentos complicados y de inigualable alegría. Muchas gracias por el cariño que he recibido de ustedes, así como también por las enseñanzas sobre como transitar por esta vida. Muchas gracias, los quiero.

A mis hermanos Josué y Diana, por su apoyo tanto en momentos de felicidad, como en aquellos que han sido difíciles, por seguir juntos para compartir todas las experiencias que se han presentado en nuestras vidas. Muchas gracias, los quiero.

Amo la tierra misma en donde trabajo,
aquella tierra en donde crece el humilde maíz.
Amo el aroma de la humedad durante la lluvia.
Amo sentir el suave viento en mi rebelde alma.
Amo caminar al atardecer, pero ahora quiero volar,
así como los sueños que parecen imposibles.

ÍNDICE

RESUMEN

Los trematodos pertenecen a la subclase Digenea y son el grupo de metazoarios parásitos más diversos en la naturaleza; éstos utilizan principalmente vertebrados como huéspedes definitivos. Los clasificación dentro de la subclase, la descripción y diferenciación de las especies se basaron durante muchos años en los caracteres morfológicos de los parásitos adultos. Sin embargo, el emplear solo las características morfológicas de éstos tiene algunas limitantes relacionadas con la complejidad de encontrar caracteres diagnósticos confiables, la plasticidad fenotípica, la similitud morfológica entre especies estrechamente relacionadas y la dificultad de asociar morfológicamente distintos estados larvarios con el estado adulto. Por ello, las limitantes del uso exclusivo de la evidencia morfológica ha tenido varias consecuencias, entre las cuales se pueden destacar la sub o sobreestimación de la diversidad de especies, la inferencia de hipótesis de relaciones filogenéticas con poca resolución y la generación de clasificaciones taxonómicas inestables, a diferentes niveles de la jerarquía. La utilización de secuencias de ADN para proponer hipótesis de relaciones filogenéticas en digéneos comenzó a partir de la década de los 90´s, y se volvió el método más común a partir del año 2000. Sin embargo, el uso de secuencias no solamente permitió inferir hipótesis de relaciones filogenéticas más robustas, sino que también resultó de gran utilidad para enlazar los distintos estados de desarrollo del ciclo de vida de las especies, para la detección de nuevas especies (incluyendo el reconocimiento de especies crípticas) y para establecer límites más precisos entre ellas; asimismo, los datos moleculares han contribuido a reconocer fenómenos de variación fenotípica, a describir procesos de especificidad con los huéspedes y los intervalos de la distribución geográfica de las especies, y a resolver problemas taxonómicos a diferentes niveles, como por ejemplo, para establecer la sinonimia entre especies, o para poner a prueba la monofilia de familias. Sin embargo, a pesar de los años que se han utilizado secuencias de ADN, hasta la fecha aún no se ha uniformizado la utilización de los diferentes marcadores moleculares, lo que ha ocasionado huecos en la información.

En la presente tesis se usan secuencias de ADN del gen nuclear 28S en conjunto con evidencia morfológica, ecológica y geográfica para investigar las relaciones filogenéticas de digéneos a diferentes escalas de la jerarquía taxonómica (especies, familias, superfamilias y suborden). Además, también se secuenciaron otros marcadores nucleares y uno mitocondrial, para poner a prueba su utilidad a diferentes niveles de la clasificación. Este trabajo de tesis está dividido en 4 capítulos. En el primero se aborda

la descripción de tres nuevas especies pertenecientes a la familia Allocreadiidae. La proposición de las nuevas especies se basó en la comparación de evidencia morfológica y en análisis filogenéticos con secuencias de 28S e ITS, para determinar las afinidades filogenéticas y distancias genéticas entre especies. También se utilizaron de datos de su distribución geográfica y afinidad hospedadora. Este capítulo contiene dos trabajos. En el primero se describe a *Auriculostoma lobata*, que parasita a *Brycon guatemalensis* en el río Usumacinta y se provee una clave taxonómica para la identificación de las especies de este género. En el segundo artículo se describen a *Auriculostoma tica*, parásito de *Gymnotus maculosus* en ríos de Costa Rica y a *Wallinia criptica* parasito de *Astyanax aeneus* en cuencas hidrológicas desde Oaxaca a Guatemala. En el segundo capítulo se presenta un análisis filogenético para corroborar la posición de los géneros *Magnivitellinum* y *Perezitrema* que parasitan peces dulceacuícolas en México. Estos géneros estaban clasificados dentro de la familia Macroderoiididae, pero los resultados de los análisis filogenéticos demostraron que Macroderoiididae constituía un grupo parafiletico, por lo que se describió una nueva familia, Alloglossiididae, para agrupar correctamente a los géneros. En el tercer capítulo esclarecieron las relaciones filogenéticas entre las familias que conforman a la superfamilia Diplostomoidea. Se obtuvieron secuencias de los genes 18S y 28S, y del gen mitocondrial *cox* 1 de ejemplares de la familia Proterodiplostomidae; la posición filogenética de esta familia era incierta pues no se contaba con secuencias de alguna de sus especies. En este capítulo también se puso a prueba y se discutió la eficiencia del gen *cox* 1 en la resolución de relaciones filogenéticas de niveles supraespecíficos. En el último capítulo se presenta un manuscrito en el que se utilizaron secuencias del gen 28S para realizar el análisis filogenético que abarca el mayor número de especies de la subclase Digenea realizado hasta la fecha (1,077 taxa); el propósito principal de este trabajo fue el de poner a prueba la robustez de los sistemas de clasificación que se han propuesto a lo largo de la historia de la clase Trematoda. Se discuten las incongruencias en las propuestas de clasificación a nivel de superfamilia y suborden que se generan con la incorporación de un mayor número de especies de diferentes familias. Finalmente, se presenta una discusión general que integra los resultados de los diferentes capítulos, atendiendo principalmente a los criterios que se utilizan en el estudio de las relaciones filogenéticas de digéneos y la importancia del uso de diferentes fuentes de caracteres, y de diferentes marcadores moleculares, en el estudio de los digéneos a diferentes niveles de la jerarquía taxonómica, desde el nivel de especie hasta el de suborden.

ABSTRACT

Trematodes belong to the subclass Digenea are represent the most diverse group of metazoan parasites in nature. Trematodes use vertebrates as definitive hosts. The classification schemes within the subclass as well as the species description and species delimitation have been based on morphological characters, particularly those of adult forms. Using only morphological characters has some disadvantages because of the problem in finding reliable diagnostic characters, phenotypic plasticity, morphological similarity among closely related species and the difficulty in linking different developmental stages of the life cycle. This disadvantages determined important consequences such as the fact that trematode diversity might be over or underestimated, and also phylogenetic inferences using only morphological characters lacked resolution, generating unstable classifications at different levels of the taxonomic hierarchy. The

use of DNA sequences to infer hypothesis of phylogenetic relationships among digeneans started in the decade of the 90´s, and it became a standars method in 2000. However, the use of DNA sequences allowed not just the inference of more robust phylogenetic relationships, but they were useful to link different developmental stages of the life cycle, to detect new species (including the recognition of cryptic species), and the establishment of boundaries among them; furthermore, DNA sequences allowed the recognition of phenotypic variation, the description of host specificity and geographic distribution patterns, as well as the resolution of taxonomic problems at different levels. For example, to establish the synonymy between two species, or to test the monophyly of genera included in certain family. However, no matter sequences have been used in the last decades, the selection of molecular markers is not standardized yet, and gaps in information are still commonly found.

In this work, 28S rDNA sequences are used in combination with other sources of information, i.e., morphology, ecology, geographical distribution, etc. to infer the phylogenetic interrelationships of digeneans at different levels of the taxonomic hierarchy (species, genera, families, superfamilies and suborders). Other molecular markers were also sequenced in particular cases to test their utility at different scales of the classification. The results of this thesis are presented in 4 chapters. Chapter I includes new species descriptions, particularly members of the family Allocreadiidae. Species recognition was based on morphological comparison and phylogenetic analyses through nuclear sequences (28S and ITS) to establish phylogenetic position and pairwise genetic distance. In addition, geographical distribution data and host

association was also use to determine species boundaries. In one of the papers we present the description of *Auriculostoma lobata*, which is a parasite of *Brycon guatemalensis* in the Usumacinta River; in that papet we also provided a taxonomic key for species in that genus. In the second paper, we described another member of the same genus, *Auriculostoma. tica*, a parasite of *Gymnotus maculosus* in rivers of Costa Rica, and *Wallinia criptica* from *Astyanax aeneus* in river basins of Oaxaca, Mexico and Guatemala. Chapter II shows a phylogenetic analysis used to corroborate the systematic position of the genera *Magnivitellinum* and *Perezitrema* which are parasites of freshwater fishes in Mexico. Both genera were classified as belonging to the family Macroderoiididae, but the results of our analyses demonstrated that the family was paraphyletic, and as a result we described a new family of digeneans, Alloglossiididae, to group the genera according with the phylogenetic classification as monophyletic assemblages. In chapter III we conducted a phylogenetic analysis of the families contained in the superfamily Dipliostomoidea to test the phylogenetic position of the family Proterodiplostomatidae because, before this study, no sequences were available for any member of the family. Ribosomal DNA sequences (18S y 28S) and the mitocondrial gene *cox* 1 were obtained for specimens of *Crocodilicola pseudeostoma*, a member of the Proterodiplostomidae; the systematic position of this family was uncertain due to the lack of molecular data. The study presented in Chapter III also tested the utility of *cox* 1 to resolve phylogenetic relationships of digeneans at supraspecific levels. Finally, in chapter IV we used 28S rDNA sequences to perform a comprehensive molecular phylogenetic analysis of the entire subclass Digenea. The analysis included 1,077 taxa and phylogenetic inferences were made through Maximum Likelihood and Bayesian Inference analyses. The analyses were conducted with the aim of testing the robustness of the current classification schemes. In that chapter, we discuss the incongruences we observed at higher levels of the hierarchy such as superfamily and suborder and we argue about the classification scheme of the Digenea and the importance of using diferent sources of characters, and different molecular markers for the study of this parasitic organisms at different scales of the taxonomic hierarchy from the species level to the suborder level.

1. INTRODUCCIÓN GENERAL

1. INTRODUCCIÓN GENERAL

La clase Trematoda es un grupo de parásitos pertenecientes al phylum Platyhelminthes que está dividida taxonómicamente en dos subclases: Aspidogastrea y Digenea (Littlewood et al., 1999a; Gibson et al., 2002). Particularmente, los digéneos son uno de los grupos de metazoarios endoparásitos de vertebrados más diversos e incluye unas 18,000 especies nominales que están agrupadas en 2 ordenes, 24 superfamilias, 150 familias y 2,800 géneros aproximadamente (Cribb et al., 2001; Olson et al., 2003; Olson y Tkach, 2005; Pérez-Ponce de León et al., 2007; Bray et al., 2008; Littlewood et al., 2015). El orden Diplostomida incluye el 12.3% de las especies de Digenea y son parásitos casi exclusivamente de tetrápodos (exceptuando a Aporocotylidae que son parásitos de sangre en peces), mientras que el orden Plagiorchiida agrupa el 87.7% de especies que parasitan tanto peces como de tetrápodos (Littlewood et al. 2015). Los digéneos presentan un par de ventosas, una oral y un acetábulo que tiene una distribución variable en el cuerpo dependiendo de las especies. Estos parásitos se caracterizan por tener ciclos de vida complejos con alternancia de generaciones entre estados de vida libre y estados dependientes de un huésped, con fases larvarias que se desarrollan en invertebrados y vertebrados, y una fase adulta sexual generalmente en vertebrados (Littlewood et al., 2015). Otros caracteres diagnósticos son: 1) tienen un ciclo de vida que involucra dos estados larvarios, miracidio y esporocisto madre, los cuales carecen de un aparato digestivo; 2) tienen un esporocisto y una redia que son larvas endoparásitas que se desarrollan en moluscos; 3) presentan una larva libre nadadora conocida como cercaria (Brooks et al., 1985; Ehlers, 1986; Gibson 1987; Pearson 1992, Cribb et al., 2001). Debido a la complejidad que involucran sus ciclos de vida, la presencia de éstos en los ecosistemas es relevante para el equilibrio del mismo (Poulin, 1997; Brooks & Hoberg, 2000). Por lo tanto, debido a la diversidad de Digenea y los grupos que han sido poco estudiados, la clasificación taxonómica de varios de los taxa ha sido poco clara. Asimismo, las relaciones entre varios de los grupos de digéneos a niveles supra e interespecíficos han sido inestables desde que comenzó su estudio desde una perspectiva filogenética. De estos estudios se desprenden las discusiones que se han derivado en preguntas evolutivas tales como el establecer cuales son las relciones más profundas dentro de los Digenea, o interpretar la evolución de los ciclos de vida a la luz de la filogenia.

1.1. Ciclo de vida general de Digenea

El ciclo de vida general de Digenea se inicia cuando los huevos se liberan al ambiente acuático a través de las heces de los huéspedes definitivos, los cuales en general son vertebrados y el los cuales los parásitos copulan. De los huevos eclosiona la primer larva conocida como miracidio, la cual infecta a moluscos por lo general. Los moluscos funcionan como los primeros huéspedes intermediarios y es en ellos en donde se desarrollan los esporocistos (segundo estado larvario), y que a su vez dan origen a las redias (tercer estado larvario). De las redias se originan las cercarias (cuarto estado larvario), que son larvas libre nadadoras. Las cercarias emergen del molusco y nadan hacia el segundo huésped intermediario que pueden ser varias especies de invertebrados, de vertebrados o bien, pueden enquistarse en la vegetación; la infección del segundo huésped generalmente es por penetración de la cercaria. Una vez en el interior de su segundo huésped intermediario, las cercarias se enquistan en diferentes órganos, transformándose en metacercarias. La metacercaria se puede enquistar en intestino, mesenterio, músculo, ojos, gónadas, riñones, cerebro e hígado. El ciclo se completa cuando el huésped definitivo consume al segundo huésped intermediario infectado (Yamaguti, 1975; Schell, 1985; Pérez-Ponce de León et al., 2007; Littlewood et al., 2015), aunque bien algunos grupos de estos parásitos pueden ser progenéticos, es decir, que en ausencia del huésped definitivo estricto, la metacercaria puede madurar dentro del huésped intermediario, razón por lo cual se pueden encontrar parásitos adultos en invertebrados (por ejemplo *Alloglossidium* en sanguijuelas) (Cribb et al., 2003; Font y Lotz, 2008). Del esquema general sobre el ciclo de vida de los digéneos puede haber varias modificaciones ya que dependiendo de la especie de parásito, se pueden omitir algunos estados larvarios o incluso se puede presentar un mayor número de etapas larvarias así como también huéspedes intermediarios (u huéspedes accidentales o paraténicos), lo cual genera ciclos de vida más complejos en estos organismos.

Debido a la compleja secuencia de etapas ontogenéticas y huéspedes que pueden llegar a tener los digéneos para completar su ciclo de vida, el estudio en este ámbito no es trivial, pues los datos sobre la ontogenia son escasos, o bien para muchas especies no existen (Yamaguti, 1975; Olson y Tkach, 2005). A pesar de que actualmente se reconoce la importancia del estudio de los ciclos de vida para la sistemática de los digéneos, fue La Rue en 1957 quién con sus trabajos sobre los ciclos de vida, recalcó la relevancia de la información de las etapas larvarias y los problemas existentes con comparaciones realizadas solamente en la morfología de los adultos (Littlewood et al., 2001). Varios autores han tratado de reconstruir la historia evolutiva de los ciclos de vida de los digéneos (Shoop, 1988), entre los cuales se pueden destacar los trabajos de Poulin y Cribb (2002), y de Cribb et al. (2003), quienes utilizaron aspectos del ciclo de vida como caracteres y estados de carácter en una matriz, para posteriormente llevar a cabo un mapeo sobre una filogenia completamente resuelta; el mapeo de los caracteres lo realizaron bajo el criterio de parsimonia. Asimismo, Cribb et al. (2003) usaron la filogenia obtenida por Olson et al. (2003) para llevar a cabo el mapeo a nivel de superfamilias.

1.2. Breve historia sobre la posición taxonómica de la subclase Digenea dentro del phylum Platyhelminthes

En 1808 Rudolphi usó el término Trematoda para agrupar a todos los parásitos de aspecto plano que no tenían forma de solitaria. Cribb et al. (2001) consideraron que la primera decisión filogenética supraespecífica dentro del grupo fue cuando van Beneden en 1858 dividió a los trematodos en “Monogénesis” y “Digénesis”, basándose en las características del ciclo de vida de los parásitos. El nombre de Digenea fue usado por primera vez por Carus en 1863, para identificar a los trematodos que tienen alternancia de generaciones durante su ciclo de vida, con larvas en moluscos y una fase sexual (estado adulto) en vertebrados (Cribb et al., 2001). Sinirsin en 1911 sugirió que Monogenea se derivó directamente de Digenea después de una simplificación en su ciclo de vida, pero Baer en 1931 y Bychowsky en 1937, argumentaron que Monogenea es el taxon hermano de Digenea + Aspidogastrea, conformando así los tres taxa, la clase Trematoda (en Cribb et al., 2001).

Al utilizar por primera vez una aproximación filogenética para el estudio de los Platyhelminthes analizando una matriz de caracteres morfológicos, Brooks (1982) propuso el subphylum Cercomeria, clado que incluyó a Trematoda, Cestoda, Monogenea y Temnocephala. Posteriormente, Ehlers (1985, 1986) también realizó un estudio filogenético con el método de parsimonia, y obtuvo un clado conformado por los principales grupos parásitos de los Platyhelminthes (Monogenea, Cestoda y Trematoda); a este clado se le dió el nombre de Neodermata, ya que sus integrantes presentan un epitelio sincicial, y quedó como el grupo hermano de Dalyellioida, el cual incluía a Temnocephalida. Así mismo, en el artículo de Ehlers, la clase Trematoda quedo dividida en Aspidogastrea y Digenea. Estos resultados, a excepción de la relación de hermandad de Dalyellioida con Neodermata que posteriormente fue refutada, fueron corroborados años más tarde con filogenias obtenidas a partir secuencias de ADN (Baverstock et al., 1991; Littlewood et al., 1999a, 1999b y 2004; Rohde, 2001; Lockyer et al., 2003; Willems et al., 2005). Cabe destacar que gracias a la contribución de las secuencias de ADN, la monofilia de Neodermata actualmente ya no es discutida, al igual que la monofilia de Digenea, la cual con excepción de la propuesta de algunos autores (Poche 1926 y Cable 1974 en Cribb et al., 2001), no ha entrado en contradicciones. Por lo tanto, los dos esquemas más apoyados sobre las relaciones filogenéticas dentro de Neodermata son los siguientes: (Monogenea + Cestoda) + Trematoda y Monogenea + (Cestoda + Trematoda), en donde Trematoda (Digenea + Aspidosgastrea) es el taxón hermano de Cestoda (Lockyer et al., 2003; Willems et al., 2005). La mayoría de la evidencia más reciente con base en secuencias de genes nuleares y mitocondriales, así como de mitogenomas apoya la monofilia de Trematoda y Cestoda (Lockyer et al., 2003; Park et al., 2007; Perkins et al., 2010; Littlewood et al., 2015)

1.3. Relaciones filogenéticas de grupos supraespecíficos dentro de la subclase Digenea

1.3.1. Trabajos con datos morfológicos

Antes de la utilización de datos moleculares, la identificación y caracterización de las especies en digéneos se basaba principalmente en los rasgos morfológicos de los adultos y en características ecológicas (tipo de huésped definitivo). Como veremos a continuación, los esquemas de clasificación dentro de Digenea propuestos únicamente en la morfología de los adultos y de sus estados larvarios también han sido cambiantes con el paso de los años, debido a varios factores: 1) la consideración y el peso taxonómico de ciertos caracteres dependiente del criterio de los diferentes investigadores, 2) la aparición de métodos de análisis más objetivos, 3) el aporte de más información sobre los ciclos de vida, 4) la aparición de técnicas que permitieron explorar con mayor precisión la morfología de los organismos (como la microscopía electrónica de barrido durante los años 70´s), y por lo tanto, 4) la incorporación de más datos sobre la morfología de los parásitos.

En 1926 Poche propuso un sistema de clasificación que contempla dos grupos principales: Gasterostomata (que contenía solo a la familia Bucephalidae) y Prosostomata, separados por la distribución de las ventosas. A su vez, Prosostomata estaba dividido en Heronimoinae (con la familia Heronimidae) y Fascioloinae (que contenía a las 30 familias restantes reconocidas hasta ese momento). Cabe destacar que bajo este esquema de clasificación, Poche incluyó a la familia Aspidogastridae dentro de Fasciolonidae (en Cribb et al., 2001). Alternativamente, La Rue (1957) también contribuyó con otra propuesta en donde consideró dos grupos a los cuales denominó como Anepithelyocistidia y Epithelyocistidia, que agrupaban 40 y 31 familias respectivamente. Esta separación de las familias en dos grandes grupos se apoyaban en la presencia/ausencia de una vesícula excretora cubierta con epitelio (figura 16.2 en Cribb et al, 2001).

Brooks et al. (1985) fueron los primeros en generar un matriz de datos morfológicos para realizar un análisis cladístico de los digéneos; los resultados de dicho estudio fueron consistentes con aquellos obtenidos por otros autores (como La Rue, 1957), pero arrojó algunas politomías y las relaciones de varias de las familias que se incluyeron quedaron sin resolver. Sin embargo, algunos de los resultados de la hipótesis filogenética de Brooks et al. (1985), por ejemplo la consideración de *Heronimus* como grupo hermano del resto de los digéneos, fueron ampliamente discutidos por investigadores como Gibson (1987) y Pearson (1992). Este último autor puso en duda la utilización de varios de los caracteres que se incluyeron en la matriz de Brooks et al. (1985).

1.3.2. Utilización de marcadores ribosomales y mitocondriales en la sistemática filogenética de Digenea

Durante mucho tiempo, la taxonomía de la subclase Digenea se basó principalmente en la descripción morfológica de los estadios adultos de estos parásitos así como en su distribución geográfica y patrones de asociación con sus hospederos (Nolan y Cribb, 2005). La utilización de caracteres morfológicos para la identificación, la descripción de especies y la reconstrucción de hipótesis filogenéticas se vio reforzada con el uso de secuencias de ADN a partir de la década de los 80´s. Las secuencias de marcadores moleculares comenzaron a popularizarse por varios motivos; unos de ellos fueron los problemas o limitantes que presentan los caracteres morfológicos durante la identificación de especies de parásitos, relacionados con la complejidad de encontrar caracteres diagnósticos adecuados, poniéndose en duda la validez taxonómica de algunos caracteres (Luton et al., 1992; Leon- Regagnon et al., 1999; Schulenburg et al., 1999); otros problemas son la plasticidad fenotípica (Galazzo et al., 2002), la similitud morfológica entre especies estrechamente relacionadas (Tkach et al., 2000b; Nolan y Cribb, 2005) y la dificultad de asociar morfológicamente distintos estados larvarios con el estado adulto (Jousson et al., 1998, 1999; Bartoli y Jousson, 2003). Todos estos problemas han tenido como consecuencia que la diversidad de este grupo de organismos pueda ser subestimada o sobreestimada (Nolan & Cribb, 2005; Sereno-Uribe et al., 2013; Sereno-Uribe et al., 2014; García-Varela et al., 2015).

Asimismo, la utilización de secuencias de ADN se volvió una práctica común en la disciplina debido a que esta nueva fuente de caracteres resultó muy útil para proponer hipótesis filogenéticas que se contrastaron con aquellas que se infirieron con caracteres morfológicos (Luton et al.,1992; Adlard et al., 1993; Bell et al., 2001; León-Régagnon y Brooks, 2003; Nolan y Cribb, 2005; Vilas et al., 2005; Pérez-Ponce de León et al., 2008; Razo-Mendivil et al., 2014). La utilización de los datos moleculares fue bien recibida por parasitólogos en las áreas de sistemática, evolución y ecología (Olson y Tchak, 2005), aún cuando existió la discusión de que el uso de caracteres moleculares para tratar de reconstruir la historia filogenética de las especies implicaba la reconstrucción de la historia de los genes y no necesariamente la historia de las especies (Sites y Crandall, 1997).

La utilización de las secuencias de ADN como caracteres para la obtención de hipótesis filogenéticas proporciono varias ventajas: 1) diferentes regiones del genoma tienen distintas tasas de mutación y por lo tanto pueden ser usadas para identificar y caracterizar poblaciones, especies, géneros etc.; 2) la generación de secuencias de ADN es relativamente rápida; 3) el número de sitios variables de la secuencia de ADN que se analizan es mucho mayor que el de los caracteres morfológicos; 4) la variación en las secuencias de ADN no está influenciada por el fenotipo, la edad ó el ambiente (McManus y Bowles, 1996; Galazzo et al., 2002).

Particularmente, para el caso de los digéneos, las secuencias se han utilizado para abordar aspectos particulares, tales como: 1) relacionar las distintas etapas larvarias de estos parásitos (Jousson et al., 1998, 1999; Bartoli y Jousson, 2003); 2) detectar especies crípticas a través de la prospección molecular de la variabilidad genética de las poblaciones, y el establecimiento de límites mas precisos entre especies, conduciendo a la inherente descripción de nuevas especies (Pérez-Ponce de León y Nadler, 2010; Nadler y Pérez-Ponce de León, 2011; Razo-Mendivil et al., 2014), así como también la resolución de problemas taxonómicos a diferentes niveles de la jerarquía taxonómica, por ejemplo, la sinonimia de especies; 3) precisar y reconocer la especificidad hospedadora de los parásitos o descartarla cuando son en realidad especies generalistas (Schulenburg y Wagele, 1998; Overstreet et al., 2002; Hernández-Mena et al., 2014); 4) precisar el intervalo de distribución geográfica a nivel de especies (Goulding y Cohen, 2014); 5) usar las filogenias de taxa supraespecíficos, como herramienta para proponer hipótesis sobre la evolución de los complejos ciclos de vida (Poulin y Cribb, 2002; Cribb et al., 2003, Littlewood et al., 2015).

El uso de marcadores con diferentes tasas de sustitución ha permitido contestar preguntas filogenéticas a distintos niveles, desde poblaciones hasta taxa supraespecíficos; y desde luego por sus altas tasas de cambio también se han utilizado mas recientemente en áreas como la genética de poblaciones y filogeografía. Tal es el caso del ADN ribosomal que tiene regiones altamente conservadas (5.8S, 18S y 28S) y otras altamente variables (espaciadores transcritos internos o ITS) (Nolan y Cribb, 2005), o bien de genes mitocondriales como la subunidad 1 de la citocromo c oxidasa (*cox*1).

La utilización de marcadores moleculares en parásitos para generar hipótesis filogenéticas ha sido creciente en los últimos años. Búsquedas de secuencias de ADN que son publicas en el GenBank, nos permite conocer algunas tendencias en el uso de los siguientes marcadores: la subunidad ribosomal mayor (28S), la subunidad ribosomal menor (18S), los ITS (que incluyen el ITS1, el 5.8S y el ITS2) del ADN nuclear y la citocromo oxidasa subunidad I (*cox* 1) del ADN mitocondrial. Los datos recabados a través de estas búsquedas nos permiten visualizar que en general hay una tendencia a generar un mayor número de secuencias de diferentes especies. En la gráfica de acumulación del número de secuencias por año, podemos observar que en los últimos diez años se presentó un mayor incremento en el número de secuencias de *cox* 1, con respecto a los otros marcadores analizados (Fig. 1). Este incremento se relaciona con dos factores principales: uno de los objetivos de la taxonomía es la identificación y delimitación de especies filogenéticamente cercanas, por lo que la generación de secuencias con una tasa de sustitución relativamente alta como el *cox* 1 permite responder a ese tipo de preguntas (Vilas et al., 2005) y por lo tanto, permite diferenciar entre especies a nivel molecular (incluso entre especies hermanas; Vilas et al., 2005; Hernández-Mena et al., 2014), por lo que fue ampliamente utilizado para establecer límites entre especies. El segundo factor se relaciona con el hecho de que *cox* 1 es lo suficientemente variable como para discernir, no solo entre especies sino también entre diferentes poblaciones (Vilas et al., 2005), lo que lo ha convertido en un marcador altamente utilizado en estudios de estructura poblacional. Los trabajos filogeográficos y de genética de poblaciones requieren del uso de un importante número de individuos secuenciados por cada especie que se analiza, para poder explorar con mayor detalle la heterogeneidad poblacional; por lo tanto, dado que este tipo de estudios en parásitos se ha incrementado durante las últimas dos décadas, el número de secuencias de los marcadores asociados a estas investigaciones también aumentó rápidamente (ver Fig. 1A). Con respecto a otros marcadores mitocondriales como la deshidrogenasa 1 (ND1 ó NADH1), se puede mencionar que también tiene una tasa de sustitución mayor a aquella de los marcadores ribosomales, e incluso es ligeramente mayor que la tasa de *cox* 1 (Vilas et al., 2005), y por ello también ha sido considerado en varios estudios poblacionales, en donde ha resultado eficaz para analizar la variabilidad intraespecífica (Crioscione y Boulin 2004; Králová-Hromadová et al., 2011; Liu et al., 2012); a pesar de su eficiencia, no ha sido un marcador de uso común entre los investigadores; lo mismo ocurre con otros marcadores mitocondriales que han sido efectivos para estudios poblacionales; tal es el caso de *cox* 3, que al parecer es más variable que *cox* 1 en digéneos (Brusentsov et al., 2013).

A pesar de la gran cantidad de secuencias que se tienen del marcador *cox* 1, la gráfica de acumulación de número de especies por año, muestra que el número de especies secuenciadas con *cox* 1 no se ha incrementado en la misma proporción que los marcadores nuleares, a pesar de la tendencia hacia el aumento debido a los estudios enfocados a la generación de códigos de barras genéticos. Uno de los principales problemas del marcador *cox* 1 es que éste no se ha secuenciado de manera homogénea es decir, que hay varios trabajos que han secuenciado fragmentos de *cox* 1 que no son homólogos entre sí lo que dificulta, o evita completamente, su comparación.

En la gráfica de acumulación de especies también se muestra que el marcador ribosomal 28S es el que cuenta con un mayor número de especies secuenciadas hasta Diciembre del 2017 (Fig. 1B). El gen 28S cuenta con regiones altamente conservadas que tienen tasas de cambio similares a las del gen 18S, y también tiene regiones con una tasa de cambio más elevada (Hillis y Dixon, 1991); esta heterogeneidad de las tasas de sustitución en el 28S ha favorecido ampliamente su elección como el marcador idóneo para investigaciones a distintas escalas taxonómicas, desde el nivel de especies (p. ej. Razo-Mendivil et al., 2014), hasta familias, superfamilias, subórdenes o incluso órdenes (Olson et al., 2003; Olson et al., 2005) (Fig. 1B) y explica porque hay un mayor número de especies secuenciadas con este marcador molecular. Sin embargo, también es necesario reconocer que el elevado número de secuencias que representan especies diferentes para estos marcadores es producto de un importante número de trabajos que han aportado información, desde los trabajos pioneros realizados por Campos et al. (1998), Littlewood et al. (1999a y 1999b), Tkach et al. (2000a), Cribb et al. (2001), Littlewood y Olson (2001), Tkach et al. (2001), Olson et al. (2003) y Tkach et al. (2003).

El gen ribosomal 18S es el marcador que tiene la tasa de cambio más lenta de todos los marcadores presentados en las Figs. 1 A,B (Hillis y Davis, 1986), y por ello su uso en digéneos se ha visto restringido casi exclusivamente al conocimiento de la posición filogenética de los taxa mayores dentro del phylum Platyhelminthes (p. ej. Baverstock et al., 1991; Rohde et al., 1995; Littlewood et al., 1999a; Willems et al., 2005) ó bien, a establecer las relaciones entre familias que componen la subclase Digena (v. gr., Cribb et al., 2001; Olson et al., 2003; Olson et al., 2005). Es probable que el menor número de especies de digéneos que se han secuenciado para el gen 18S se deba a que la tasa de sustitución es mas lenta. A pesar de ello, también se ha explorado su eficiencia para inferir relaciones filogenéticas entre especies de la misma familia (v. gr., Kaukas y Rollinson, 1997; Schulenburg y Wagele, 1998; Snyder y Tkach, 2001; León-Régagnon y Paredes, 2002).

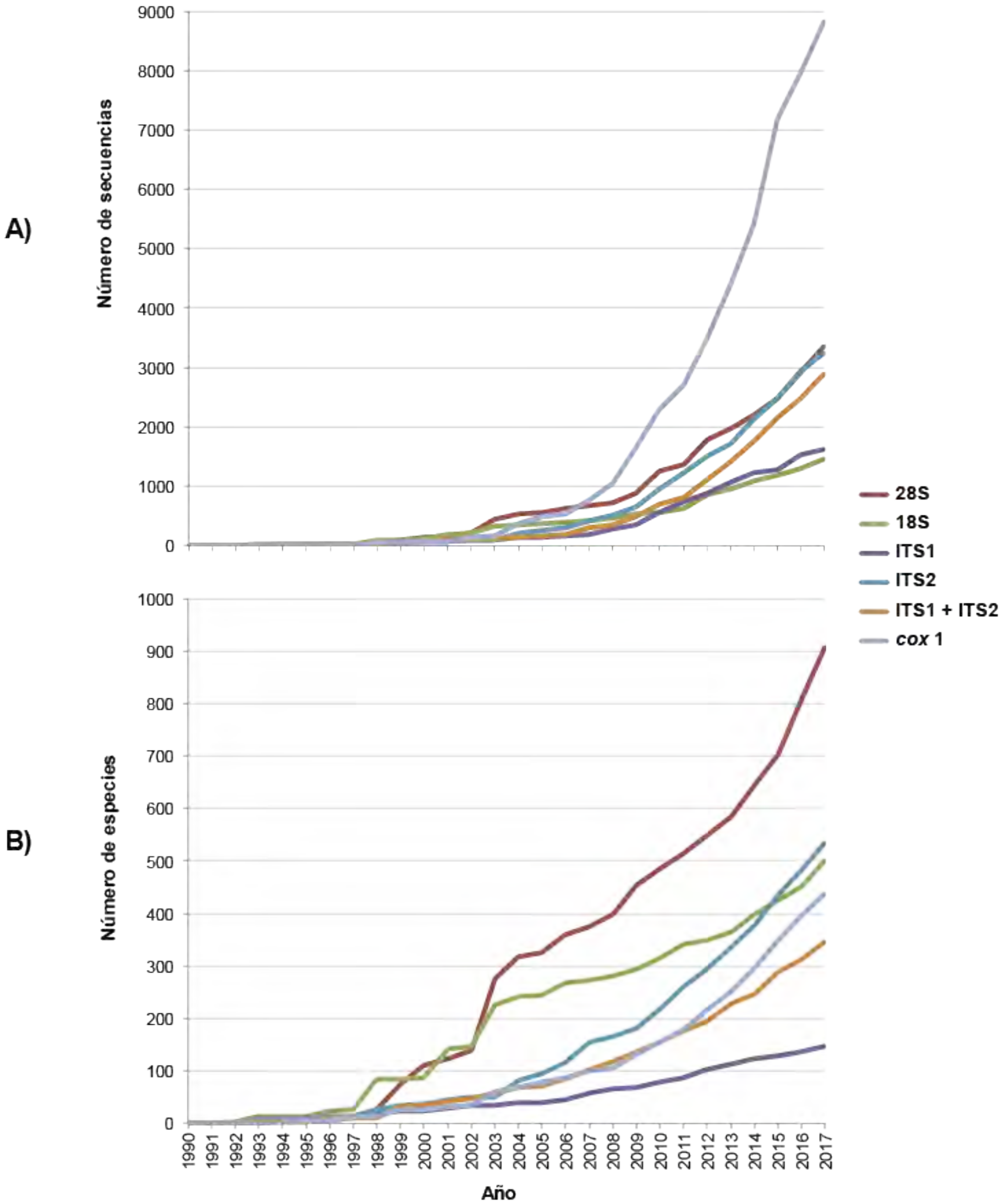


Figura 1. Gráficas de acumulación anuales de A) número de secuencias y B) número de especies por cada uno de los marcadores moleculares que se utilizan con mayor frecuencia en trematodos.

1.3.3. Uso de secuencias de ADN a niveles supraespecíficos

El primer trabajo publicado que incorporó secuencias de especies de trematodos fue realizado por Hu Qu et al. (1986) utilizando el gen ribosomal 18S. Este trabajo pretendía establecer las relaciones filogenéticas entre grupos de helmintos. En los años siguientes, se obtuvieron secuencias de especies digéneos de interés médico y veterinario, como algunas especies en los géneros *Schistosoma*, *Clonorchis*, *Fasciola* y *Fasciolopsis* entre otros (van Herwerden et al., 1998). Sin embargo, la secuenciación de especies que se encontraban parasitando a la fauna silvestre comenzó a realizarse hasta inicios de los años 90´s. A partir de esa década y hasta la mitad de la década pasada, fueron múltiples los estudios que utilizaron secuencias de ADN para esclarecer las relaciones filogenéticas entre los grupos mayores que conforman el phylum Platyhelminthes; éstos incluyeron algunos representantes de la sublcase Digenea (v. gr., Baverstock et al., 1991; Blair, 1993 Blair y Barker, 1993; Rohde et al., 1993 y 1995; Campos et al., 1998; Littlewood et al., 1999a y 1999b; Litvaitis y Rohde, 1999; Littlewood y Olson, 2001; Lockyer et al., 2003, Willems et al., 2005 entre otros). A diferencia de la clase Cestoda, en la que los esfuerzos primero se centraron en la resolución de las interrelaciones de los taxa a un nivel superior de la jerarquía, los estudios sobre la sistemática de los digéneos se concentraron inicialmente en los taxa inferiores (Olson y Tkach, 2005), y se usaron marcadores moleculares que debido a su tasa de cambio mas acelerada, como los ITS y el *cox* 1, por ser mas apropiados para resolver preguntas sobre las relaciones filogenéticas a nivel de géneros, especies y aún dentro de las especies (Nolan & Cribb, 2005; Vilas et al., 2005). No fue sino hasta inicios del siglo 2000 cuando se intensificaron los esfuerzos para generar secuencias de ADN de una mayor diversidad de digéneos, con el propósito de inferir las relaciones filogenéticas de las familias que conforman la subclase Digenea.

Cribb et al. (2001) fueron de los primeros en proponer una hipótesis filogenética de las familias de digéneos utilizando una matriz en donde combinaron caracteres morfológicos con secuencias del gen ribosomal 18S, para un total de 75 especies pertenecientes a 55 familias de digéneos. Estos autores combinaron ambas fuentes de información (morfológica y molecular) en un análisis de evidencia total, y utilizaron los métodos de inferencia de mínima evolución y de parsimonia para sus reconstrucciones filogenéticas, los cuales resultaron en topologías similares. Asimismo, Cribb et al. (2001) concluyeron que la familia Heronimidae no representaba el grupo hermano del resto de Digenea, contrario a lo que anteriormente se había sugerido debido a la simplicidad de su ciclo de vida (Brooks et al., 1985). Independientemente de que Cribb et al. (2001) encontraron topologías resueltas, los internodos son relativamente cortos y los valores de soporte de los clados son bajos, hecho que se atribuye a que el marcador utilizado (18S) es muy conservado aún a este nivel. La menor variación del gen 18S en digéneos contrasta con la mayor variabilidad del mismo marcador en cestodos (Olson y Tkach, 2005), y por lo tanto, un par de años más tarde se decidió también adicionar secuencias del 28S del DNA ribosomal, que es más variable y aporta una mejor señal filogenética para las comparaciones entre familias.

Olson et al. (2003) propusieron una nueva hipótesis filogenética que confirmó los patrones generales encontrados previamente por Cribb et al. (2001). En dicho trabajo, Olson et al. (2003) ampliaron el muestreo de taxones y el número de marcadores moleculares y utilizaron una combinación de secuencias del 18S y del 28S para 163 especies representantes de 77 familias de la sublcase Digenea, y además agregaron siete especies de la subclase Aspidosgastrea como grupo externo. En este estudio, ya no se utilizó una matriz de caracteres morfológicos para realizar las inferencias filogenéticas. Olson et al. (2003) realizaron inferencias filogenéticas basadas en Parsimonia e Inferencia Bayesiana, para cada marcador de manera independiente y además construyeron una matriz concatenada de ambos marcadores. Basados en las topologías obtenidas, los autores encontraron que la clasificación tradicional de los digéneos que contemplaba ordenes como Echinostomida La Rue, 1957, Plagiorchiida La Rue, 1957 y Strigeida Poche,1926 resultaron parafiléticos y propusieron una nueva clasificación filogenética de las familias en dos grandes ordenes, Diplostomida (=Strigeida *sensu lato*) que fue propuesto como nuevo orden en dicho trabajo, y Plagiorchiida. Estos ordenes quedaron subdivididos en 22 superfamilias que contenían a su vez a 8 familias en Diplostomida y 69 en Plagiorchiida (Olson et al., 2003). De acuerdo con Olson et al. (2003) el orden Diplostomida agrupa a las superfamilias Brachylaimoidea, Diplostomoidea, y Schistosomatoidea; y el orden Plagiorchiida agrupa a Bivesiculoidea, Transversotrmatoidea, Azygioidea, Hemiuroidea, Heronimoidea, Bucephaloidea, Gymnophalloidea, Paramphistomoidea, Pronocephaloidea, Haplosplanchnoidea, Echinostomatoidea, Opisthorchioidea, Apocreadioidea, Lepocreadioidea, Monorchioidea, Gorgoderoidea, Allocreadioidea, Plagiorchioidea y Microphalloidea (ver también, Cribb et al., 2003). Ellos también concluyen con sus análisis que hay 18 familias con problemas taxonómicos, ya que en los filogramas son recobradas como parafiléticas (Brachylaimidae + Leucochloridiidae, Diplostomidae + Strigeidae, Hemiuridae + Lecithasteridae, Microscaphidiidae + Mesometridae, Echinostomidae + Fasciolidae, Heterophyidae + Opisthorchiidae, Haploporidae + Atractotrematidae, Opecoelidae + Opistholebetidae y Zoogonidae + Faustulidae).

El estudio más reciente de las relaciones globales dentro de Digenea fue realizado por Littlewood et al. (2015). Ellos mencionaron que hasta el 2015 había descritas 150 familias *versus* las 148 que fueron reconocidas en la serie de libros "Keys to Trematoda" (Gibson et al., 2002; Jones et al., 2005; Bray et al., 2008). Estos autores usaron secuencias de ADN del 18S y 28S que estaban publicadas en el GenBank hasta enero del 2013, e incluyeron solo aquellas secuencias con una longitud mayor a 500 pares de bases. En total, incluyeron secuencias de 92 familias de Digenea y sus matrices de datos quedaron conformadas por 202 especies para el 18S y 556 especies para el 28S. Gracias a su análisis, precisaron que la subclase Digenea se conforma de 2 ordenes y 24 superfamilias, 2 superfamilias más que Olson et al. (2003). La superfamilia Allocreadioidea *sensu* Olson et al. (2003) no la reconocieron y en su lugar la dividieron en las dos superfamilias Brachycladioidea y Opecoeloidae; adicionalmente también reconocen la superfamilia Haploporoidea, la cual había sido incluida por Olson et al. (2003) dentro de Gorgoderoidea.

De todo lo anterior se desprende que la clasificación de los digéneos aún requiere hacer revisiones de los grupos supraespecíficos para establecer con claridad las relaciones entre sus miembros y para resolver todos aquellos casos en los que hay grupos parafiléticos. La idea central del presente estudio es precisamente la de contribuir en esa dirección, aprovechando que las tecnologías para generar secuencias que se han desarrollado en las últimas décadas han permitido incrementar la librería genética para este grupo de parásitos y por ello, resulta relevante realizar nuevas inferencias filogenéticas en la búsqueda de una clasificación más estable. Con base en ello, en el presente trabajo de tesis se plantearon los siguientes objetivos:

2. OBJETIVOS

2. OBJETIVOS

2.1. Objetivo general

Utilizar secuencias del gen ribosomal 28S en conjunto con evidencia morfológica, ecológica y geográfica para investigar las relaciones filogenéticas de digéneos (Trematoda) a diferentes escalas de la jerarquía taxonómica (especies, familias, superfamilias y suborden) y proveer de clasificaciones que reflejen las relaciones entre los grupos.

2. 2. Objetivos particulares

1. Formular hipótesis sobre la posición filogenética de especies de digéneos pertenecientes a 26 familias, las cuales no han sido secuenciadas hasta la fecha y que se encuentran parasitando distintas especies de vertebrados que se distribuyen en México, y en su caso, proponer nuevas especies y taxones supraespecíficos.

2. Poner a prueba la eficiencia del gen citocromo oxidasa subunidad I (*cox* 1) para la inferencia filogenética a niveles supraespecíficos.

3. Poner a prueba la robustez de las hipótesis filogenéticas de Cribb et al. (2001), Olson et al. (2003) y de Littlewood et al. (2015) con la incorporación de un mayor número de terminales pertenecientes a diferentes especies y familias de la subclase Digenea utilizando el gen ribosomal 28S.

3. CAPÍTULO I

3. CAPÍTULO I. DESCRIPCIÓN DE NUEVAS ESPECIES Y SUS RELACIONES FILOGENÉTICAS

En este capítulo se aborda uno de los objetivos particulares de esta tesis, referente a la descripción de nuevas especies. Durante la colecta de distintos vertebrados que actúan como huéspedes definitivos de varios grupos de digéneos, se capturaron peces dulceacuícolas pertenecientes al orden Characiformes. En éstos se recolectaron parásitos pertenecientes a la familia Allocreadiidae de los géneros: *Auriculostoma*, *Creptotrematina* y *Wallinia*. Entre los digéneos recolectados se detectó la presencia de tres especies nuevas, mismas que fueron descritas en dos publicaciones. La descripción de las nuevas especies se basó en la comparación de caracteres cualitativos y cuantitativos con especies previamente descritas. Adicionalmente se obtuvieron secuencias de los genes 28S e ITS para determinar las afinidades filogenéticas y las distancias genéticas para estos marcadores. Además de las comparaciones morfológicas, genéticas, y de los análisis filogenéticos, la descripción de las especies se complemento con la evidencia de su distribución geográfica y afinidad hospedadora.

En el primer artículo de este capítulo se abordó la descripción de una especie perteneciente al género *Auriculostoma*, la cual parasita al pez *Brycon guatemalensis* en la cuenca del río Usumacinta, y se incluyó una clave taxonómica para la identificación de las especies pertenecientes a este género de digéneo. Este artículo ya se encuentra publicado en la revista Zootaxa. En el segundo artículo del capítulo, se realiza la descripción de dos especies: una del género *Wallinia* que parasita al pez *Astyanax aeneus* en cuencas hidrológicas desde Oaxaca a Guatemala, y otra perteneciente nuevamente al género *Auriculosotoma* pero que parasita al pez *Gymnotus maculosus* en ríos del noroeste de Costa Rica. Este segundo artículo será enviado para su publicación a la revista Zookeys.

Zootaxa 4196 (2): 261–277
http://www.mapress.com/j/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

http://doi.org/10.11646/zootaxa.4196.2.5
http://zoobank.org/urn:lsid:zoobank.org:pub:87539887-8919-45C1-9326-EACB8B9283D9

A new species of *Auriculostoma* (Trematoda: Allocreadiidae) from the intestine of *Brycon guatemalensis* (Characiformes: Bryconidae) from the Usumacinta River Basin, Mexico, based on morphology and 28S rDNA sequences, with a key to species of the genus

DAVID IVÁN HERNÁNDEZ-MENA[1,2], CHRISTINA LYNGGAARD[1], BERENIT MENDOZA-GARFIAS[1] & GERARDO PÉREZ-PONCE DE LEÓN[1*]

[1]*Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), Ap. Postal 70-153, C.P. 04510, México D.F., México. E-mail: dahernandez.243@gmail.com; lynggaardc@gmail.com; berenit@ib.unam.mx; ppdleon@ib.unam.mx*

[2]*Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, México, D.F., México*

Abstract

We describe a new species of *Auriculostoma* Scholz, Aguirre-Macedo & Choudhury, 2004 based on several sources of information including morphology (light and scanning electron microscopy [SEM]), sequences of two nuclear genes, host association, and geographical distribution. Morphologically, the new species most closely resembles *Auriculostoma astyanace* Scholz, Aguirre-Macedo & Choudhury, 2004, but differs by having deeply lobated testes and cirrus-sac extending posteriorly to seminal receptacle level. *Auriculostoma lobata* **n. sp.** can be readily distinguished from all the other congeners by the combination of the following characters: testes located in tandem, testes deeply lobated, and larger body size. A phylogenetic analysis using 28S rDNA sequences along with those available for other allocreadiid trematodes, revealed that the new species is a sister taxon of *A. astyanace*, a species described from the banded astyanax, *Astyanax fasciatus* (Cuvier) in Nicaragua. *Auriculostoma totonacapanensis* Razo-Mendivil, Mendoza-Garfias, Pérez-Ponce de León & Rubio-Godoy, 2014 from the Mexican tetra, *Astyanax mexicanus* (De Filippi) in Mexico is the sister taxon of *A. astyanace* plus the new species. Genetic divergence levels for the 28S rDNA and ITS2 were estimated among the Middle-American species of *Auriculostoma* infecting characiforms. The validity of the new species is then established by reliable morphological differences, its host association to bryconids (*Brycon guatemalensis* Regan), restricted geographical distribution (Usumacinta and Lacantun River basins), and genetic divergence levels, albeit relatively low. A morphometric comparison between the new species and the other seven congeneric species was undertaken and, in addition, a taxonomic key to identify the species contained in the genus *Auriculostoma*, widely distributed across the Americas, is provided.

Key words: Digenea, 28S rDNA, ITS, scanning electron microscopy, integrative taxonomy, Mexico, *Auriculostoma lobata* **n. sp.**, Allocreadiidae, *Brycon guatemalensis*, Tabasco, Chiapas, Mexico

Introduction

Members of the Allocreadiidae Looss, 1902 are the most widely distributed freshwater fish trematodes across the Americas. Among allocreadiids, the genus *Auriculostoma* Scholz, Aguirre-Macedo & Choudhury, 2004 contain seven species exclusively distributed in the Neotropical biogeographical region (see Choudhury *et al*. 2016). Two of these species, *Auriculostoma platense* (Szidat, 1954) Scholz, Aguirre-Macedo & Choudhury, 2004, and *Auriculostoma macrorchis* (Szidat, 1954) Scholz, Aguirre-Macedo & Choudhury, 2004 are found in siluriforms and characiforms in South America. The other five species are only found in characiforms, and include three species from South America, i.e., *Auriculostoma stenopteri* (Mañé-Garzón & Gascón, 1973) Scholz, Aguirre-Macedo & Choudhury, 2004, *Auriculostoma foliaceum* Curran, Tkach & Overstreet, 2011, and *Auriculostoma diagonale* Curran, Tkach & Overstreet, 2011, and two species from Middle-America, i.e., *Auriculostoma astyanace* Scholz, Aguirre-Macedo & Choudhury, 2004, the type species of the genus, and *Auriculostoma totonacapanensis* Razo-Mendivil, Mendoza-Garfias, Pérez-Ponce de León & Rubio-Godoy, 2014 (Szidat 1954;

Accepted by N. Dronen: 12 Oct. 2016; published: 22 Nov. 2016

Mañé-Garzón & Gascón 1973; Scholz *et al*. 2004; Kohn *et al*. 2007; Curran *et al*. 2011; Razo-Mendivil *et al*. 2014).

Species of *Auriculostoma* are characterized by having a funnel-shaped oral sucker with a single narrow muscular lobe on either side, spherical and pre-equatorial ventral sucker, uterus usually pre-testicular, vitelline follicles laterally distributed along the body, from the anterior third to the posterior end, and testes distributed in tandem or oblique, located in the posterior part of the body (Razo-Mendivil *et al*. 2014). The major diagnostic character of this genus is the presence of a single pair of muscular lobes on either side of the oral sucker, with a broad base that extends from the ventrolateral to the dorsolateral side of the oral sucker (Razo-Mendivil *et al*. 2014).

The two species currently occurring in Middle-America are parasites of small-bodied characids. *Auriculostoma astyanace* is a parasite of the banded astyanax, *Astyanax fasciatus* (Cuvier) from Nicaragua (Scholz *et al*. 2004), whereas *A. totonacapanensis* is found in the Mexican tetra, *Astyanax mexicanus* (de Fillipi) from Veracruz, Mexico. During the course of an investigation aimed at describing the genetic variation of the helminth fauna of characiforms across Middle-America, specimens of the Machaca, *Brycon guatemalensis* Regan, were collected in the El Mangal Lagoon within the Usumacinta River basin, in southern Mexico, and studied for helminth parasites. The species *A. astyanace* had been previously recorded as a parasite of *B. guatemalensis* by Salgado-Maldonado *et al*. (2011) in the Usumacinta and Lacantun Rivers in Chiapas, Mexico, however the authors overlooked important morphological characteristics that set this species apart, and the assignment as *A. astyanace* we believe was erroneous. A close morphological examination of these specimens, and the morphological and molecular study of the material we sampled from the same host species and river system (Usumacinta), allowed us to determine that they in fact represent an undescribed species. The new species is herein described, a molecular phylogenetic analysis is undertaken to establish the sister group relationships along with other allocreadiid trematodes, and a key to identify the species of the Neotropical genus *Auriculostoma* is presented.

Material and methods

Specimen collection. Adult specimens of the genus *Auriculostoma* were collected from the intestine of *Brycon guatemalensis*, in El Mangal Lagoon, Tenosique, Tabasco, Mexico in October, 2014 (Table 1). Worms recovered from their hosts were rinsed in 0.6% saline; five specimens were fixed in 100% ethanol for molecular analysis and 10 specimens were fixed in hot (steaming) 4% formalin for morphological study.

Morphological study. Specimens were stained with Mayer's paracarmine, dehydrated in a graded ethanol series, cleared in methyl salicylate, and mounted as permanent slides with Canada balsam. A single individual was used for scanning electron microscopy (SEM) study. The specimen was dehydrated through a graded series of ethyl alcohol, and then critical point dried with carbon dioxide. Specimens were mounted on a metal stub with carbon adhesive tabs, then gold coated, and examined at 15 kV in a Hitachi Stereoscan Model SU1510 SEM (Hitachi Ltd., Tokyo, Japan). Drawings were made with a drawing tube attached to the light microscope. Measurements are presented in micrometers (µm) with the range followed by the mean in parentheses (Table 2). Specimens were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM). For morphological comparison, type and voucher specimens of the following allocreadiid trematodes were studied: 1 holotype and 1 paratype of *A. astyanace* from *A. fasciatus*, Loonku Creek, Bluefields, Región Autónoma del Atlántico del Sur (RAAS), Nicaragua (CNHE 4923, 4924), 6 voucher specimens of *A. astyanace* from *B. guatemalensis*, Usumacinta, Chiapas, Mexico (CNHE 7505), and 1 holotype and 7 paratypes of *A. totonacapanensis* from *A. mexicanus* from Filipinas, Veracruz, Mexico (CNHE 8471), and 1 paratype U.S. National Parasite Collection (USNPC 107880).

DNA extraction, amplification, and sequencing. Five specimens were digested overnight at 56 °C in a solution containing 10 mM Tris–HCl (pH 7.6), 20 mM NaCl, 100 mM Na2 EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. DNA was extracted from the supernatant using the DNAzol (Molecular Research Center, Cincinnati, Ohio). The domains D1-D3 of the large subunit from nuclear ribosomal DNA (28S) and the internal transcribed spacers (ITS1-5.8S-ITS2) from nuclear ribosomal DNA were amplified using polymerase chain reaction (PCR) using the forward primer 502 5′-CAAGTACCGTGAGGGAAAGTTGC-3′ (García-Varela & Nadler 2005) and the reverse primer 536 5′-CAGCTATCCTGAGGGAAAC-3′ (García-Varela & Nadler 2005) for

TABLE 1. Genbank accesion number of the digenean species used for phylogenetic analysis in alphabetical order.

Digenean species	GenBank accession numbers	Host species	Locality	Reference
Allocreadiidae Looss, 1902				
Allocreadium isoporum (Looss, 1894) Looss, 1900	GU462125	*Alburnus alburnus* (Linnaeus)	Lake Oster, Karelia, Russia	Petkevičiūtė *et al.* (2010
Allocreadium lobatum Wallin, 1909	EF032693	*Semotilus corporalis* (Mitchill)	Moosehead Lake, Maine, USA	Curran *et al.* (2006)
Allocreadium neotenicum Peters, 1957	JX977132	*Hydroporus rufifrons* (Duftschmid)	Lake District, Cumbria, United Kingdom	Bray *et al.* (2012)
Auriculostoma astyanace Scholz, Aguirre-Macedo & Choudhury, 2004	HQ833707	*Astyanax aeneus* (Günther)	Tempisquito River, Guanacaste, Costa Rica	Curran *et al.* (2011)
	KF631422	*Astyanax fasciatus* (Cuvier)	Sapoa River, Guanacaste, Costa Rica	Razo-Mendivil *et al.* (2014)
Auriculostoma lobata **n. sp.**	KX954170-74	*Brycon guatemalensis* Regan	El Managal Lagoon, Tenosique, México	Present study
Auriculostoma totonacapanensis Razo-Mendivil, Mendoza-Garfias, Pérez-Ponce de León & Rubio-Godoy 2014	KF631417 KF631418	*Astyanax mexicanus* (De Filippi)	Filipinas, Veracruz, Mexico	Razo-Mendivil *et al.* (2014)
Bunodera eucaliae (Miller, 1936) Brooks, 1992	DQ029329	*Culaea inconstans* (Kirtland)	Brokenhead River, Manitoba, Canada	Choudhury & León-Règagnon (2005)
Bunodera inconstans (Lasee, Font & Sutherland, 1988) Brooks, 1992	DQ029330	*Culaea inconstans* (Kirtland)	Brokenhead River, Manitoba, Canada	Choudhury & León-Règagnon (2005)
Bunodera luciopercae (Müller, 1776) Lühe, 1909	GU462113	*Sphaerium rivicola* (Lamarck)	Tvertsa River, upper Volga River basin, Russia	Petkevičiūtė *et al.* (2010)
Bunodera mediovitellata Tsimbaliuk & Roytman, 1966	DQ029332	*Gasterosteus aculeatus* Linnaeus	Little Campbell River (Camp- bell Creek) B. C., Canada	Choudhury & León-Règagnon (2005)
Bunodera sacculata Van Cleave & Mueller, 1932	DQ029333	*Perca fluviatilis* Linnaeus	Lake Sasajewun, Algonquin Park, Ontario, Canada	Choudhury & León-Règagnon (2005)
Crepidostomum cooperi Hopkins, 1931	DQ029328	*Percopsis omiscomaycus* (Walbaum)	Lake Winnipeg, Manitoba, Canada	Platta & Choudhury (2006)
Crepidostomum cornutum (Osborn, 1903) Stafford, 1904	EF032695	*Lepomis gulosus*(Cuvier)	Pascagoula River, Mississippi, USA	Curran *et al.* (2006)
Creptotrema funduli Mueller, 1934	JQ425256	*Fundulus notatus* (Rafinesque)	Biloxi River, Mississippi, USA	Curran *et al.* (2012)
Creptotrematina aguirrepequenoi Jiménez-Guzmán, 1973	HQ833708 HQ833709	*Astyanax aeneus* (Günther)	Tempisquito River, Guanacaste, Costa Rica	Curran *et al.* (2011)
Margotrema bravoae Lamothe-Argumedo, 1970	KT833277	*Ilyodon furcidens* (Jordan & Gilber)	Ahuacapán, Jalisco, Mexico	Pérez-Ponce de León *et al.* (2015)

……continued on the next page

TABLE 1. (Continued)

Digenean species	GenBank accession numbers	Host species	Locality	Reference
Margotrema resolanae Pérez-Ponce de León, Martínez-Aquino & Mendoza-Garfias, 2013	KT833271	*Xenotaenia resolanae* Turner	Cuzalapa River, Jalisco State, Mexico	Pérez-Ponce de León *et al.* (2015)
Megalogonia ictaluri Surber, 1928	EF032694	*Ictalurus punctatus* (Rafinesque)	Pearl River, Mississippi, USA	Curran *et al.* (2006)
Paracreptotrema blancoi Choudhury, Pérez-Ponce de León, Brooks & Daverdin, 2006	KT833285	*Priapichthys annectens* (Regan)	Orosí River, Costa Rica	Pérez-Ponce de León *et al.* (2015)
Paracreptotrema heterandriae Salgado-Maldonado, Caspeta-Mandujano & Vázques, 2012	KF697693	*Heterandria bimaculata* (Heckel)	Agua Bendita, Xico, Veracruz, Mexico	Razo-Mendivil *et al.* (2014)
Paracreptotrema rosenthali Bautista-Hernández, Monks, Pulido-Flores & Miranda, 2015	KT833287	*Xiphophorus malinche* Rauchenberger, Kallman & Morizot	Malila River, Hidalgo, Mexico	Pérez-Ponce de León *et al.*, (2015)
Pseudoparacreptotrema macroacetabulata Pérez-Ponce de León, Pinacho-Pinacho, Mendoza-Garfias, Choudhury & García-Varela, 2016	KT833322	*Profundulus kreiseri* Matamoros, Schaefer, Hernández & Chakrabarty	Quebrada el Paraiso, Honduras	Pérez-Ponce de León *et al.* (2015)
Pseudoparacreptotrema profundulusi (Salgado-Maldonado, Caspeta-Mandujano & Martínez-Ramírez, 2011) Pérez-Ponce de León, Pinacho-Pinacho, Mendoza-Garfias, Choudhury & García-Varela, 2016	KT833290	*Profundulus* sp. Hubbs	Templo River, San Juan del Río, Oaxaca, Mexico	Pérez-Ponce de León *et al.* (2015)
Wallinia chavarriae Choudhury, Daverdin & Brooks, 2002	HQ833703	*Astyanax aeneus* (Günther)	Animas River, Guanacaste, Costa Rica	Curran *et al.* (2011)
Wallinia mexicana Pérez-Ponce de León, Razo-Mendivil, Mendoza-Garfias, Rubio-Godoy & Choudhury, 2015	KJ535504	*Astyanax mexicanus* (De Filippi)	Covadonga River, Durango, Mexico	Pérez-Ponce de León *et al.* (2015)
	KJ535505	*Astyanax mexicanus*	Huichihuayan River, San Luis Potosí, Mexico	
Callodistomidae Odhner, 1910				
Prosthenhystera caballeroi Jiménez-Guzmán, 1973	KM871185	*Astyanax aeneus* (Günther)	Tampisquto River, Guanacaste, Costa Rica	Tkach & Curran (2015)
Prosthenhystera obesa (Diesing, 1850) Travassos, 1922	AY222206	*Hoplias* sp. Gill	Rio Itaya, Iquitos, Peru	Olson *et al.* (2003)
	EF032690	*Ictalurus punctatus* (Rafinesque)	Pearl River, Mississippi, USA	Curran *et al.* (2006)
Prosthenhystera oonastica Tkach & Curran, 2015	KM871181	*Pylodictis olivaris* (Rafinesque)	Pearl River, Mississippi, USA	Tkach & Curran (2015)

28S, and the forward primer BD1 5′-GTCGTAACAAGGTTTCCGTA-3′ (Bowles & MacManus 1993) and the reverse primer BD2 5′-ATCTAGACCGGACTAGGCTGTG-3′ (Luton *et al.* 1992) for ITS region. PCR reactions (25 µl) consisted of 10 µl of each primer, 2.5 µl of 10x buffer, 2 mM $MgCl_2$, and 1 U of TaqDNA polymerase (Invitrogen Corporation, São Paulo, Brazil). PCR cycling parameters consisted of denaturation at 94 °C for 5 min, followed by 36 cycles of 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min, followed by a post-amplification incubation at 72 °C for 10 min. The same PCR primers plus the following internal primers were used for sequencing reactions: BD2 5′-TATGCTTAAATTCAGCGGGT-3′ (Luton *et al.* 1992), 502 5′-CAAGTACCGTGAGGGAAAGTTGC-3′ (García-Varela & Nadler 2005), 503 5′-CCT TGGTCCGTGTTTCAAGACG-3′ (Stock *et al.* 2001) and 504 5′-CGTCTTGAAACACGGACTAAGG-3′ (García-Varela & Nadler 2005). Sequencing reaction of the ITS was performed using two initial external primers plus two internal primers: BD3 5′-GAACATCGACATCTTGAACG-3′ and 5′-ATAAGCCGACCCTCGGC-3′ (Hernández-Mena *et al.* 2014). Reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contigs were assembled and base-calling differences resolved using Geneious Pro 4.8.4 (Biomatters Ltd.). Sequences were deposited in the GenBank database (Table 1).

Alignment, phylogenetic analyses and sequence variation. Sequences of the 28S rRNA gene of the new species were aligned with those of other congeneric species and with those of species belonging to 11 other genera of the family Allocreadiidae available in Genbank (see Table 1). Sequences were aligned with the software ClustalW (Thompson *et al*. 1997), implemented in the website http://www.genome.jp/tools/clustalw/. Trees search was performed with Maximum Likelihood method (ML). The ML trees were inferred using the program RAxML 7.0.4 (Stamatakis 2006). Analyses were carried with 100 replicates and GTR+GAMMA+I model was used. Each analysis was initiated with a random starting tree, employing the rapid hill-climbing algorithm (−x) (Stamatakis *et al*. 2007). Clade support was assessed with 10,000 bootstrap replicates, implementing the rapid-hill climbing algorithm (Stamatakis *et al*. 2008). Species of the genus *Prosthenhystera* Travassos, 1922 (Callodistomidae) were used as an outgroup for rooting the trees. The phylogenetic tree obtained was drawn with the software FigTree version 1.4.2. Molecular variation of 28S data set was estimated using uncorrected *p* distances (p-distances) with the software PAUP* 4.0b10 (Swofford 2003). In addition, molecular variation of ITS2 was estimated to compare the five newly sequenced individuals with their close relatives, *A. astyanace* and *A. totonacapanensis* (GenBank accession numbers KX954165- KX954169, KF631428 and HQ833707, KF631423-26, respectively).

Results

Family Allocreadiidae Looss 1902

Genus *Auriculostoma* Scholz, Aguirre-Macedo & Choudhury, 2004

Auriculostoma lobata n. sp.

(Figs. 1–2)

Synonym. *Auriculostoma astyanace* of Salgado-Maldonado *et al.* (2011)

Type-host. machaca, *Brycon guatemalensis* Regan (Actinopterygii: Bryconidae).

Type-locality. El Mangal Lagoon, Usumacinta River basin, Tenosique, Tabasco, Mexico (17°38'46.99" N, 91°22'58.0" W).

Other localities. Lacantun River, Chiapas, Mexico (16°06′03″ N, 90°57′30″ W and 16° 14′ 46″ N, 90° 50′ 08″ W); Usumacinta River, Chiapas, Mexico (16°49′24″ N, 90°53′18″ W).

Site of infection. Intestine.

Specimens deposited. Holotype (CNHE 1081); paratypes: 8 specimens from Tabasco (CNHE 1082) and 6 specimens from Chiapas (CNHE 7505).

Etymology. The specific name *lobata* is the nominative feminine singular of the Latin adjective "lobatus" and is referring to the lobated form of the testes, in this case with 5 to 6 deep lobes.

Description. (Based on 15 adult specimens, nine from Tabasco, and six from Chiapas—measurements in Table 2). Body elongate, slightly narrowing at pharyngeal level. Tegument unspined. Oral sucker subterminal, funnel-shaped, well-developed, with 19 dome-like papillae arranged in 4 rows: 4 apical, 6 anterior (slightly noticeable), 4 on inner surface (two near mouth), and 5 on outer surface (Fig. 2B,D). Oral sucker with a single pair of muscular lobes located on either side of oral sucker, with a broad base. Muscular lobes stretching from ventrolateral to dorsolateral side and slightly differentiated of apical region; dorsolateral side of lobes narrow with triangle-shaped "free" ends. Mouth subterminal. Prepharynx absent. Pharynx muscular, ovoid and well developed. Esophagus curved. Intestinal cecal bifurcation between pharynx and ventral sucker. Intestinal ceca long and narrow, reaching near to posterior extremity. Ventral sucker spherical, strongly muscular, and slightly larger than oral sucker. Testes in tandem, multilobated (deeply lobed), in posterior half of body. Anterior testis usually with 5 lobes and posterior testis with 6 lobes. Post-testicular space representing 21.4–29.4% (24.5%) of body length. Cirrus sac very long, forming several loops (3 to 5), extending to surpass the posterior border of ovary, containing elongate internal seminal vesicle, pars prostatica (bulb shaped), and wide muscular ejaculatory duct. Genital pore median, opening on ventral surface, between intestinal bifurcation and anterior margin of ventral sucker. Ovary pre-testicular, pre-equatorial, sinistral or dextral, oval and entire. Seminal receptacle saccate, post-ovarian. Laurer's canal not observed. Mehlis' gland posterior, between ovary and anterior portion of seminal receptacle. Vitelline follicles small, numerous, forming intra- and extracecal lateral fields along body, extending from cecal bifurcation to posterior end of body, and confluent at post-testicular area. Vitelline reservoir ventral to Mehlis' gland. Uterus completely pre-testicular, intercecal, between anterior testis and genital pore. Eggs moderately small. Excretory vesicle I-shaped, reaching to posterior margin of posterior testis. Excretory pore terminal.

Remarks. *Auriculostoma lobata* **n. sp.** conforms to the diagnosis of *Auriculostoma* as recently amended by Razo-Mendivil *et al*. (2014), including the presence of a single pair of muscular lobes on either side of the oral sucker. The new species most closely resembles the type species of the genus, *A. astyanace* as they have similar morphological characteristics such as testes in tandem, a genital pore located between the intestinal bifurcation and ventral sucker, a curved esophagus, vitelline follicles extending from the cecal bifurcation to the posterior end of the hindbody, a cirrus sac forming several loops (up to 5), and an excretory pore terminal. However, they differ in that the new species possesses strongly lobated testes (anterior testis with 5 major lobes and posterior testis with 6 major lobes), and the cirrus sac extends to the post-ovarian region. Instead, *A. astyanace* possesses smooth (entire) testes, and cirrus sac does not surpass the ovarian region. Unfortunately, SEM microphotographs are not available for this species to be compared with those of the new species, and ultrastructure of the body surface may reveal additional differences between the two species.

The new species can be distinguished from the other congeners by the combination of some morphological characters. In particular, the new species differs from three of the other six congeneric species, i.e., *A. diagonale, A. platense* and *A. totonacapanensis* by having testes deeply lobed located in tandem rather than entire testes in oblique postion. Even though in some specimens of *A. totonacapanensis* testes seem to be in tandem (Razo-Mendivil *et al*. 2014), in most of them testes are oblique. Additionally, *A. lobata* **n. sp**. differs from *A. diagonale* and *A. platense* in the anterior extension of the vitelline follicles; in the new species, the vitelline follicles reach the cecal bifurcation, whereas in *A. diagonale* they reach the pharynx level and in *A. platense* follicles reach the mid-level of the esophagus. *Auriculostoma lobata* **n. sp.** differs further from *A. totonacapanensis* by having a genital pore located between the cecal bifurcation and the ventral sucker, and not at the level of the cecal bifurcation as in *A. totonacapanensis*. In addition, the ultrastructure of the body surface of both species provide additional information. We obtained SEM photographs of our specimens and compared them with those from Razo-Mendivil *et al.* (2014). Both species possess 19 dome-like papillae on the oral sucker surface arranged in 4 rows (4 apical, 6 anterior, 4 on inner surface, and 5 on the outer surface) and both have a single pair of muscular lobes located on either side of the oral sucker. However, they can be readily distinguished by the shape of the dorsolateral side of the oral lobes, which in the new species, are "free" and triangular-shaped (see Fig. 2B–C).

Finally, the new species is similar to the other three congeneric species, *A. foliaceum, A. macrorchis* and *A. stenopteri* by possessing multilobated testes. However, the new species is readily distinguished by the anterior extension of the vitelline follicles; in *A. lobata* **n. sp.**, the vitelline follicles reach the cecal bifurcation, whereas in *A. foliaceum*, follicles are absent in the forebody while in *A. macrorchis* and *A. stenopteri* follicles extend up to the level of the oral sucker. Additionally, the new species differs from *A. foliaceum* by having a ventral sucker larger than the oral sucker, an excretory pore terminal, and a smaller oral sucker on average; in *A. foliaceum* the oral sucker is 1.5 times larger than the ventral sucker, the excretory pore is subterminal, and the oral sucker is larger. From *A. macrorchis*, the new species differs further because vitelline follicles are intra- and extracecal in the pre-testicular region and not largely extracecal.

TABLE 2. Comparative measurements among species of the genus *Auriculostoma*.

Character	*A. lobata* **n. sp.**	*A. astyanace*	*A. totonacapanensis*	*A. diagonale*	*A. foliaceum*	*A. macrorchis*	*A. platense*	*A. stenopteri*
n*	14	5	11	2	1	1	1	2
Body L	2,539–3,010 (2,735)	1,900–2,900 (2,900)	1,028–2,003 (1,402)	1,207–1,600	1,993	1,500	800	750–1,320
Body W	502–639 (581)	400–488 (488)	287–568 (398)	352–474	450	500	200	216–308
FB L	457–525 (477) (16–19% (17) of body length)	(25–32%) (25) of body length)	–	(27–28% of body length)	430 (22% of body length)	–	–	–
OS L	153–234 (204)	202–234 (234)	117–212 (156)	170–190	279	180	120	–
OS W (excluding dorsal lobes)	191–245 (206)	208– 246 (246)	128–223 (176)	170–208	374	200	–	–
OS W (including dorsal lobes)	243–335 (267)	256–304 (301)	121– 268 (214)	–	–	–	–	–
PH L	114–139 (124)	93–115 (115)	39–92 (69)	45–57	95	70	–	60–96
PH W	82–112 (99)	90–118 (112)	44–101 (74)	63–68	100	50	–	60–88
ES L	71–141 (112)	70–141 (102)	71–96 (84)	–	111	–	–	40–80
VS L	238–319 (260)	250–285 (275)	153–240 (193)	213–221	207	150	120	148–190
VS W	233–324 (273)	250–330 (330)	154–259 (198)	213–227	202	–	–	140–240
OS L: VS L	1:1.08–1.66 (1.29)	1:1.10–1.17 (1.13)	1:1.03–1.62 (1.25)	1: 1.16–1.25 (1.21)	1: 0.7	1: 0.83	1: 1	–
OS W: VS W (exluding lobes)	1:1.21–1.52 (1.34)	1:1.21–1.29 (1.23)	1:1.01–1.54 (1.13)	1:1.1–1.25	1:0.54	1:1–1.3	1:1	1:1.3
AT L	267–352 (312)	166–266 (256)	103–219 (149)	193–261	106	–	–	60–120
AT W	167–274 (206)	157–218 (218)	62–218 (130)	110–159	134	–	–	15–72
PT L	318–396 (360)	186–349 (349)	118–259 (175)	221–304	167	–	–	80–132
PT W	191–234 (215)	173–211 (205)	62–239 (130)	139–142	145	–	–	80–100

……continued on the next page

TABLE 2. (Continued)

Character	*A. lobata* **n. sp.**	*A. astyanace*	*A. totonacapanensis*	*A. diagonale*	*A. foliaceum*	*A. macrorchis*	*A. platense*	*A. stenopteri*
OV L	164–217 (193)	160–230 (218)	93–210 (134)	156–161	145	–	–	52–120
OV W	92–165 (120)	144–202 (182)	68–167 (107)	102–145	117	–	–	32–52
SR L	150–201 (181)	157– 205	55–135 (84)	85–108	40	–	–	–
SR W	101–115 (115)	102–115 (115)	39–137 (75)	59–77	45	–	–	–
PSTS L	456–793 (647) (21–29% (25) of body length)	–	250–275 (268)	(28% of body length)	560 (28% of body length)	–	–	–
PCS L	116–171 (142) (4–6% (5) of body length)	–	–	204– 245 (15–17% of body length)	141 (7% of body length)	–	–	52–108
GPD	373–461 (411)	–	239–362 (284)	–	–	–	–	–
Eggs L	46–58 (52)	55–59	48–65 (54)	54–57	58–63	55	75	68–76
Eggs W	23–35 (27)	34–41	30–41 (34)	28–32	28–29	29	39	28–40
Reference	This paper	Scholz *et al.* 2004	Razo–Mendivil *et al.* 2014	Curran *et al.* 2011	Curran *et al.* 2011	Szidat 1954	Szidat 1954	Mané–Garzón & Gascón 1973

FB= Forebody, OS= oral sucker, PH= pharynx, ES= Esophagus, VS= ventral sucker, AT= anterior testis, PT= Posterior testis, OV= ovary, SR= seminal receptacle, PSTS= Post-testicular space, PCS= Post-cecal space, GPD= genital pore distance from anterior end of body. L = Length, W = Width. * Number of specimens measured in the original description.

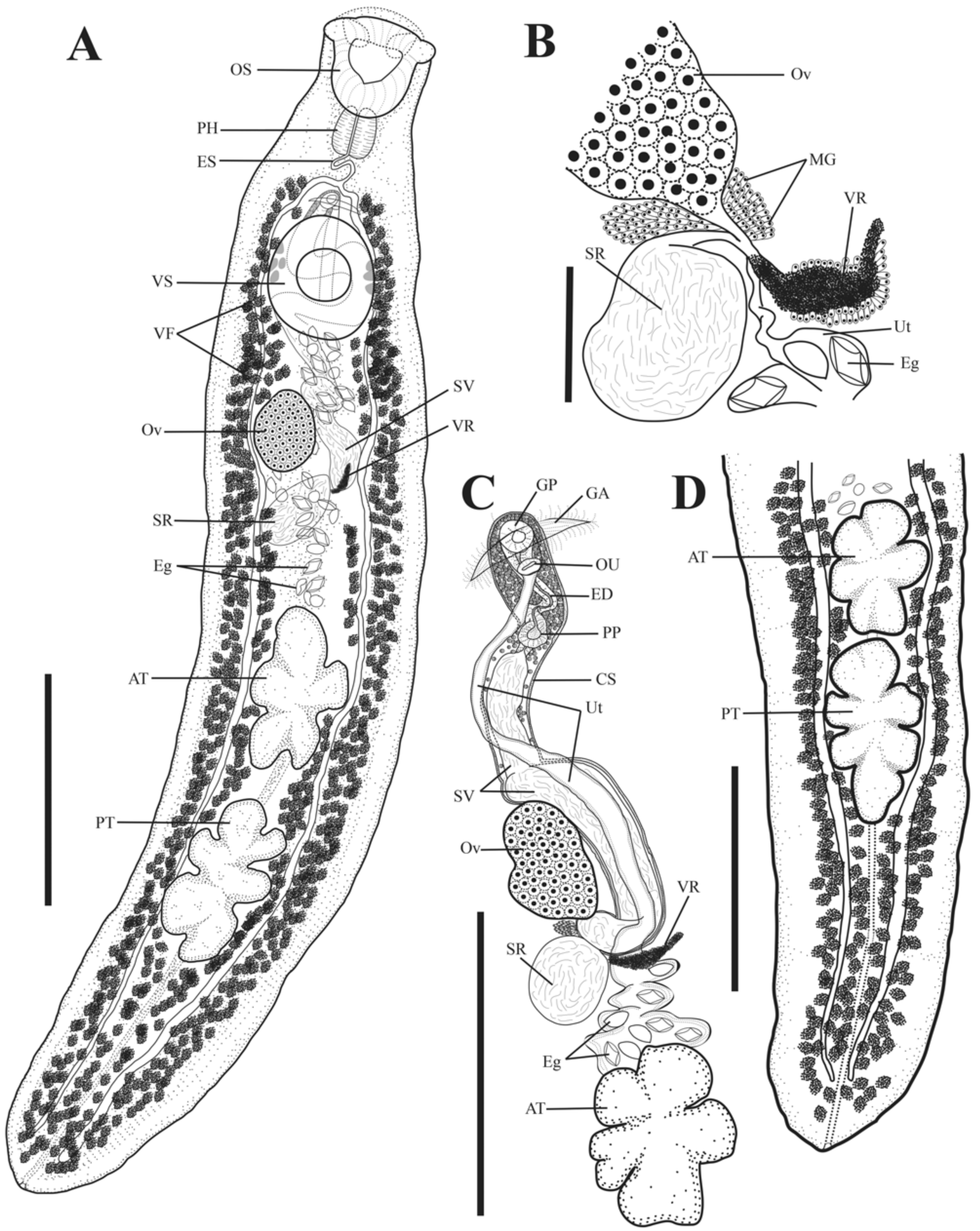


FIGURE 1. *Auriculostoma lobata* **n. sp.** (A) Ventral view of holotype. Scale bar = 500 µm. (B) Ventral view of ovarian complex of paratype. Scale bar = 100 µm. (C) Ventral view of terminal genitalia showing that the ejaculatory duct and the uterus open to the genital atrium (reconstruction made from the observation of several specimens). Scale bar = 500 µm. (D) Detail of the anterior and posterior testes of paratype. Scale bar = 500 µm. Abbreviations: AT= anterior testis, CS= cirrus sac, ED= ejaculatory duct, Eg= eggs, ES= esophagus, GA= genital atrium, GP= genital pore, MG= Mehlis´gland, OS= oral sucker, OU= opening of the uterus into genital atrium, Ov= ovary, PH= pharynx, PP= pars prostatica, PT= posterior testis, SR= seminal receptacle, SV= seminal vesicle, Ut= uterus, VF= vitelline follicles, VS= ventral sucker, VR= vitelline reservoir.

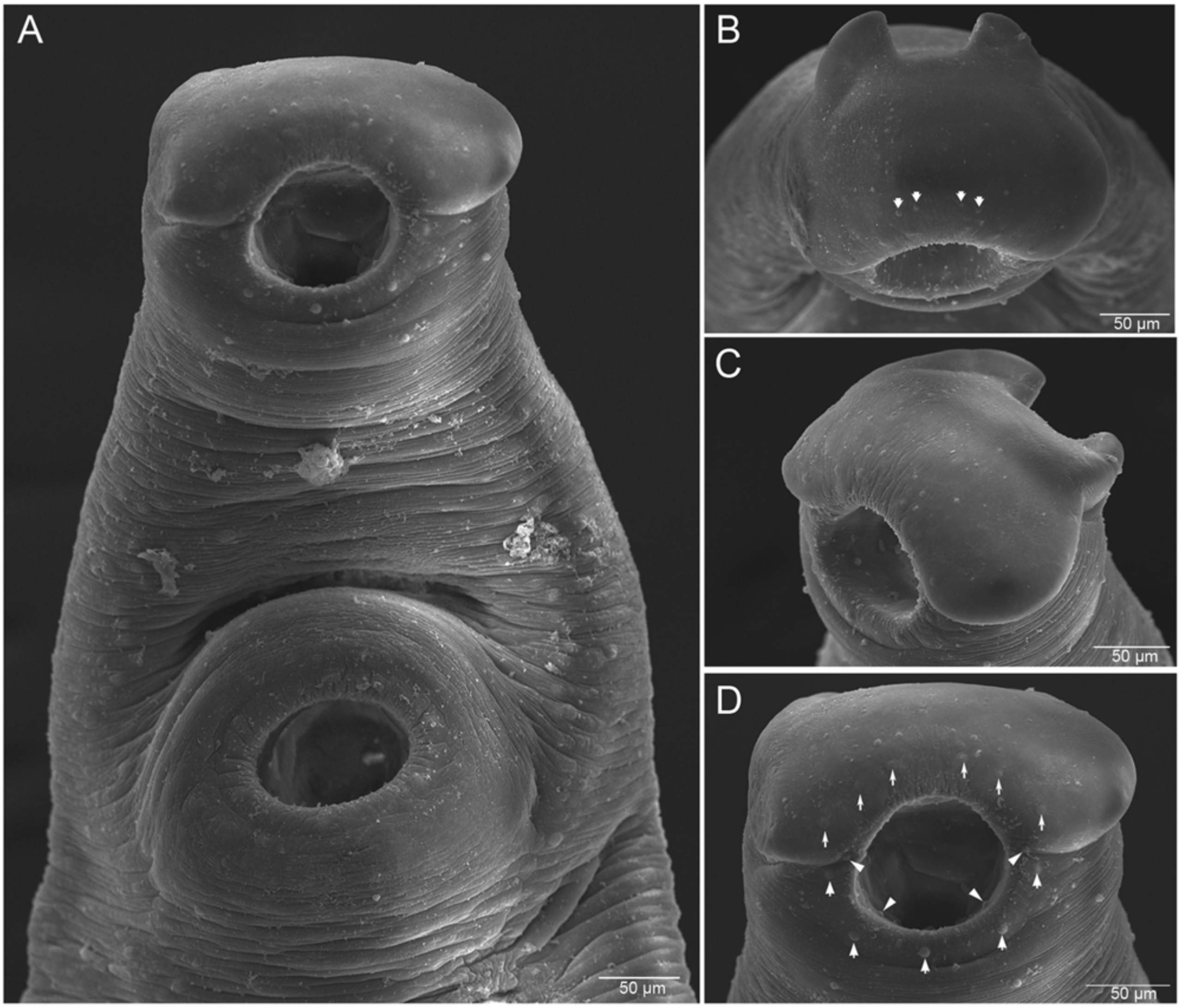


FIGURE 2. Scanning electron micrographs of *Auriculostoma lobata* **n. sp.** (A) Anterior end with oral sucker bearing muscular lobe on either side, genital atrium and ventral sucker. (B) Pair of muscular lobes with a short posterior ''free'' end, and 4 apical dome-like papillae (white arrows). (C) Lateral view of muscular lobe. (D) Distribution of dome-like papillae over oral sucker, 6 anterior (white arrows), 4 on the inner surface (white triangle), and 5 on the outer surface (white arrows).

Phylogenetic analyses and molecular divergence. The final data set for the 28S rRNA gene consisted of 36 sequences, 33 of them representing 25 species of the Allocreadiidae and 3 species of the Callodistomidae as outgroups. Final alignment contained 1,776 base pairs. Nucleotide frequencies were A=0.213, C= 0.215, G= 0.312 and T= 0.261. The selected model for the ML analysis was GTR+GAMMA+I. ML yielded a single tree with ln= -7038.128347. The ML phylogenetic tree showed an unresolved clade that contains the three genera of the Allocreadiidae commonly found as parasites of neotropical characids: *Creptotrematina, Auriculostoma* and *Wallinia* (Fig. 3). The phylogenetic analysis also showed that the genus *Auriculostoma* is monophyletic, with high bootstrap support value (Fig. 3). Each of the three congeneric species are recovered as monophyletic assemblages, with moderate bootstrap support. The five isolates of *Auriculostoma lobata* **n. sp.** are reciprocally monophyletic and are nested as the sister taxa of *A. astyanace*, and this clade as sister group of *A. totonacapanensis*. Intraspecific genetic divergence of the 28S rDNA sequences among individuals of *A. lobata* **n. sp.** was null. Genetic divergence between *A. lobata* **n. sp.** and *A. astyanace*, its sister species, was relatively low (0.29%), and between the new species and *A. totonacapanensis* reached 2.33%. As expected, intergeneric genetic divergence was higher. Between species of *Auriculostoma* and species of *Creptotrematina*, values ranged from 4.07 to 4.80%, and between species of *Auriculostoma* and species of *Wallinia* values varied between 2.47 and 5.38%.

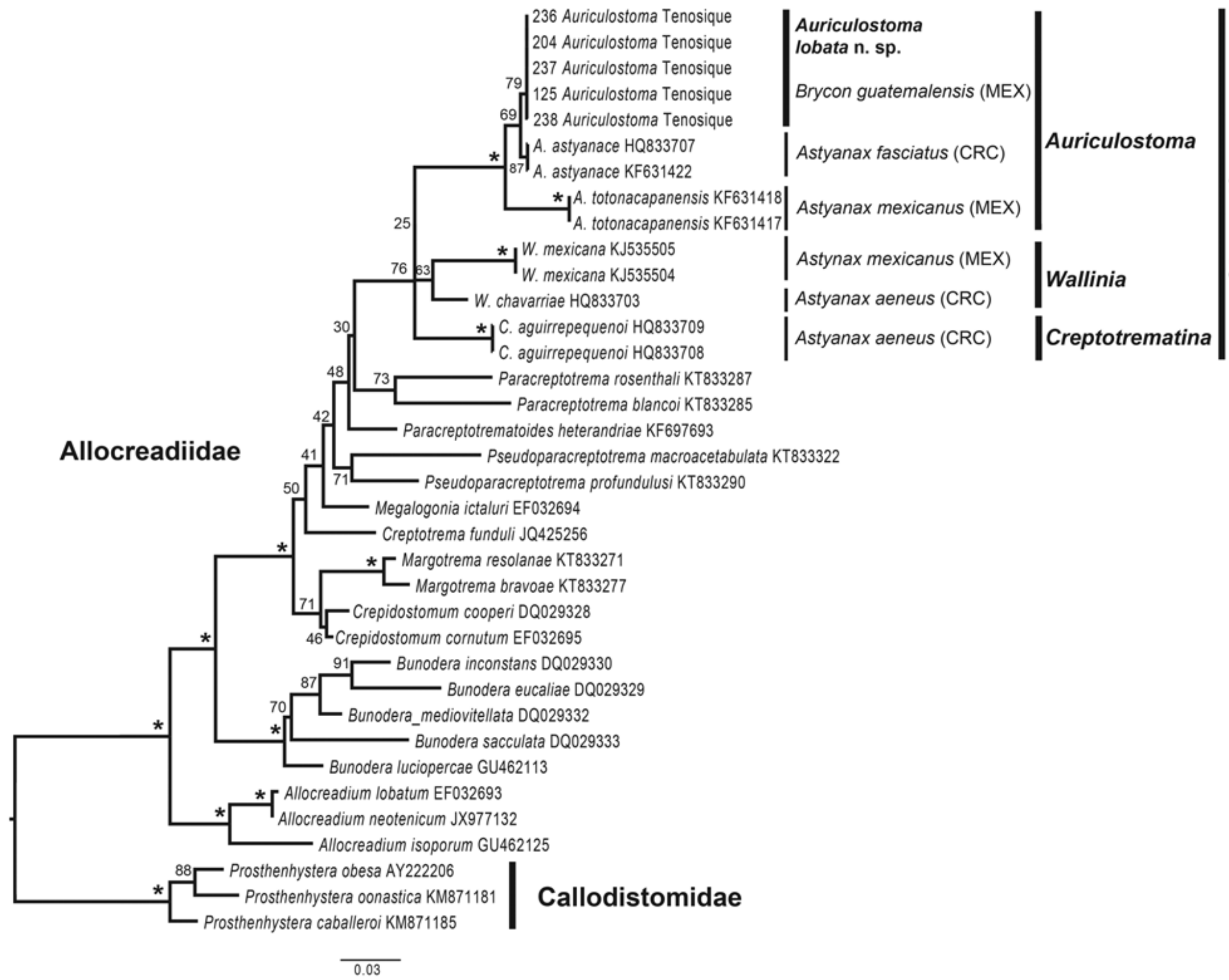


FIGURE 3. Phylogram representing a hypothesis for the relationships of *Auriculostoma lobata* **n. sp.** with other allocreadiids. The tree represents the single best tree obtained from maximum likelihood analysis based on 28S rDNA sequences from allocreadiids and rooted using species of *Prosthenhystera* (Callodistomidae). Numbers near internal nodes indicate bootstrap support values (*= values 100%).

Discussion

An integrative taxonomy approach was taken in this study to describe a new species of *Auriculostoma* whose species are parasites of freshwater fishes across the Americas and are characteristically Neotropical (Choudhury *et al*. 2016). Integrative taxonomy offers a powerful tool to shed light on hidden biodiversity, combining multiple operational criteria in an evolutionary context in order to delineate species boundaries (Dayrat 2005). Morphologically, the new species can be distinguished from other congeners by the combination of several traits, although the major distinguishing character is the presence of multilobated testes, a constant character among sampled individuals. The hosts of other Middle-American species of *Auriculostoma* are members of the family Characidae, particularly tetras of the genus *Asytanax* Baird & Girard. In contrast, the host, *Brycon guatemalensis*, of the new species, belongs to the family Bryconidae, also within the order Characiformes. This fish species is distributed across the Atlantic slope of Middle-America, from Grijalva-Usumacinta River systems, in southern Mexico to eastern Panama, while in the Pacific slope, it is only found in the Choluteca River basin of Honduras (Lima 2003). In this case, host association plays an important role, in addition to morphology in the distinction of the new species from its close relatives. To the best of our knowledge, no other species of *Auriculostoma* has been found in any of the other 42 species of *Brycon* Müller & Troschel, across its distributional range in the neotropics (Angulo & Gracián-Negrete 2013). The presence of the new species in *B. guatemalensis* can be hypothesized to be

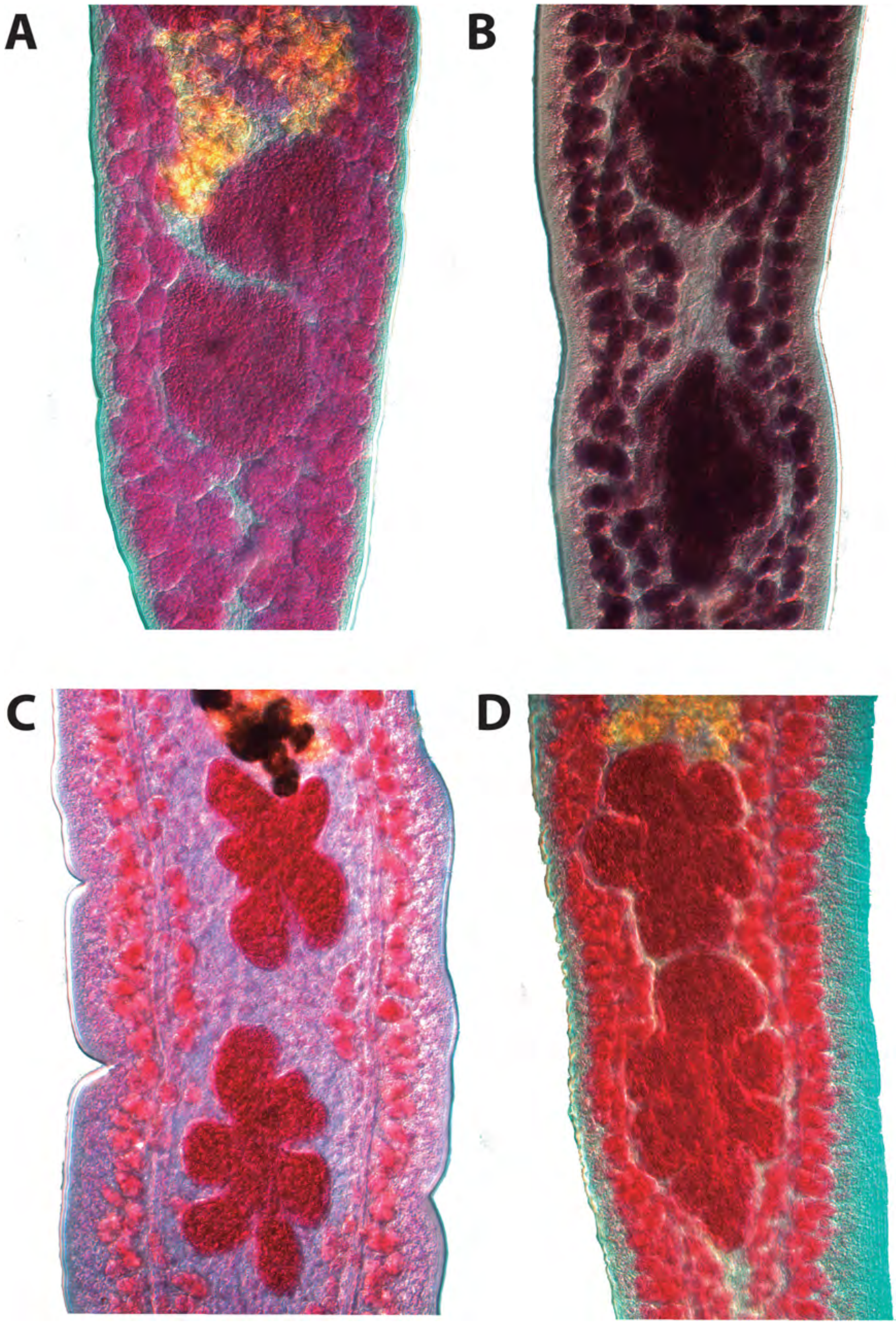


FIGURE 4. Testes shape of the three species of *Auriculostoma* in Middle-American characiforms: A) *A. totonacapanensis* (holotype) from *Astyanax mexicanus*: testes oblique, with entire (smooth) margins. B) *A. astyanace* (holotype) from *A. aeneus*: testes in tandem, with entire (smooth) margins. C) *A. lobata* **n. sp.** from *Brycon guatemalensis,* Usumacinta River, Chiapas, Mexico, and D) *A. lobata* **n. sp.** from *B. guatemalensis,* Tenosique, Tabasco, Mexico: testes in tanden, with deeply lobated testes.

a result of a speciation event via host-switching since the banded tetra, *Astyanax aeneus* occurs sympatrically in the same river basin (Miller *et al*. 2005). Characidae and Bryconidae are not sister taxa to each other (Abe *et al*. 2014), so co-diversification of *Auriculostoma* with these fish lineages can be ruled out, rather differences in biology and feeding habits between species of *Asytanax* and *Brycon* may have played a role in the speciation event. Interestingly, this difference in trematode-host associations in species of *Brycon* and *Asytanax* is also reflected in the differences between species of *Rhabdochona* Railliet, 1916 that parasitise them. *Brycon guatemalensis* is parasitised by the nematode *Rhabdochona acuminata* (Molin, 1860) Gendre, 1921 in the Usumacinta River (Caspeta-Mandujano *et al*. 2005) and the nematode is also found in *Brycon* in South America [and in other fish hosts according to Pinto *et al*. (2010), and references therein]. In contrast, *Astyanax aeneus* and *A. mexicanus* are parasitised by the congener *R. mexicana* Caspeta-Mandujano, Moravec & Salgado-Maldonado, 2000 in several localities of southern and central Mexico (Garrido-Olvera *et al*. 2006).

Sequences of the 28S rRNA gene revealed that the new species is the sister taxon of *A. astyanace*. In this case, however, the level of genetic divergence among species is relatively low, in comparison with divergence levels found among species of other allocreadid trematodes for the same molecular marker. For instance, Razo-Mendivil *et al*. (2014) obtained 2% divergence of the 28S rRNA gene between two species of *Auriculostoma*, *A. totonacapanensis* and *A. astyanace* which is almost the same divergence level between the new species and *A. totonacapanensis* as we show in our study. Pérez-Ponce de León *et al*. (2015) obtained 3.3% divergence between *Wallinia mexicana* Pérez-Ponce de León, Razo-Mendivil, Mendoza-Garfias, Rubio-Godoy & Choudhury, 2015 and *W. chavarriae* Choudhury, Daverdin & Brooks, 2002. Interestingly, in other trematode families, species have been distinguished morphologically even though relatively low values of genetic divergence were found for 28S rDNA sequences. For example, Curran *et al*. (2013) recognized three species of *Homalometron* Stafford, 1904 with divergence levels between 0.5 and 1.6%. Actually, these authors obtained a divergence of 0.5% between two cryptic species of *Homalometron*. Also, Kasl *et al*. (2014) distinguished a new species of *Alloglossidium* Simer, 1929, *A. floridense* Kasl, Fayton, Font & Criscione, 2014, from four other congeneric species with divergence values varying from 0.3 to 2.5%. In the latter case, sufficient morphological differences were found to support the species delimitation, as in our study, and the new species of *Alloglossidium* was described. Unfortunately, at the moment no mitochondrial sequences are available for *A. astyanace* and *A. totonacapanensis*. In addition, the ITS1 sequence of both species available in GenBank is incomplete. However, we obtained sequences of ITS2 for the new species to compare with those from the aforementioned species. The new species differ from *A. astyanace* by 1.1%, while it differs from *A. totonacapanensis* by 4%. Between *A. totonacapanensis* and *A. astyanace* genetic divergence is 3.7%. In comparison, Kasl *et al*. (2014) found even lower divergence values of ITS2 between the new species they discovered and two congeners, 0.3% between *Alloglossidium floridense* and *A. kenti*, and 0.6% between *A. floridense* and *A. fonti*.

In conclusion, the common grounds of all the aforementioned examples is that new species are recognised irrespective of a relatively low genetic variation, at least for the 28S rDNA and ITS2 ribosomal genes. We could argue that these low genetic divergence values may indicate a recent or incipient speciation event. However, contrary to the common assumption that the degree of morphological differentiation between related species should be proportional to the time since their divergence, we show here that such differentiation occurs between recently formed species. The new species is specific to *Brycon guatemalensis* and its distribution range seems to be restricted to southern Mexico, across the Usumacinta and Lacantun River basins. Morphologically, the three species of *Auriculostoma* in Middle-American characiforms are similar, but distinguishing characters are reliable (Fig. 4), and additional sources of information confirm the species separation. We conclude that the specimens from *B. guatemalensis* can be reliably distinguished and should be considered a distinct species. The record of *A. astyanace* by Salgado-Maldonado *et al*. (2011), as parasites of the same host species, in the same river basin, correspond with the new species herein described. This study raises the number of *Auriculostoma* species to eight, and the following identification key separates them.

Key to species of *Auriculostoma*

1. Testes with entire (smooth) margins 2
- Testes with irregluar or lobulated margins 5
2. Genital pore located at the level of the cecal bifurcation. Parasite of Characiformes: *Astyanax mexicanus*, Mexico . . . .

. . . . *A. totonacapanensis*

- Genital pore located between the anterior margin of the ventral sucker and cecal bifurcation 3

3. Testes in tandem. Parasite of Characiformes: *Astyanax aeneus,* Costa Rica and Nicaragua. . . . . *A. astyanace*

- Testes oblique 4

4. Vitelline follicles extending anteriorly to the mid-level of esophagus. Eggs large (75 µm long). Parasite of Siluriformes: *Pimelodus blochii* Cuvier & Valenciennes, *Iheringichthys labrosus* (Lütken) and *Rhinodoras dorbignyi* (Kner), Argentina and Brazil . . . . *A. platense*

- Vitelline follicles extending anteriorly to pharynx level. Eggs relatively small (55 µm long). Parasite of Characiformes: *Stethaprion erythrops* Cope*,* Peru . . . . *A. diagonale*

5. Testes with lobulated margins. Parasite of Characiformes: *Brycon guatemalensis,* in Mexico . . . . *A. lobata* **n. sp.**

- Testes with irregular margins 6

6. Oral sucker smaller than ventral sucker. Parasite of Characiformes: *Charax stenopterus* Cope, Uruguay . . . .*A. stenopteri*

- Oral sucker larger than ventral sucker 7

7. Vitelline follicles extending anteriorly to poterior margin of oral sucker. Parasite of Characiformes and Siluriformes: *Roeboides bonariensis* (Steindachner), *Ageneiosus inermis* (Linnaeus), *Auchenipterus nuchalis* Spix & Agassiz and *Auchenipterus nigripinnis* (Boulenger), Argentina . . . . *A. macrorchis*

- Vitelline follicles extending anteriorly to mid-level of ventral sucker. Parasite of Characiformes: *Bryconops caudomaculatus* (Günther)*,* Peru . . . . *A. foliaceum*

Acknowledgments

This paper is part of the requirements to fulfil the PhD program of D.I.H.M in the Posgrado en Ciencias Biológicas, UNAM. D.I.H.M thanks the graduate program and the Consejo Nacional de Ciencia y Tecnología for providing a scholarship. We thank Carlos Pedraza, Patricia Ornelas, Rocío Rodiles, Alfonso Castillo and Alfonso González for their help during field work. Luis García provided specimens from the Colección Nacional de Helmintos for comparison, Jesus Hernández-Orts helped with observation of some morphological traits, and the support of Laura Márquez processing samples for the automated sequencer is greatly appreciated. Financial support for this research was obtained through a grant from the Dirección General de Asuntos del Personal Académico (PAPIIT-UNAM IN204514) to G.P.P.dL. We sincerely thank two anonymous reviewers whose comments greatly improved the quality of the paper in terms of writting and content. This paper was written during the sabbatical leave of G.P.P.dL to the University of Otago, New Zealand. Thanks are due to Dr. Robert Poulin for facilities provided in his laboratory, and to DGAPA-UNAM and CONACyT for their support through their sabbatical programs.

References

Abe, K.T., Mariguela, T.C., Avelino, G.S., Foresti, F. & Oliveira, C. (2014) Systematic and historical biogeography of the Bryconidae (Ostariophysi: Characiformes) suggesting a new rearrangement of its genera and an old origin of Mesoamerican ichthyofauna. *BMC Evolutionary Biology*, 14, 152–152.
http://dx.doi.org/10.1186/1471-2148-14-152

Angulo, A. & Gracián-Negrete, J.M. (2013) A new species of *Brycon* (Characiformes: Characidae) from Nicaragua and Costa Rica, with a key to the lower Mesoamerican species of the genus. *Zootaxa*, 3731 (2), 255–266.
http://dx.doi.org/10.11646/zootaxa. 3731.2.6

Bautista-Hernández, C. E., Monks, S., Pulido-Flores, G. & Miranda, R. (2015) A new species of *Paracreptotrema* (Digenea, Plagiorchiformes, Allocreadiidae) infecting two species of poeciliids in Río Malila of the Río Pánuco basin, Hidalgo, México, with a key to the species of the genus. *ZooKeys*, 482, 55–66.
http://dx.doi.org/10.3897/zookeys.482.8144

Bowles, J. & McManus, D. P. (1993) Rapid discrimination of Echinococcus species and strains using a PCR-based method. *Molecular and Biochemical Parasitology*, 57, 231–239.
http://dx.doi.org/10.1016/0166-6851(93)90199-8

Bray, R.A., Foster, G.N., Waeschenbach, A. & Littlewood, D.T.J. (2012) The discovery of progenetic *Allocreadium neotenicum* Peters, 1957 (Digenea: Allocreadiidae) in water beetles (Coleoptera: Dytiscidae) in Great Britain. *Zootaxa,* 3577, 58–70.

Brooks, D.R. (1992) New distribution records for *Bunodera eucaliae* and *Bunodera inconstans* n. comb. (Digenea: Allocreadiidae: Bunoderinae) with discussion of their phylogenetic relationships. *Journal of Parasitology*, 78, 779–783.
http://dx.doi.org/10.2307/3283303

Caspeta-Mandujano, J.M., Moravec, F. & Salgado-Maldonado, G. (2000) *Rhabdochona mexicana* sp. n. (Nematoda: Rhabdochonidae) from the intestine of characid fishes in Mexico. *Folia Parasitologica*, 47, 211–215.
http://dx.doi.org/10.14411/fp.2000.039

Caspeta-Mandujano, J.M., Cabañas-Carranza, G., Salgado-Maldonado, G., Gosztonyi, A.E. & Cremonte, S. (2005) Nematode parasites of the characid freshwater fish *Brycon guatemalensis* in the Usumacinta River, Chiapas, Mexico. *Helminthologia*, 42, 41–44.

Choudhury, A., Daverdin, R.H., & Brooks, D.R. (2002) *Wallinia chavarriai* n. sp. (Trematoda: Macroderoididae) in *Astyanax aeneus* (Gunther, 1860) and *Bryconamericanus sceloparius* (Regan, 1908) (Osteichthyes: Characidae) from the Area de Conservación de Guanacaste, Costa Rica. *Journal of Parasitology*, 137, 287–302.

Choudhury, A. & León-Règagnon, V. (2005) Molecular phylogenetics and biogeography of *Bunodera* spp. (Trematoda: Allocreadiidae), parasites of percid and gasterosteid fishes. *Canadian Journal of Zoology*, 83, 1540–1546.
http://dx.doi.org/10.1139/z05-153

Choudhury, A., Pérez-Ponce de León, G., Brooks, D. R. & Daverdin, R. (2006) *Paracreptotremata blancoi* sp. n. (Digenea: Plagiorchiformes), in the olomina, *Priapichthys annectens* (Osteichthyes: Poeciliidae) from the Área de Conservación Guanacaste, Costa Rica. *Journal of Parasitology*, 92, 565–568.

Choudhury, A., Aguirre-Macedo, M.L., Curran, S.S., Ostrowski de Núñez, M., Overstreet, R.M., Pérez-Ponce de León, G. & Portes Santos, C. (2016) Trematode diversity in freshwater fishes of the Globe II: 'New World'. *Systematic Parasitology*, 93, 271–282.
http://dx.doi.org/10.1645/GE-3540.1

Curran, S.S., Pulis, E.E., Hugg, D.O., Brown, J.P., Manuel, L.C., & Overstreet R.M. (2012) Phylogenetic position of *Creptotrema funduli* in the Allocreadiidae based on partial 28S rDNA Sequences. *Journal of Parasitology*, 98, 873–875.
http://dx.doi.org/10.1645/GE-3066.1

Curran, S.S., Tkach, V.V. & Overstreet, R.M. (2006) A review of *Polylekithum* Arnold, 1934 and its familial affinities using morphological and molecular data, with description of *Polylekithum catahoulensis* sp. nov. *Acta Parasitologica*, 51, 238–248.
http://dx.doi.org/10.2478/s11686-006-0037-1

Curran, S.S., Tkach, V.V. & Overstreet, R.M. (2011) Phylogenetic affinities of *Auriculostoma* (Digenea: Allocreadiidae), with descriptions of two new species from Peru. *Journal of Parasitology*, 97, 661–670.
http://dx.doi.org/10.1645/GE-2641.1

Curran, S.S., Tkach, V. & Overstreet, R.M. (2013) Molecular evidence for two cryptic species of *Homalometron* (Digenea: Apocreadiidae) in freshwater fishes of the Southeastern United States. *Comparative Parasitology*, 80, 186–195.
http://dx.doi.org/10.1654/4626.1

Dayrat, B. (2005) Towards integrative taxonomy. *Biological Journal of the Linnean Society*, 85,407–415.
http://dx.doi.org/10.1111/j.1095–8312.2005.0503.x

García-Varela, M. & Nadler, S.A. (2005) Phylogenetic relationships of Palaeacanthocephala (Acanthocephala) inferred from SSU and LSU rDNA gene sequences. *Journal of Parasitology*, 91, 1401–1409.

Garrido-Olvera, L., García-Prieto, L. & Pérez- Ponce de León, G. (2006) Checklist of the adult nematode parasites of fishes in freshwater localities from Mexico. *Zootaxa*, 1201, 1–45.

Gendre, E. (1921) Notes d'Helminthologie africaine (sixième note). *Procès-Verbaux de la Société Linnéenne de Bordeaux*, 73, 148–156.

Hernández-Mena, D.I., García-Prieto, L. & García-Varela, M. (2014) Morphological and molecular differentiation of *Parastrigea* (Trematoda: Strigeidae) from Mexico, with the description of a new species. *Parasitology International*, 63, 315–323.
http://dx.doi.org/10.1016/j.parint.2013.11.012

Hopkins, S.H. (1931) Studies on *Crepidostomum*. II. The "*Crepidostomum laureatum*" of A. R. Cooper. *Journal of Parasitology*, 18, 79–91.
http://dx.doi.org/10.2307/3271966

Jiménez-Guzmán, F. (1973) Tremátodos digeneos de peces dulceacuícolas de Nuevo León, México. I. Dos nuevas especies y un registro nuevo en el caracido *Astyanax fasciatus mexicanus* (Filippi).*Cuadernos del Instituto de Investigaciones Cientificos, Universidad Autónoma de Nuevo León, México*, 17, 1–19.

Kasl, E.L., Fayton, T.J., Font, W.F. & Criscione, C.D. (2014) *Alloglossidium floridense* n. sp. (Digenea: Macroderoididae) from a spring run in North Central Florida. *Journal of Parasitology*, 100, 121–126.
http://dx.doi.org/10.1645/13–252.1.

Kohn, A., Fernandes, B.M. & Cohen, S.C. (2007) *South American trematodes parasites of fishes*. Ministerio da Saúde, FIOCRUZ, Rio de Janeiro, Brasil, 318 pp.

Lamothe-Argumedo, R. (1970) Tremátodos de peces VI. *Margotrema bravoae* gen., nov., sp., nov., (Trematoda:Allocreadiidae) parásito de *Lermichthys multiradiatus* Meek. *Anales del Instituto de Biología, Universidad Nacional Autónoma de México*, 41, 87–92.

Lima, F.C.T. (2003) Characidae - Bryconinae (Characins, tetras). *In:* Reis, R.E., Kullander, S.O. & Ferraris, C.J. Jr. (Eds.), *Checklist of the Freshwater Fishes of South and Central America*. Porto Alegre, EDIPUCRS, Brasil, pp. 174–181.

Looss, A. (1900) Nachträgliche Bemerkungen zu den Namen der von mir vorgeschelagenen Distomengattungen. *Zoologischer Anzeiger*, 23, 601–608.

Lühe, M. (1909) Parasitische Plattwürmer. I. Trematoden. *Die Süsswasserfauna Deutschland*, 17, 215–215.

Luton, K., Walker, D. & Blair, D. (1992) Comparisons of ribosomal internal transcribed spacers from two congeneric species of flukes (Platyhelminthes: Trematoda: Digenea). *Molecular & Biochemical Parasitology*, 56, 323–327. http://dx.doi.org/10.1016/0166-6851(92)90181-I

Mañé-Garzón, F. & Gascón, A. (1973) Digenea de peces de agua dulce de Uruguay, I. Una Nueva especie del genero *Crepidostomun* Braum, 1900 del intestino de *Asiphonichthys stenopterus*. *Revista de Biología del Uruguay*, 1, 11–14.

Miller, R.R., Mickley, W.L. & Norris, S.M. (2005) *Freshwater Fishes of Mexico*. University of Chicago Press, Chicago, 490 pp.

Mueller, J.F. (1934) Parasites of Oneida Lake fishes. Part IV. Additional notes on parasites of Oneida Lake fishes, including descriptions of new species. *Roosevelt Wild Life Annals*, 3, 335–373.

Olson, P.D., Cribb, T.H., Tkach, V.V., Bray, R.A. & Littlewood, D.T. (2003) Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal for Parasitology,* 33, 733–755. http://dx.doi.org/10.1016/S0020-7519

Pérez-Ponce de León, G., Martínez-Aquino, A. & Mendoza-Garfias, B. (2013) A new species of *Margotrema* (Digenea, Allocreadiidae) from the Leopard splitfin *Xenotaenia resolanae* (Cyprinodontiformes, Goodeidae) from west-central Mexico. *Zootaxa*, 3670 (1), 94–96. http://dx.doi.org/10.11646/zootaxa.3670.1.10

Pérez-Ponce de León, G., Razo-Mendivil, U., Mendoza-Garfias, B., Rubio-Godoy, M. & Choudhury, A. (2015) A new species of *Wallinia* Pearse, 1920 (Digenea: Allocreadiidae) in *Astyanax mexicanus* (Characidae) from Mexico revealed by morphology and sequences of the 28S ribosomal RNA gene. *Folia Parasitologica*, 62, 18–18. http://dx.doi.org/10.14411/fp.2015.018

Pérez-Ponce de León, G., Pinacho-Pinacho, C.D., Mendoza- Garfias, B., Choudhury, A. & García-Varela, M. (2016) Phylogenetic analysis using the 28S rRNA gene reveals that the genus *Paracreptotrema* (Digenea: Allocreadiidae) is not monophyletic; description of two new genera and one new species. *Journal of Parasitology*, 102, 131–142.

Peters, L.E. (1957) An analysis of the trematode genus *Allocreadium* Looss with the description of *Allocreadium neotenicum* sp. nov. from water beetles. *Journal of Parasitology*, 43, 136–142. http://dx.doi.org/10.1645/15-815

Petkevičiūtė, R., Stunzenas, V., Stanevičiūtė, G. & Sokolov, S.G. (2010) Comparison of the developmental stages of some European allocreadiid trematode species and a clarification of their life-cycles based on ITS2 and 28S sequences. *Systematic Parasitology*, 76, 169–178. http://dx.doi.org/10.1007/s11230-010-9249-8

Pinto, M.R., Noronha, D., Marcelo Knoff, M. & Gomes, D.C. (2010) *Rhabdochona (R.) uruyeni* (Nematoda, Rhabdochonidae) in Brazil: present status of South American *Rhabdochona* Railliet with a worldwide bibliographical survey of the genus from 1845 to 2010. *Neotropical Helminthology*, 4, 49–69.

Platta, C.S. & Choudhury, A. (2006) Systematic position and relationships of *Paracreptotrematina limi,* based on partial sequences of 28S rRNA and cytochrome c oxidase subunit 1 genes. *Journal of Parasitology*, 92, 411–413. http://dx.doi.org/10.1645/GE-3521RN.1

Razo-Mendivil, U., Mendoza-Garfias, B., Ponce-Pérez de León, G. & Rubio-Godoy, M. (2014) A new species of *Auriculostoma* (Digenea: Allocreadiidae) in the Mexican tetra *Astyanax mexicanus* (Actinopterygii: Characidae) from central Veracruz, Mexico, described with the use of morphological and molecular data. *Journal of Parasitology*, 100, 331–337.

Salgado-Maldonado, G., Caspeta-Mandujano, J.M., Moravec, F., Soto-Galera, E., Rodiles-Hernández, R., Cabañas-Carranza, G. & Montoya-Mendoza, G. (2011) Helminth parasites of freshwater fish in Chiapas, Mexico. *Parasitology Research,* 108, 31–59. http://dx.doi.org/10.1007/s00436-010-2035-3

Scholz, T., Aguirre-Macedo, M.L. & Choudhury, A. (2004) *Auriculostoma astyanace* n. gen., n. sp. (Digenea: Allocreadiidae), from the banded astyanax, *Astyanax fasciatus* (Characiformes: Characidae), from Nicaragua, with a reevaluation of Neotropical *Crepidostomum* spp. *Journal of Parasitology,* 90, 1128–1132. http://dx.doi.org/10.1645/GE-3275

Stafford, J. (1904) Trematodes from Canadian fishes. *Zoologischer Anzeiger*, 27, 481–495.

Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatic,* 22, 2688–90. http://dx.doi.org/10.1093/bioinformatics/btl446

Stamatakis, A., Blagojevic, F., Nikolopoulos, D. & Antonopoulos, C. (2007) Exploring new search algorithms and hardware for phylogenetics: RAxML meets the IBMcell. *Journal of VLSI Signal Processing*, 48, 271–286. http://dx.doi.org/10.1007/s11265-007-0067-4

Stamatakis, A., Hoover, P. & Rougemont, J. (2008) A rapid bootstrap algorithm for the RAxML web servers. *Systematic Biology*, 57, 758–71. http://dx.doi.org/10.1080/10635150802429642

Stock, S.P., Campbell, J.F. & Nadler, S.A. (2001) Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology*, 87, 877–889. http://dx.doi.org/10.1645/0022–3395

Surber, E.W. (1928) *Megalogonia ictaluri*, a new species of trematode from the channel cat-fish, *Ictalurus punctatus*. *Journal of Parasitology*, 14, 269–269.

http://dx.doi.org/10.2307/3271386
Swofford, D.L. (2003) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.
Szidat, L. (1954) Trematodes nuevos de peces de agua dulce de la República Argentina y un intento para aclarar su carácter marino. *Revista del Instituto de Investigación de las Ciencias Naturales y Museo Argentino de Ciencias Naturales "Bernardino Rivadavia",* 3, 1–85.
Thompson, J., Gibson, T., Plewnlak, F., Jeanmougin, F. & Higgins, D. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25, 4876–4882. http://dx.doi.org/10.1093/nar/25.24.4876
Tkach, V.V. & Curran, S.S. (2015) *Prosthenystera oonastica* n. sp. (Digenea: Callodistomidae) from ictalurid catfishes in southeastern United States and molecular evidence differentiating species in the genus across Americas. *Systematic Parasitology*, 90, 39–51.
http://dx.doi.org/10.1007/s11230-014-9531-2
Travassos, L. (1922) Contribuições para o conhecimento da fauna helmintológica brasileira - XIV. Espécies brasileiras da família Gorgoderidae Looss, 1901. *Brasil Médico*, 36 , 17–20.
Tsimbaliuk, A.K. & Roitman, V.A. (1966) Trematode *Bunodera mediovitellata* parasite of stickleback in Commodore Islands. *Trudy Gel'mint. Lab.*, *Akad Nauk SSSR*, 17, 290–296.
Van Cleave, H.J. & Mueller, J.E (1932) Parasites of Oneida Lake Fishes. Part I. Description of new genera and new species. *Roosevelt Wild Life Annals*, 3, 5–71.
Wallin, I.E. (1909) A new species of the trematode genus *Allocreadium*, with a revision of the genus and a key to the sub-familyAllocreadiinae. *Transactions of the American Microscopical Society*, 29, 50–66.
http://dx.doi.org/10.2307/3220971

Two new species of allocreadiid trematodes (Digenea: Allocreadiidae) parasitising freshwater fishes of Mexico and Costa Rica revealed by 28S rDNA sequences and morphology.

David Iván Hernández-Mena[1,2], Carlos Daniel Pinacho-Pinacho[3], Martín García-Varela[1], Berenit Mendoza-Garfias[1], Gerardo Pérez-Ponce de León[1*]

***1** Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Apartado Postal 70-153, C.P. 04510, Ciudad de México, Mexico. **2** Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Apartado Postal 70-153, C.P. 04510, Ciudad de México. **3** Instituto de Ecología, A.C., Red de Biología Evolutiva, km 2.5 Ant. Carretera a Coatepec, Xalapa, C. P. 91070, Veracruz,Mexico*

*Corresponding author: Gerardo Pérez-Ponce de León (ppdleon@ib.unam.mx)

Abstract

Using DNA sequences from the 28S ribosomal gene, we detected two new species of allocreadiids, which were collected in freshwater fish that are distributed in Middle-America. We describe two new species, one belonging to the genus *Auriculostoma* Scholz, Aguirre-Macedo & Choudhury, 2004 and another of the genus *Wallinia* Pearse, 1920, and their delimitation criteria were based on 28S rDNA sequences, morphological data (light and scanning electron microscopy [SEM]), host association, and geographical distribution. *Auriculostoma tica* sp. n. was found parasitizing *Gymmnotus maculosus* in Costa Rica, while *Wallinia criptica* sp. n. was found parasitizing *Astyanax aeneus* in Oaxaca and Chiapas, Mexico, and in Guatemala. A phylogenetic analysis using 28S rDNA sequences along with those available for other allocreadiid trematodes, revealed the relationships of the new species: *A. tica* sp. n. is a sister taxon of *A. totonacapanensis* Razo-Mendivil, Mendoza-Garfias, Pérez-Ponce de León & Rubio-Godoy, 2014 and *W. criptica* sp. n. is a sister of *W. brasiliensis* Dias, Müller, de Almeida, da Silva, de Azevedo, Pérez-Ponce de León & Abdallah, 2018. Genetic distances for the 28S rDNA were estimated among the American species of allocreadiids. Reliable morphological differences, its host association, restricted geographical distribution, and genetic distances supported the validity of the new species.

Keywords

Middle-America, 28S, Molecular Phylogeny, *Auriculostoma*, *Wallinia*.

Introduction

The knowledge about the diversity and phylogenetic relationships of allocreadiid trematodes in Middle American freshwater fishes has increased in the last 15 years. A large diversity has been documented for species occurring primarily in small-bodied characids (tetras), and cyprinodontiforms of the families Poeciliidae Garman (guppies) and Profundulidae Hoedeman & Bronner (killifishes) (e.g., Choudhury et al. 2002, 2006; Scholz et al. 2004; Razo-Mendivil et al. 2014a, 2014b; Bautista-Hernández et al. 2015; Pérez-Ponce de León et al. 2015, 2016; Hernández-Mena et al. 2016). The first species descriptions of allocreadiids in that geographical area, were solely based on morphological characters (Choudhury et al. 2002; Scholz et al. 2004), however most new species have been described following an integrative taxonomy approach (Dayrat 2005) where DNA sequence data has been used in combination with morphological analysis of phenotypic differences, mostly through scanning electron microscopy observations (SEM), host association and geographic distribution, to assess more robust species limits (see Razo-Mendivil et al. 2014a; Pérez-Ponce de León et al. 2015; Hernández-Mena et al. 2016). Three additional Middle American species have been described following that approach within the genera *Wallinia* Pearse, 1920 and *Auriculostoma* Scholz, Aguirre-Macedo & Choudhury, 2004, i.e., *A. totonacapanensis* Razo-Mendivil, Mendoza-Garfias, Pérez-Ponce de León & Rubio-Godoy, 2014 as a parasite of *A. mexicanus* (De Filippi) from Veracruz, Mexico; *A. lobata* Hernández-Mena, Lyngaard, Mendoza-Garfias & Pérez-Ponce de León in *Brycon guatemalensis* Regan, from Chiapas, Mexico (Razo-Mendivil et al. 2014a; Hernández-Mena et al. 2016); and one species of *Wallinia*, *W. mexicana* Pérez-Ponce de León, Razo-Mendivil, Mendoza-Garfía, Rubio-Godoy & Choudhury, 2015 in *A. mexicanus* from central Mexico (Pérez-Ponce de León et al. 2015). In continuation with our efforts to uncover and describe the allocreadiid diversity in Neotropical freshwater fishes, a new phylogenetic analysis of the Allocreadiidae was performed, and sequences of the 28S ribosomal rDNA gene of newly sampled specimens were added, from our field collections of 2015 and 2016 in several localities of southern Mexico, Guatemala and Costa Rica, resulting in the discovery of two new species, one belonging to *Wallinia*, as a parasite of *Astyanax aeneus* Günther and one to *Auriculostoma*, from *Gymnotus maculosus* Albert & Miller. Specimens processed for morphology and SEM showed distinctive characters when compared with congeneric species allocated into these two genera and therefore, in this study we describe the two new species; the species delimitation criteria are then based on information from 28S rDNA sequences, morphological data, geographical distribution, and host association.

Material and methods

Sample collections. Specimens of allocreadiids were collected in freshwater fishes from two locations in Costa Rica (*Gymnotus maculosus* n= 3), and three in Mexico (*Astyanax aeneus*, n= 15), one in Guatemala (*A. aeneus*, n= 11), in 2015 and 2016 (Table 1). Fish were collected with seine nets and electrofishing, kept alive and necropsied 4 hr after capture. The gastrointestinal tract was removed from each host and placed in Petri dishes with 6.5% saline solution and were examined under a stereomicroscope. Some specimens were fixed in hot (nearly boiling) tap water and kept in vials with 4% formalin for subsequent morphological study. Other specimens were placed directly in vials with 100% ethanol for molecular analyses.

Molecular work. Specimens were individually digested overnight at 56°C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM Na2 EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. DNA was isolated from the supernatant using the DNAzol (Molecular Research Center, Cincinnati, Ohio) following the manufacturer’s instructions. The domains D1-D3 of the 28S rRNA gene were amplified using the forward primer 391 (5′-AGC GGA GGA AAA GAA ACT AA-3′; Nadler & Hudspeth 1998) and the reverse primer 536 (5'-CAG CTA TCC TGA GGG AAA C-3'; García-Varela & Nadler 2005). Polymerase chain reaction (PCR) amplifications were carried out with 2 µl of DNA extraction supernatant, 1µl of each PCR primer (10 µM), 2.5 µl of 10× buffer, 1.5 µl of 25 mM of $MgCl_2$, 0.5 µl of dNTPs (10 mM) and 1 U of Taq DNA polymerase. The following amplification profile was used: 95°C for 5 min (denaturation); 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min; and a final extension of 72 °C for 10 min. PCR products for 28S rRNA were sequenced with PCR primers plus internal primers 503 (5'-CCT TGG TCC GTG TTT CAA GAC G-3'; Stock et al. 2001) and 504 (5'-CGT CTT GAA ACA CGG ACT AAG G-3'; García-

Varela & Nadler 2005). PCR products were sequenced using an ABI 3730xl Genetic Analyser (Applied Biosystems). The consensus sequences were obtained from the individual sequences amplified from each primer using the software Geneious Pro 4.8.4® (Biomatters Ltd. Auckland, New Zealand). Sequences were submitted to GenBank (Table 1).

Phylogenetic analyses. The newly generated sequences of the nuclear 28S rDNA were aligned with available sequences of other species of allocreadiids and callodistomids using ClustalW (Thompson et al. 1997), implemented in the web http://www.genome.jp/tools/clustalw/. Based on previously phylogenetic trees of Allocreadiidae (Hernández-Mena et al. 2016; Pérez-Ponce de León et al. 2016), sequences of species of *Prosthenhystera* Travassos, 1922 (Callodistomidae), *Paracreptotrematoides heterandriae* (Salgado-Maldonado, Caspeta-Mandujano & Vázquez, 2012) Pérez Ponce de León, Pinacho-Pinacho, Mendoza-Garfias, Choudhury & García-Varela, 2016, *Paracreptotrema blancoi* Choudhury, Pérez Ponce de León, Brooks & Daverdin, 2006, and *P. rosenthali* Bautista-Hernández, Monks, Pulido-Flores & Miranda, 2015 were used as outgroup for rooting the trees. Phylogenetic analyses were run under maximum likelihood (ML) and Bayesian inference (BI), employing the model of nucleotide evolution GTR+GAMMA-I selected with jModelTest v2 (Darriba et al. 2012). ML inference (20 replicates), model parameters and bootstrap (Bt) support (1,000 repetitions) were estimated with RAxML v. 7.0.4 (Stamatakis 2006). BI analysis was carried out using MrBayes v. 3.2 (Ronquist et al. 2012), running two independent Markov Chain Monte Carlo runs of two chains each run (a heating parameter value of 0.5) for 10 million generations and sampling tree topologies every 1000 generations (printfreq=1000 samplefreq=1000 diagnfreq=10000). Burn-in periods were set to the first 500 generations. A 50% majority-rule consensus tree and nodal support estimated as posterior probability (PP) values were calculated from the remaining trees. The phylogenetic trees obtained from both analyses were visualized in FigTree v. 1.4.2. Genetic distances for the 28S dataset were calculated as uncorrected *p*-distance using MEGA v6 (Tamura et al. 2013).

Morphological study. Specimens were stained with Mayer's paracarmine and Gomori´s trichrome, dehydrated in a graded ethanol series, cleared in methyl salicylate, and examined as permanent mounts in Canada balsam. Specimens were examined using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) with differential interference contrast optics. Measurements were taken using the Olympus Quick-Photo Image-Program, and are presented in micrometers (µm) with the range followed by the mean in parentheses. Drawings were made with a drawing tube attached to the microscope. Representative specimens were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM). For morphological comparison, the following type specimens were studied: *A. astyanace* ex *A. fasciatus* 1 holotype (CNHE 4923) and 1 paratype (CNHE 4924), from Loonku Creek, Bluefields, Región Autónoma del Atlántico del Sur (RAAS), Nicaragua; *A. lobata* ex *B. guatemalensis* 1 holotype (CNHE 1081) and eigth paratypes (CNHE 1082), from El Mangal Lagoon, Tenosique, Tabasco, Mexico plus six voucher specimens from Río Usumacinta, Chiapas, Mexico (CNHE 7505); *A. totonacapanensis* ex *A. mexicanus* 1 holotype and 7 paratypes Mexico (CNHE 8471), from Filipinas, Veracruz, and 1 paratype U.S. National Parasite Collection (USNPC 107880); *W. mexicana* ex *A. mexicanus* 1 holotype (CNHE 8453), 15 paratypes from Río Covadonga at Peñon Blanco, Durango, Mexico (CNHE 6311-6313), and six specimens from Río Huichihuayán, San Luis Potosí, Mexico (CNHE 7411).

A single individual of *Auriculostoma* and three specimens of *Wallinia* were used for scanning electron microscopy (SEM) study. The specimens were dehydrated through a graded series of ethyl alcohol, and then critical point dried with carbon dioxide. Specimens were mounted on a metal stub with carbon adhesive tabs, then gold coated, and examined at 15 kV in a Hitachi Stereoscan Model SU1510 SEM (Hitachi Ltd., Tokyo, Japan).

Results

Phylogenetic relationships and genetic divergence

The data set of the 28S rRNA gene consisted of 34 sequences representing 15 species, four of which are members of *Auriculostoma* and four of *Wallinia*; the remaining species represented four species of allocreadiids, and three of the callodistomid genus *Prosthenhystera*. The final alignment consisted of 1,424 bp. Nucleotide frequencies were A=0.218, C= 0.212, G= 0.314 and T= 0.256. The ML tree had a value of ln=-3949.293223. Both the ML and BI analyses yielded the same phylogenetic relationships. The phylogenetic tree (Fig. 1) shows that allocreadiids are a monophyletic group, with high nodal support. Within allocreadiids, a monophyletic group including species of *Creptotrematina* Yamaguti, 1954, *Auriculostoma* and *Wallinia,* typical inhabitants of small-bodies characids, are formed with low bootstrap and posterior probability support values. Both phylogenetic trees shows *Creptotrematina aguirrepequenoi* Jiménez, 1973 as the sister species of members of *Auriculostoma* and *Wallinia*, both recovered as monophyletic assemblages. The new species, *Auriculostoma tica* sp. n (two samples) is recovered as the sister species of *A. totonacapanensis*, with *A. astyanace* Scholz, Aguirre-Macedo & Choudhury, 2004 and *A. lobata* as the sister species of this clade. The 13 samples of *Wallinia criptica* sp. n. are reciprocally monophyletic, with the highest values of nodal support, and are nested as the sister species of *W. brasiliensis* Dias, Müller, de Almeida, da Silva, de Azevedo, Pérez-Ponce de León & Abdallah, 2018. *Wallinia mexicana* and *W. chavarriae* Choudhury, Daverdin & Brooks, 2002 are their sister group.

Genetic distance of the 28S rDNA sequences among genera is relatively high, varying from 2.66 to 5.51% between *Auriculostoma* and *Wallinia*, 3.76 to 4.49% between *Auriculostoma* and *Creptotrematina*, and 3.67 to 5.33% between *Wallinia* and *Creptotrematina*. Likewise, the interspecific genetic distance between *A. tica* sp. n. and *A. totonacapanensis*, its sister species, is 2.11%, whereas between *A. tica* sp. n. and *A. lobata* is 1.47, and between the new species and *A. astyanace*, divergence is 1.38%. The genetic distance between the sister species pair *W. criptica* sp. n. and *W. brasiliensis* is 1.19%, meanwhile between the new species and *W. chavarriae* it reaches 3.03%, and between the new species and *W. mexicana* is 4.04%. Intraspecific genetic divergence of the 28S rDNA sequences between the two isolates of *A. tica* sp. n. and among the 13 isolates of *W. criptica* sp. n. was null (Table 2).

Family Allocreadiidae Looss, 1902

Genus *Auriculostoma* Scholz, Aguirre-Macedo & Choudhury, 2004

***Auriculostoma tica* sp. n.**

Figs 2 and 3

Type material. Holotype (CNHE XXXX); paratypes: 1 specimen from Orosí River (CNHE XXXX) and 2 specimens from Pitaya River (CNHE XXX).

Type host. *Gymnotus maculosus* Albert & Miller (Gymnotidae)

Type locality. Orosí River, Costa Rica (11°02'50" N, 85°22'48" W).

Other Localities. Creek at Pitaya, affluent to the Orosí River, Costa Rica (11°03'05" N, 85°24'30" W).

Site in host. Intestine.

Etymology. This specific epithet refers to the Costa Rican people, who refer to themselves as "ticos", in recognition of their friendship while we conducted field work in particular areas of the country.

Description. [Based on 4 adult specimens from Orosí River] Body elongate, 1,551–2,118 (1,778) long, narrow at the pharynx level; maximum width 424 –568 (478) in the region between ovary and anterior testis. Tegument without spines. Forebody length 563–654 (600). Oral sucker subterminal, funnel-shaped, well-developed; single pair of dorsolateral muscular lobes located on either side, with an inconspicuous base, stretching from ventrolateral to dorsolateral side, forming short rounded "free" ends. Oral sucker 240–270 (260) long, 216–260 (241) wide, excluding lateral lobes, and 285–335 (316) wide including lateral lobes; oral sucker with 21 dome-like papillae arranged in 4 rows: 4 apical, 8 anterior (Figs. 3B,D), 4 on inner surface, and 5 on outer surface. Ventral sucker spherical, muscular, 143–179 (164) long, 161–192 (181) wide, and slightly smaller than oral sucker; ratio of oral sucker length to ventral sucker length 1:0.57–0.74 (1:0.68); ratio of oral sucker width to ventral sucker width not considering lateral lobes 1: 0.62–0.85 (1: 0.75), and ratio of oral sucker width to ventral sucker width considering lateral lobes 1: 0.48–0.67 (1: 0.57). Mouth subterminal. Prepharynx absent. Pharynx muscular, globular, 74–98 (86) long, 81–115 (100) wide. Esophagus curved. Intestinal bifurcation at anterior margin of ventral sucker. Intestinal ceca long and wide, reaching near to posterior extremity. Post-cecal space length: 59–198 (132).

Two testes of similar size, oval, entire, always oblique, in posterior half of body. Anterior testis submedial, 135-172 (149) long by 83–110 (97) wide. Posterior testis medial, 135–182 (158) long by 109–144 (121) wide. Post-testicular space length: 386–642 (487), representing 25–30% (27%) of body length. Cirrus-sac relatively long, dorsal, median, straight, extending from genital pore to mid-level between ventral sucker and ovary, containing wide muscular ejaculatory duct, pars prostatica, and bi-partite internal seminal vesicle; cirrus not observed. Genital pore median, opening on ventral surface, between pharynx (in the middle of esophagus), and intestinal bifurcation; genital pore distance from anterior end of body: 363–454 (400). Ovary bean-shaped, entire, posterior to ventral sucker, pre-equatorial, in middle line of body, 90–112 (103) long by 75–129 (105) wide. Seminal receptacle sac-shaped, post-ovarian, 61 long by 95 wide. Mehlis' gland posterior to ovary and seminal receptacle. Laurer's canal not observed. Vitelline follicles small, numerous, intra- and extracecal along body, extending from genital pore to posterior end of body, confluent at post-testicular area. Vitelline reservoir ventral to Mehlis' gland. Uterus pretesticular, between posterior border of anterior testis and genital pore. Metraterm relatively short. Eggs 41–70 (54) long by 23–39 (29) wide. Excretory vesicle I-shaped, reaching to anterior margin of anterior testis. Excretory pore terminal.

Remarks

Auriculostoma tica sp. n. conforms to the diagnosis of *Auriculostoma*, in having a single pair of muscular lobes on either side of the oral sucker (Scholz et al. 2004; Razo-Mendivil et al. 2014a). Overall, the new species is morphologically more similar to *A. diagonale* Curran, Tkach & Overstreet, 2011 *A. platense* (Szidat, 1954) Scholz, Aguirre-Macedo & Choudhury, 2004, and *A. totonacapanensis* by possessing entire testes in oblique position; in some specimens of *A. totonacapanensis* testes seem to be in tandem, but in most of them testes are oblique (see Razo-Mendivil et al. 2014a). In both *A. tica* sp. n. and *A. platense* the anterior extension of the vitelline follicles extends to the mid-level of the esophagus, whereas in *A. diagonale* they reach the pharynx level, and in *A. totonacapanensis* follicles reach the intestinal bifurcation. Additionally, the new species differs from these species by the posterior extension of the cirrus-sac and the position of the genital pore: in *A. tica* sp. n. the cirrus-sac extends to the mid-level of the region between the ventral sucker and ovary and the genital pore reaches the mid level of the esophagus; in *A. platense* the cirrus-sac does not extend posteriorly beyond the ventral sucker and the genital pore is located between the anterior margin of the ventral sucker and caecal bifurcation; in *A. diagonale* the cirrus-sac extends to the anterior margin of the ovary and the genital pore is located between the anterior margin of the ventral sucker and caecal bifurcation; finally, in *A. totonacapanensis*, the cirrus-sac extends to the mid-level or to the posterior margin of the ovary but does not surpass the ovarian region, and the genital pore is located at the level of the caecal bifurcation.

The new species can be distinguished from the other congeners by a combination of several morphological characters and measurements; *A. tica* sp. n. differs from the remaining species, except from *A. stenopteri* (Mañé-Garzón & Gascón, 1973) Scholz, Aguirre-Macedo & Scholz, 2004 because the genital pore reaches the mid-level of the esophagus length; in *A. foliaceum* Curran, Tkach & Overstreet, 2011, *A. lobata*, and *A. macrorchis* (Szidat, 1954) Scholz, Aguirre-Macedo & Choudhury, 2004 the genital pore is located between the anterior margin of the ventral sucker and ceacal bifurcation. *Auriculostoma tica* sp. n. further differs from these four species, by having testes with entire margins located in oblique position rather than testes in tandem, with irregular or lobulated margins, by the anterior extension of the vitelline follicles, and by the difference in OS/VS width ratio; in *A. tica* sp. n. the vitelline follicles reach the mid-level of the esophagus, the genital pore is also located in that position, and the OS/VS width ratio is 1: 0.48–0.67, whereas in *A. foliaceum* follicles are absent in the forebody, and the ratio is 1:0.5; in *A. lobata* follicles reach the caecal bifurcation and the ratio is 1:1.21-1-52, while in *A. macrorchis* and *A. stenopteri* follicles extend up to the level of the oral sucker with ratios of 1:1-1.3 and 1:1.3, respectively. Additionally, the new species differs from *A. foliaceum* by having an excretory pore terminal, while in *A. foliaceum* the excretory pore is subterminal. From *A. macrorchis*, the new species differs further by having vitelline follicles intra- and extracecal and not largely extracecal as in *A. macrorchis*.

Even though the new species and *A. astyanace* are distributed in the same geographic area in Lower Central America, they are morphologically different. *Auriculostoma tica* sp. n. possesses oblique testes while in *A. astyanace* testes are in tandem position. Furthermore, the cirrus-sac in the new species is shorter than in *A. astyanace*; and in terms of host association, the new species is a parasite of gymnotids while *A. astyanace* is a common parasite of characids. Differences in morphology and host association may be the cause that they are not each other sister species, even occurring in sympatry (Fig. 1). Finally, the ultrastructure of the body surface of *A. tica* sp. n., *A. lobata* and *A. totonacapanensis* provide additional information for species delimitation; unfortunately, the ultrastructure of *A. astyanace* has not been described yet. The SEM photographs of our specimens possess 21 dome-like papillae on the oral sucker surface arranged in 4 rows, 2 apical papillae more than in *A. lobata* and *A. totonacapanensis*. The three species possess a single pair of muscular lobes located on either side of the oral sucker. However, they can be readily distinguished by the shape of the base of the dorsolateral oral lobes, which in the new species is inconspicuous (see Fig. 3).

Genus *Wallinia* Pearse, 1920

***Wallinia criptica* sp. n.**

Figs 4 and 5

Type material. Holotype (CNHE XXXX); paratypes: 11 specimens from San Juan del Río, Oaxaca, Mexico (CNHE XXXX) and 5 specimens from Río Las Cabezas, Saranate, Guatemala (CNHE XXX).

Type host. *Astyanax aeneus* (Günther).

Type locality. San Juan del Río, Oaxaca, México (16°53'57" N, 96°09'57" W).

Other localities. Río San Juan, Chiapas, Mexico (16°21'00" N, 93°30'54" W); Río las Cabezas at Sanarate, El Progreso, Guatemala (14°44'23" N, 90°04'52" W).

Site in host. Intestine.

Etymology. The specific name reflects the fact that the species is morphologically almost indistinguishable from their congeners, even though the genetic divergence for the molecular marker used shows it represents an independent evolutionary significant unit.

Diagnosis and description. [Based on 10 adult specimens from San Juan del Río, Oaxaca, Mexico and 5 adult specimens from Río las Cabezas at Saranate, El Progreso, Guatemala] Body elongate, 2,045–3,346 (2,740) long, maximum width 585–807 (699) at posterior third of body; anterior end bluntly rounded. Body widening gradually behind oral sucker region, terminating in narrower bluntly rounded end. Tegument without spines. Remnants of eyespots present at level of pharynx. Forebody 415–659

(533) long; forebody length: total body length ratio 0.16–0.21 (0.20); forebody without tegumental papillae on lateral sides. Oral sucker spherical, subterminal, well-developed, 241–332 (276) long, 246–331 (281) wide. Oral sucker with a variable number of papillae (Fig. 5C, D). Ventral sucker subspherical, smaller than oral sucker, muscular, 203–339 (265) long, 229–338 (277) wide. Ratio of oral sucker length to ventral sucker length 1:0.81–1.13 (1: 0.95); ratio of oral sucker width to ventral sucker width 1:0.83–1.17 (1: 0.99). Mouth subterminal. Prepharynx absent. Pharynx muscular, well-developed 93–143 (120.5) long, 110–163 (144) wide. Oesophagus curved and narrow 51–113 (78) long. Cluster of gland cells situated laterally on either side of pharyngeal and esophageal region. Intestinal bifurcation at anterior margin of ventral sucker, 366–518 (450) from anterior end of body; ceca terminating blindly in region between testes and posterior end of body, 548–797 (668) from posterior end. Testes two, oblique, rounded, entire, submedial. Anterior testis 190–384 (264) long by 128–262 (207) wide. Posterior testis contiguous with anterior testis, 192–337 (276) long by 140–272 (228) wide, 692–1453 (1029) from posterior end of body. Intertesticular space 99–368 (213). Cirrus-sac elongate, median, dorsal, and extending beyond posterior margin of ventral sucker; cirrus sac containing: distal, tubular and muscular ejaculatory duct surrounded by scattered gland cells; ovoid pars prostatica obscured by surrounding gland cells; unipartite folded seminal vesicle continuous occupying most of cirrus sac with narrower distal tubular portion; cirrus not observed. Genital pore median at intestinal bifurcation. Ovary 183–274 (211) long by 126–215 (168) wide, rounded, margin smooth, entire, post-acetabular, not overlapping ventral sucker, pre-testicular with posterior border at the level of the anterior border of the anterior testis, pre-equatorial, ventrally situated, in sinistral position (or median), with distinct distal region containing larger oocytes. Seminal receptacle ovoid 118–153 (129) long by 84.7–144.9 (115) wide, immediately

posterior to ovary, not overlapping it. Mehlis' gland composed of scattered gland cells obscuring ootype, median, between ovary, anterior testis and seminal receptacle. Laurer's canal traversing dorsal side of body laterally and opening to dorsal surface just past outer edge of cecum at level of ovary, seminal receptacle, or anterior testis; opening dextrally. Vitellarium follicular in two ventrolateral fields extending usually from anterior margin of ventral sucker to halfway between posterior margin of posterior testis and end of ceca; follicles overlapping cecal field ventrally; vitelline ducts uniting immediately posterior to ootype to form vitelline reservoir. Uterus extending posteriorly between intertesticular space and alongside testes, forming convoluted loops, mostly intercecal, filling post-testicular space and extending to posterior end of body; distal arm of uterus winding anteriorly, traversing pretesticular region medially, forming ventral metraterm leading to genital pore. Eggs numerous, ovoid, operculate, embryonated, 47–64 (51) long, 24–39 (29) wide. Excretory bladder narrow, I-shaped, Due to the amount of eggs in the uterus is not able to see until it extends anteriorly; excretory pore slightly dorso-terminal.

Remarks

Wallinia criptica sp. n. is placed *Wallinia* because it shows the diagnostic characters that distinguish this genus from other allocreadids, i.e., an unspined body, well-developed cirrus-sac, and medium genital pore (Choudhury et al. 2002). The new species is morphologically more similar to *W. mexicana*, a parasite of the Mexican tetra fish, *Astyanax mexicanus* (De Filippi) in Mexico, although they are not each other the closest relatives; however, they differ in the anterior extension of the vitelline follicles and ovary shape; in *W. criptica* sp. n. the ovary is oval and the vitelline follicles do not extend anteriorly to the anterior margin of ventral sucker, while in *W. mexicana* the ovary is triangular shaped and and vitelline follicles reach the intestinal bifurcation. Even though these two species are morphologically similar, the genetic divergence for the 28S rRNA gene is very high, reaching values of 4%. The new species can be readily distinguished from the other three congeners. From *W. chavarriae*, a parasite of *A. aeneus* and *Bryconamericus scleroparius* (Regan) in Costa Rica, the new species can be distinguished by having more restricted vitellarium; vitelline follicles extend posteriorly to reach half the distance between the posterior testis and the end of the caeca, by having a longer esophagus, and oblique testes, instead of having vitelline follicles reaching the posterior margin of posterior testis, short oesophagus and testis in tandem (or slightly oblique in some specimens), almost contiguous, as in *W. chavarriae.* The closest relative to the new species is *W. brasiliensis* (Fig. 1), a species recently described from the intestine of two species of tetras, *Astyanax fasciatus* (Cuvier) and *Astyanax lacustris* Lucena and Soares, 2016 from the Batalha River in São Paulo State, Brazil. This species pair exhibits the smallest genetic divergence for the 28S rRNA gene, with 1.19%. The Brazilian species differs from *W. criptica* sp. n. by having a shorter body (1,136 vs 2,749), by the extension of the vitelline follicles almost reaching the posterior end of the body, by the testes shape (elongated rather than rounded), and by having relatively larger eggs. Finally, the type species for which sequence data has not been obtained yet, *W. valenciae* Pearse, 1920, a parasite of *Gephyrocharax valenciae* (Eiganmann) in Venezuela differs from the new species by having tubular vitelline follicles rather than rounded and by possessing a smaller oral sucker (relative to the ventral sucker).

Discussion

In this paper we present the description of two new species, which increase the diversity of fresh parasites of freshwater fish in Middle-America. With the addition of the two new species, the number of congeneric species raises to five for the genus *Wallinia*, and to nine in *Auriculostoma* (Hernández-Mena et al. 2016; Dias et al. 2018). In addition A. *tica* is the first species of allocreadiids described in Costa Rica.

The diversity of allocreadiids on Neotropical freshwater fishes has increased in the last years as a result of a more detailed study of the morphological traits in combination with DNA sequences and molecular phylogenies. New species and even new genera have been uncovered (e.g., Pérez-Ponce de León et al. 2016). Particularly within the genus *Auriculostoma* and *Wallinia*, six additional species have been described following that approach, four in *Auriculostoma*, i.e., *A. foliaceum* in *Bryconops caudomaculatus* (Günther); *A. diagonale* in *Stethaprion erythrops* Cope, both from Peru; *A. totonacapanensis* in *A. mexicanus* from Veracruz, Mexico, and *A. lobata* in *Brycon guatemalensis* from Chiapas, Mexico (Curran et al., 2011; Razo-Mendivil et al. 2014; Hernández-Mena et al., 2016); and two species in *Wallinia* were also described, including *W. mexicana* in *A. mexicanus* from central Mexico, and *W. brasiliensis* from *Astyanax fasciatus* and *A. lacustris* in the State of São Paulo, Brazil (Pérez-Ponce de León et al. 2015; Dias et al., 2018). Species in both genera are mainly parasites of characids across the Neotropical biogeographical region. The five species of *Wallinia* and six of the nine species of *Auriculostoma* are exclusively parasites of characids. Two species of the latter genera, *A. platense* (Szidat, 1954) Scholz, Aguirre-Macedo & Choudhury, 2004, and *A. macrorchis* (Szidat, 1954) Scholz, Aguirre-Macedo & Choudhury, 2004 are parasites of siluriforms in Argentina (see Kohn et al. 2007; Ostrowski de Nuñez et al. 2017) and the new species we describe herein is a parasite of a gymnotid in Costa Rica. These results indicate that it would be likely to find a higher diversity of *Auriculostoma* in other neotropical freshwater fishes.

Acknowledgements

This study is part of the fulfillment of DIHM for the requirements to obtain PhD degree in the Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México. DIHM also thanks the Consejo Nacional de Ciencia y Tecnología (CONACyT) for student grant 245193. We thank Luis García for providing specimens from the Colección Nacional de Helmintos for comparison and for providing catalog numbers for the specimens studied in the present study. We thank Patricia Ornelas for her help in the collection of fish in Mexico. We also thank Laura Márquez for the support of processing samples for the automated sequencer is greatly appreciated. This project was funded by the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-UNAM) IN202617 to GPPL, and PAPIIT-UNAM IN206716 to M.G.V.

References

Bautista-Hernández C, Monks S, Pulido-Flores G, Miranda R (2015) A new species of *Paracreptotrema* n. sp. (Digenea, Plagiorchiformes, Allocreadiidae) from *Xiphophorus malinche* (Teleostei,Poeciliidae) in Río Malila of the Río Pánuco basin,Hidalgo, México with a key to the species of the genus. ZooKeys 482: 55–66. https://doi.org/10.3897/zookeys.482.8144

Choudhury A, Daverdin RH, Brooks DR (2002) *Wallinia chavarriae* n. sp. (Trematoda: Macroderoididae) in *Astyanax aeneus* (Günther, 1860) and *Bryconamericus scleroparius* (Regan, 1908) (Osteichthyes: Characidae) from the Area de Conservación Guanacaste, Costa Rica. Journal of Parasitology 88(1): 107–112. https://doi.org/10.1645/0022-3395(2002)088[0107:WCNSTM]2.0.CO;2

Choudhury A, Pérez-Ponce de León G, Brooks DR, Daverdin RH (2006) *Paracreptotrema blancoi* N. gen., N. sp (Digenea : Plagiorchiformes : Allocreadiidae) in the Onomina, *Priapichthys annectens* (Osteichthyes :

Poeciliidae), from the area de Conservacion Guanacaste, Costa Rica. Journal of Parasitology 92(3): 565–568. https://doi.org/10.1645/GE-3540.1

Curran SS, Tkach VV, Overstreet RM (2011) Phylogenetic affinities of *Auriculostoma* (Digenea: Allocreadiidae), with descriptions of two new species from Peru. Journal of Parasitology 97(4): 661–670. https://doi.org/10.1645/GE-2641.1

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9(8): 772. https://doi.org/10.1038/nmeth.2109

Dayrat B (2005). Towards integrative taxonomy. Biological Journal of the Linnean Society 85: 407–415. https://doi.org/10.1111/j.1095-8312.2005.00503.x

Dias KGA, Müller MI, de Almeida AC, da Silva RJ, de Azevedo RK, Pérez-Ponce de León G, Abdallah BD (2018) A New Species of *Wallinia* Pearse, 1920 (Digenea: Allocreadiidae) collected from *Astyanax fasciatus* (Cuvier, 1819) and *A. lacustris* Lucena and Soares, 2016 (Characiformes: Characidae) in Brazil based on morphology and DNA sequences. Parasitology Research, XXX, XX-XX.

García-Varela M, Nadler SA (2005) Phylogenetic relationships of Palaeacanthocephala (Acanthocephala) inferred from SSU and LSU rDNA gene sequences. Journal of Parasitology 91(6): 1401–1409. https://doi.org/10.1645/GE-523R.1

Kohn, A., Fernandes, B.M. & Cohen, S.C. (2007) South American trematodes parasites of fishes. Ministerio da Saúde, FIOCRUZ, Rio de Janeiro, Brasil, 318 pp.

Hernández-Mena D, Lynggaard C, Mendoza-Garfias B, Perez-Ponce de León G (2016) A new species of *Auriculostoma* (Trematoda: Allocreadiidae) from the intestine of *Brycon guatemalensis* (Characiformes: Bryconidae) from the Usumacinta River Basin, Mexico, based on morphology and 28S rDNA sequences, with a key to species of the genus. Zootaxa 4196(2): 261–277. http://doi.org/10.11646/zootaxa.4196.2.5

Nadler SA, Hudspeth DSS (1998) Ribosomal DNA and phylogeny of the Ascaridoidea (Nemata: Secernentea): Implications for morphological evolution and classification. Molecular Phylogenetic & Evolution 10(2): 221–236. https://doi.org/10.1006/mpev.1998.0514

Ostrowski de Núñez M, Arredondo NJ, Gil de Pertierra AA (2017) Adult Trematodes (Platyhelminthes) of freshwater fishes from Argentina: a checklist. Revue Suisse de Zoologie 124(1): 91–113. https://doi.org/10.5281/zenodo.322669

Pearse AS (1920) The fishes of Lake Valencia, Venezuela. University of Wisconsin Studies in Science 1: 1–51.

Pérez-Ponce de León G, Razo-Mendivil U, Mendoza-Garfias B, Rubio-Godoy M, Choudhury A (2015) A new species of *Wallinia* Pearse, 1920 (Digenea: Allocreadiidae) in *Astyanax mexicanus* (Characidae) from Mexico revealed by morphology and sequences of the 28S ribosomal RNA gene. Folia Parasitologica 62: 018. https://doi.org/10.14411/fp.2015.018.

Pérez-Ponce de León G, Pinacho-Pinacho CD, Mendoza-Garfias B, Choudhury A, García-Varela M (2016) Phylogenetic analysis using the 28S rRNA gene reveals that the genus *Paracreptotrema* (Digenea: Allocreadiidae) is not monophyletic; Description of two new genera and one new species. Journal of Parasitology 102(1): 131–142. https://doi.org/10.1645/15-815

Razo-Mendivil U, Mendoza-Garfias B, Ponce-Pérez de León G, Rubio-Godoy M (2014a) A new species of *Auriculostoma* (Digenea: Allocreadiidae) in the Mexican tetra *Astyanax mexicanus* (Actinopterygii: Characidae) from central

Veracruz, Mexico, described with the use of morphological and molecular data. Journal of Parasitology 100(3): 331–337. https://doi.org/10.1645/13-352.1

Razo-Mendivil U, Mendoza-Garfias B, Ponce-Pérez de León G, Rubio-Godoy M (2014b) Testing the systematic position and relationships of *Paracreptotrema heterandriae* within the Allocreadiidae through partial 28s rRNA sequences. Journal of Parasitology 100(4): 537–541. https://doi.org/10.1645/13-421.1

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, et al. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Scholz T, Aguirre-Macedo ML, Choudhury A (2004) *Auriculostoma astyanace* n. gen., n. sp. (Digenea: Allocreadiidae), from the banded astyanax, *Astyanax fasciatus* (Characiformes: Characidae), from Nicaragua, with a reevaluation of Neotropical *Crepidostomum* spp. Journal of Parasitology 90(5): 1128–1132. https://doi.org/10.1645/GE-3275

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22(21): 2688–2690. https://doi.org/10.1093/bioinformatics/btl446

Stock SP, Campbell JF, Nadler SA (2001) Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. Journal of Parasitology 87(4): 877–889. https://doi.org/10.1645/0022-3395(2001)087[0877:POSTCS]2.0.CO;2

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30(12): 2725–2729. https://doi.org/10.1093/molbev/mst197

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 15(24): 4876–4882.

Figure legends

Fig. 1. Phylogenetic relationships among allocreadiid trematodes resulting from Maximum Likelihood based on the partial sequence of the 28S rRNA gene. Bootstrap support values and posterior probability values are shown near the nodes. The two new species described here are highlighted in bold. Host species and geographical distribution of each species on the tree is indicated. Mex = Mexico, CR = Costa Rica, Bra = Brazil; Gtm = Guatemala. The scale-bar indicates the number of substitutions per site.

Fig. 2. *Auriculostoma tica* sp. n. (A) Ventral view of holotype. Scale bar = 500 µm. (B) Ventral view of terminal genitalia showing that the ejaculatory duct and the uterus open to the genital atrium (reconstruction made from the observation of several specimens). Scale bar = 200 µm.

Fig. 3 Scanning electron micrographs of *Auriculostoma tica* sp. n. (A) Anterior end with oral sucker bearing muscular lobe on either side, genital atrium and ventral sucker showing 6 dome-like papillae (white arrows). (B) Pair of muscular lobes with a short posterior ‘free’ end, and 4 apical dome-like papillae. (C) Lateral view of muscular lobe. (D) Distribution of dome-like papillae over oral sucker, 8 anterior, 4 on the inner surface, and 5 on the outer surface (white arrows).

Fig. 4 *Wallinia criptica* sp. n. (A) Ventral view of holotype. Scale bar = 500 µm. (B) Ventral view of terminal genitalia showing that the ejaculatory duct and the uterus open to the genital atrium (reconstruction made from the observation of several specimens). Scale bar = 200 µm.

Fig. 5 Scanning electron micrographs of *Wallinia criptica* sp. n. (A) Whole specimens, ventral view. (B), (C) Oral sucker showing an irregular arrangment of dome-like papillae. (D) Ventral sucker showing 6 dome-like papillae (white arrows).

Figure 1

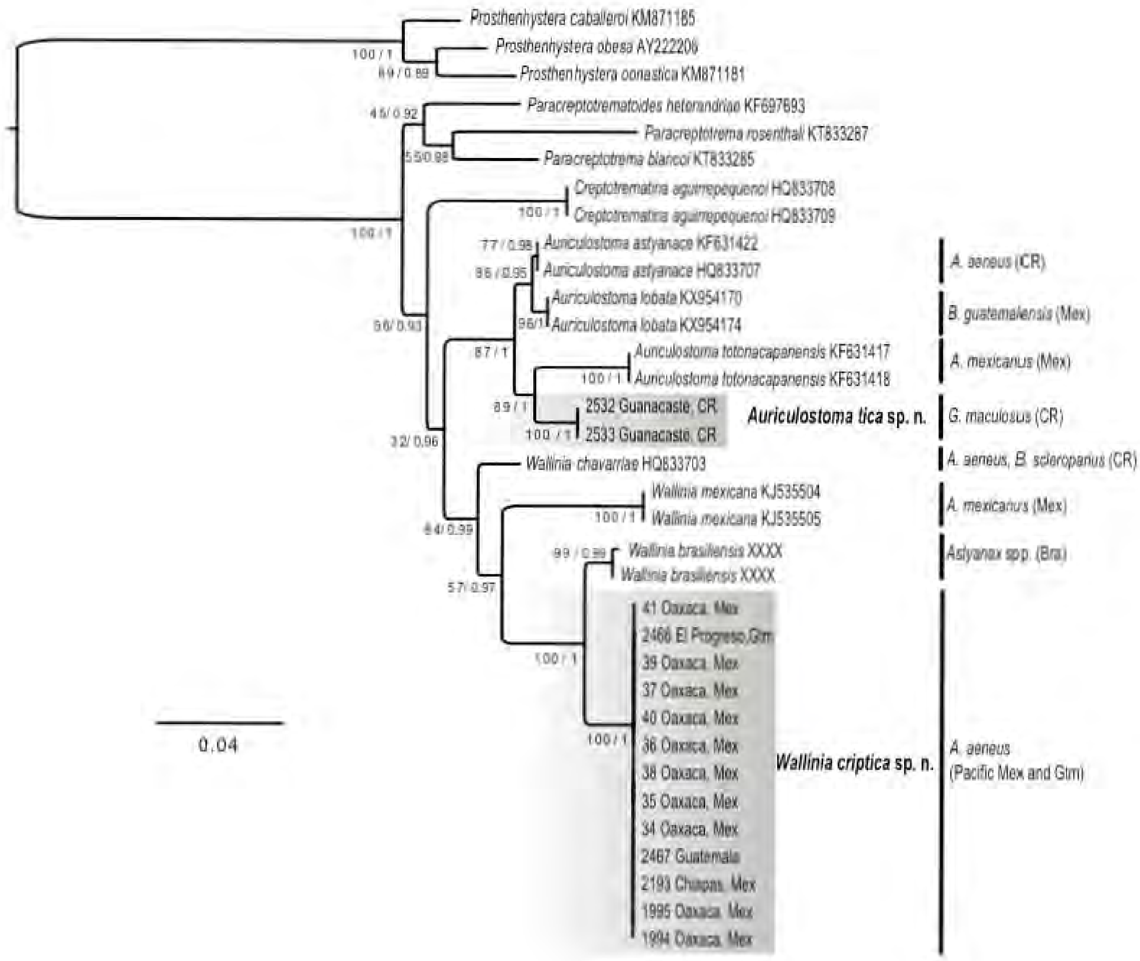

Prosthenhystera caballeroi KM871185
Prosthenhystera obesa AY222206
Prosthenhystera oonastica KM871181
Paracreptotrematoides heterandriae KF697693
Paracreptotrema rosenthali KT833287
Paracreptotrema blancoi KT833285
Creptotrematina aguirrepequenoi HQ833708
Creptotrematina aguirrepequenoi HQ833709
Auriculostoma astyanace KF631422
Auriculostoma astyanace HQ833707
Auriculostoma lobata KX954170
Auriculostoma lobata KX954174
Auriculostoma totonacapanensis KF631417
Auriculostoma totonacapanensis KF631418
2532 Guanacaste, CR
2533 Guanacaste, CR
Auriculostoma tica sp. n.
Wallinia chavarriae HQ833703
Wallinia mexicana KJ535504
Wallinia mexicana KJ535505
Wallinia brasilensis XXXX
Wallinia brasilensis XXXX
41 Oaxaca, Mex
2466 El Progreso,Gtm
39 Oaxaca, Mex
37 Oaxaca, Mex
40 Oaxaca, Mex
36 Oaxaca, Mex
38 Oaxaca, Mex
35 Oaxaca, Mex
34 Oaxaca, Mex
2467 Guatemala
2193 Chiapas, Mex
1995 Oaxaca, Mex
1994 Oaxaca, Mex
Wallinia criptica sp. n.
A. aeneus (CR)
B. guatemalensis (Mex)
A. mexicanus (Mex)
G. maculosus (CR)
A. aeneus, B. scleroparius (CR)
A. mexicanus (Mex)
Astyanax spp. (Bra)
A. aeneus
(Pacific Mex and Gtm)
0.04

Figure 2

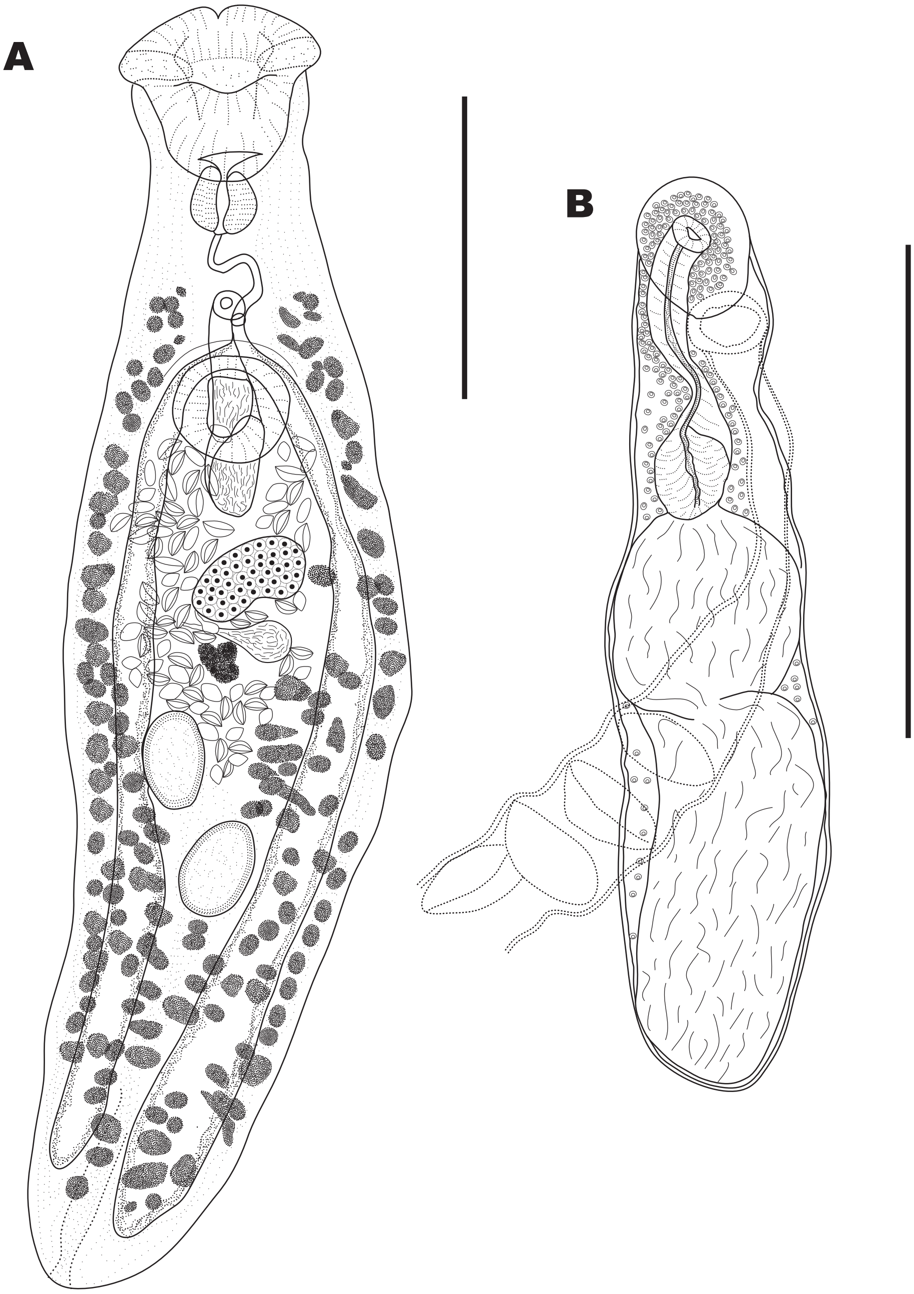


Figure 3

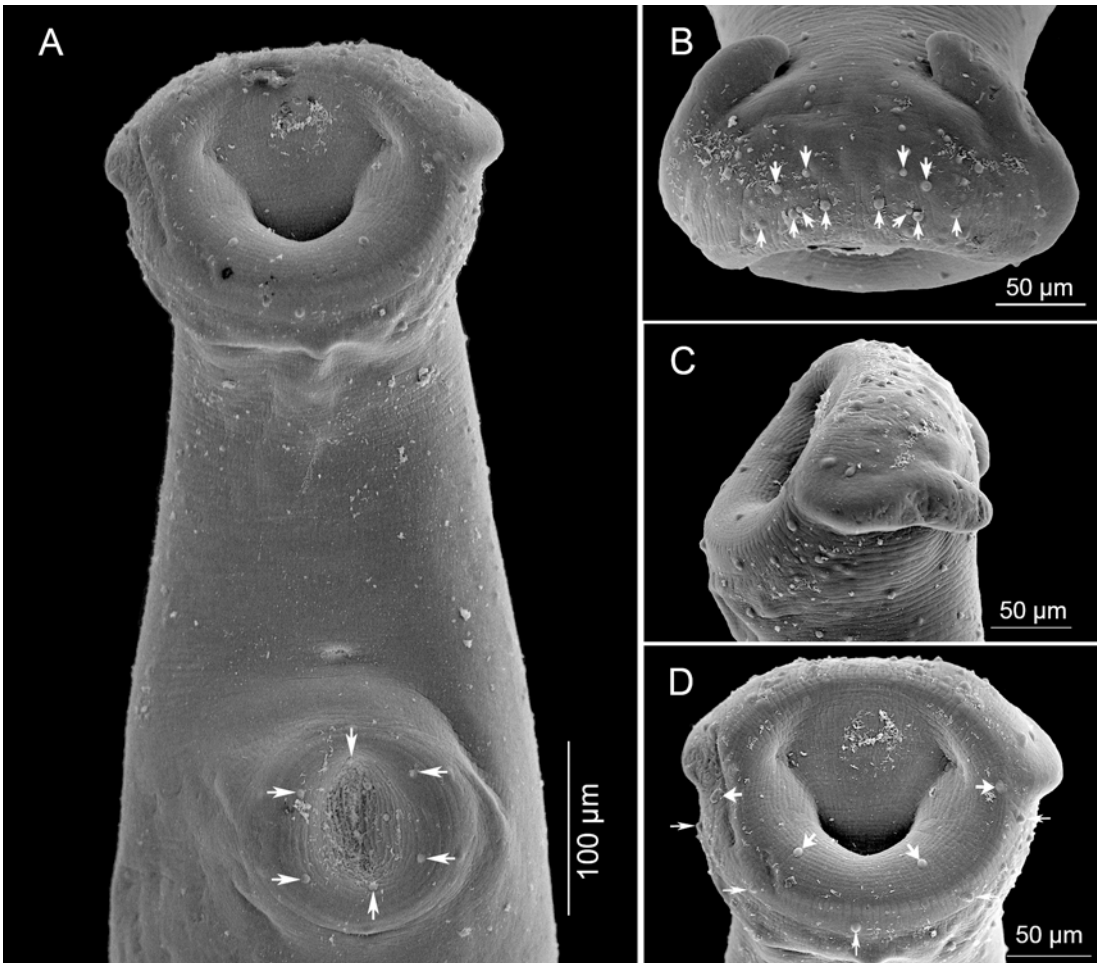


Figure 4

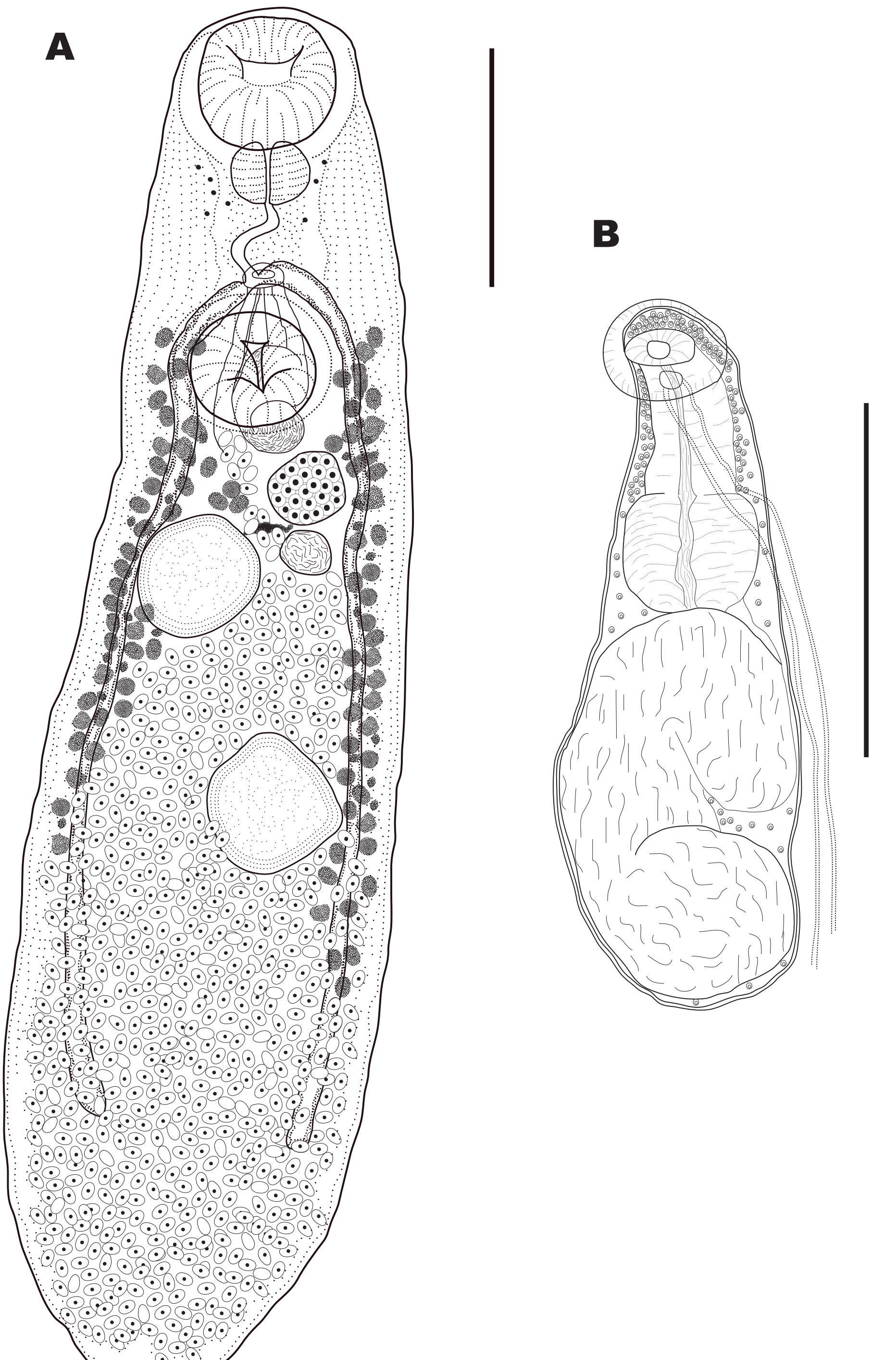
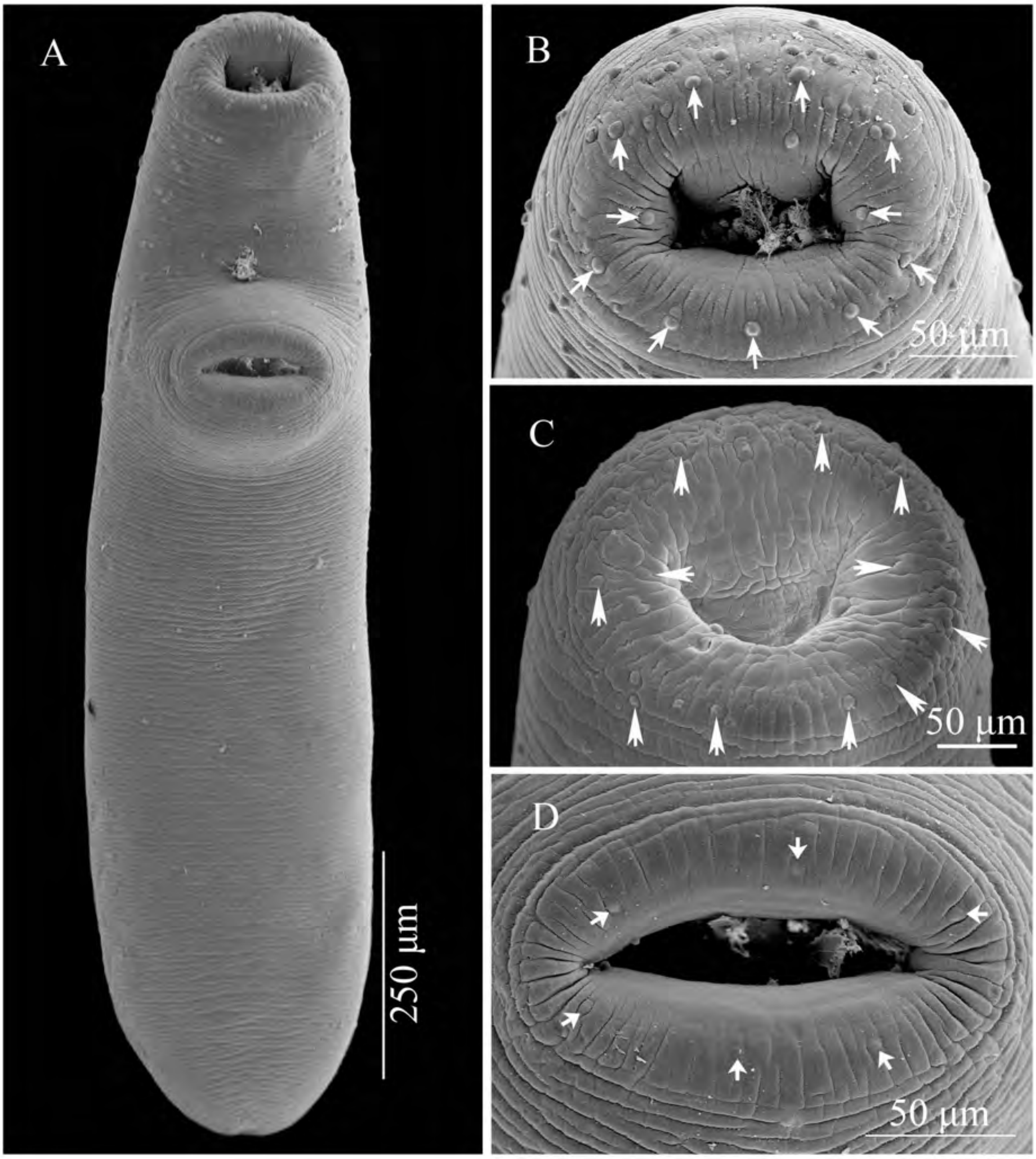


Figure 5



4. CAPÍTULO II

4. CAPÍTULO II. POSICIÓN FILOGENETICA Y DESCRIPCIÓN DE UNA NUEVA FAMILIA

En este capitulo se presenta una hipótesis filogenética en la que se investiga la posición de dos géneros de trematodos parásitos de peces dulceacuícolas endémicos de México, los cuales originalmente estaban clasificados dentro de la misma familia Macroderoiididae. Análisis filogenéticos usando el marcador nuclear 28S utilizando Parsimonia, Máxima Verosimilitud e Inferencia Bayesiana, demostraron que Macroderoiididae, así como *Macroderoides* son grupos parafileticos, por lo que surgió el establecimiento de una nueva familia que agrupa a las especies y géneros. La información se presenta en un artículo publicado en la revista Systematic Parasitology titulado "**Phylogenetic position of *Magnivitellinum* Kloss, 1966 and *Perezitrema* Baruš & Moravec, 1967 (Trematoda: Plagiorchioidea: Macroderoididae) inferred from partial 28S rDNA sequences, with the establishment of Alloglossidiidae n. fam.**".

Syst Parasitol (2016) 93:525–538
DOI 10.1007/s11230-016-9645-9

CrossMark

Phylogenetic position of *Magnivitellinum* Kloss, 1966 and *Perezitrema* Baruš & Moravec, 1967 (Trematoda: Plagiorchioidea: Macroderoididae) inferred from partial 28S rDNA sequences, with the establishment of Alloglossidiidae n. fam.

David Iván Hernández-Mena · Berenit Mendoza-Garfias · Claudia Patricia Ornelas-García · Gerardo Pérez-Ponce de León

Received: 25 January 2016 / Accepted: 17 May 2016

Abstract The systematic position of two genera of Macroderoididae McMullen, 1937, *Perezitrema* Baruš & Moravec, 1967 and *Magnivitellinum* Kloss, 1966 is reviewed based on a phylogenetic analysis of the interrelationships of 15 species of the family allocated into six genera, along with 44 species of plagiorchioid trematodes, using partial sequences of the 28S rRNA gene. Sequences were analysed through parsimony, maximum likelihood and Bayesian inference. The obtained topologies show *Perezitrema* as the sister taxon of three species of *Macroderoides* Pearse, 1924; the latter genus appears to be paraphyletic since another three species are not included in this group. Instead, *Magnivitellinum* was placed as the sister taxon of *Alloglossidium* Simer, 1929. These relationships are well supported by high bootstrap and posterior probability values. The resulting trees demonstrate that the family Macroderoididae, as currently conceived in taxonomic treatments, is not monophyletic. *Magnivitellinum simplex* Kloss, 1966 and *Alloglossidium* spp. were nested as sister taxa of members of the family Leptophallidae Dayal, 1938, whereas *Perezitrema bychowskii* Baruš & Moravec, 1967 and species of *Macroderoides* and *Paramacroderoides* Venard, 1941 were grouped with *Auridistomum chelydrae* (Stafford, 1900), a monotypic member of Auridistomidae Stunkard, 1924. Based on our results, a new family, Alloglossidiidae n. fam. was established to accommodate the genera *Magnivitellinum* and *Alloglossidium*.

D. I. Hernández-Mena · B. Mendoza-Garfias · G. Pérez-Ponce de León (✉)
Laboratorio de Helmintología, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico, DF, Mexico
e-mail: ppdleon@ib.unam.mx

D. I. Hernández-Mena
Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Mexico, DF, Mexico

C. P. Ornelas-García
Laboratorio de Ictiología, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico, DF, Mexico

Introduction

The family Macroderoididae McMullen, 1937 was erected to include intestinal parasites of freshwater fish, although the group now includes forms that mature in marine fish, and some even in crustaceans and leeches (Font & Lotz, 2008a). The assignment of genera into this family of plagiorchioid trematodes, and as a consequence its species composition, is still not settled and has been controversial due to the inadequate choice of morphological characters (e.g. excretory vesicle shape), and insufficient information regarding their life-cycles (Font & Lotz, 2008a). The current classification of the Macroderoididae includes trematodes that mostly infect freshwater fish and are diagnosed by having a spined tegument, elongate, blind caeca, usually extending to near posterior extremity, I-shaped excretory vesicle, and exceptionally forming cyclocoel. However, as discussed by Kostadinova & Pérez-del-Olmo (2014) the search of apparent non-homoloplasious morphological characters at the higher taxonomic levels of trematodes has been controversial, and requires the addition of molecular data to obtain a more accurate classification scheme. This seems to be the case for the family Macroderoididae.

Font & Lotz (2008a) discussed in detail the complex taxonomic history of the family and recognised only nine valid genera: *Alloglossidium* Simer, 1929; *Cirkennedya* Gibson & Bray, 1979; *Gauhatiana* Gupta, 1955; *Macroderoides* Pearse, 1924; *Rauschiella* Babero, 1951; *Magnivitellinum* Kloss, 1966; *Paramacroderoides* Venard, 1941; *Perezitrema* Barus & Moravec, 1967; and *Malawitrema* Bray & Hendrix, 2007. Likewise, Font & Lotz (2008a) also recognised that the assignment of genera such as *Gauhatiana* and *Rauschiella* to Macroderoididae, both of which possess Y-shaped vesicles, should be considered provisional. Further, Pojmańska et al. (2008) considered some genera of trematodes that have been included at different times and by different authors in the family as *incertae sedis*, i.e. *Wallinia* Pearse, 1920; *Glossidium* Looss, 1899, *Vietosoma* Van Cleave & Mueller, 1932; and *Pseudomagnivitellinum* Dronen & Underwood, 1980. Razo-Mendivil et al. (2006) obtained molecular evidence that the genus *Rauschiella* does not belong to the Macroderoididae. Pérez-Ponce de León et al. (2007a) used 28S rDNA sequence data to show that *Wallinia* belongs to the Allocreadiidae Looss, 1902. The other three genera, i.e. *Glossidium*, *Vietosoma* and *Pseudomagnivitellinum* exhibit morphological characters of the family Macroderoididae, and are parasites of siluriform freshwater fishes (Khalil, 1972; Hoffman, 1999; Dronen & Underwood, 1980).

In this study, we incorporate 28S rDNA sequences for species of two genera of the Macroderoididae (*Magnivitellinum* and *Perezitrema*) to the database that has been built for different members of the family (see Tkach et al., 1999, 2001, 2008, 2010; Tkach & Mills, 2011; Tkach & Kinsella, 2011; Kasl et al., 2014). We investigate the systematic position of these two genera of macroderoidids through rDNA sequences in the context of the phylogeny of the Plagiorchioidea Lühe, 1901, and we discuss the implications of our findings for the classification scheme of the family that resulted in the establishment of a new family to accommodate some of the genera, and we further discuss the host and historical biogeography of the host-parasite associations.

Materials and methods

Specimen collection

Adult specimens of two species belonging to the genera *Magnivitellinum* and *Perezitrema*, were collected from freshwater fishes in seven localities across Mexico, between October 2013 and March 2015 (Table 1). Worms recovered from their hosts were rinsed in 0.6% saline solution; some individuals were fixed in 100% ethanol for molecular analyses and some individuals were fixed in hot (steaming) 4% formalin for further morphological study. Some specimens were stained with Mayer's paracarmine and mounted in Canada balsam for identification; voucher specimens were deposited in the Colección Nacional de Helmintos (CNHE), Mexico City, with the accession numbers 9974-9976 (*Magnivitellinum simplex* Kloss, 1966) and 9973 (*P. bychowskyi* Baruš & Moravec, 1967).

Light and scanning electron microscopy

Specimens were stained with Gomori's trichrome or Mayer's paracarmine, dehydrated in a graded ethanol series, cleared in methyl salicylate, and mounted as permanent slides in Canada balsam. Four individuals (two of each species) fixed in 4% formalin were used for scanning electron microscopy (SEM) studies. Worms were dehydrated through a graded ethanol series, critical point-dried with carbon dioxide, mounted on metal stubs with carbon adhesive tabs, gold coated and examined at 15 kV in a Hitachi Stereoscan Model SU1510 SEM (Hitachi Ltd., Tokyo, Japan).

DNA extraction, amplification and sequencing

Eight trematodes identified as *Magnivitellinum simplex*, from five localities and ten identified as *Perezitrema bychowskyi* from two localities, were individually digested overnight at 56°C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl,

Table 1 Digenean species used in phylogenetic analyses (in alphabetical order by family and species) with GenBank accesion numbers, hosts and localities of the isolates sequenced

Species	GenBank acc. no.	Host species (Order)	Locality
Allocreadiidae Looss, 1902			
Auriculostoma astyanace Scholz, Aguirre-Macedo & Choudhury, 2004	HQ833707	*Astyanax fasciatus* (Cuvier) (Characiformes)	Río Animas, Costa Rica
Auridistomidae Stunkard, 1924			
Auridistomum chelydrae (Stafford, 1900)	AY116872	*Chelydra serpentina* (L.) (Testudines)	Jackson County, USA
Brachycoeliidae Looss, 1899			
Brachycoelium salamandrae (Frölich, 1789)	AF151935	*Salamandra salamandra* (L.) (Caudata)	Zakarpatska, Ukraine
Parabrachycoelium longicaecum Pérez-Ponce de León, Mendoza-Garfias, Razo & Parra-Olea, 2011	HQ165754	*Chiropterotriton* Taylor, 1944 (Caudata)	Tlaquilpa, Mexico
Cephalogonimidae Looss, 1899			
Cephalogonimus americanus Stafford, 1902	HM137615	*Ambystoma velasci* Dugès (Caudata)	Quechulac, Mexico
Cephalogonimus retusus (Dujardin, 1845)	AY222276	*Pelophylax ridibundus* (Pallas) (Caudata)	Kokaljane, Bulgaria
Choanocotylidae Jue Sue & Platt, 1998			
Choanocotyle nematoides Jue Sue & Platt, 1998	AY116862	*Emydura macquarii* Gray (Testudines)	MacCleay River, Australia
Glypthelminthidae Cheng, 1959			
Glypthelmins brownorumae Razo-Mendivil, León-Règagnon & Pérez-Ponce de León, 2004	AY875674	*Rana brownorum* (Sanders) (Anura)	Villahermosa, Mexico.
Haematoloechidae Freitas & Lent, 1939			
Haematoloechus abbreviatus (Bychowsky, 1932)	AF184251	*Bombina variegata* (L.) (Anura)	Zakarpatska region, Ukraine
Leptophallidae Dayal, 1938			
Leptophallus nigrovenosus (Bellingham, 1844)	AF151914	*Natrix natrix* (L.) (Squamata)	Kiev region, Ukraine
Macrodera longicollis (Abildgaard, 1788)	AF151913	*Natrix natrix* (L.) (Squamata)	Kiev region, Ukraine
Metaleptophallus gracillimus (Lühe, 1909)	AF151912	*Natrix natrix* (L.) (Squamata)	Kiev region, Ukraine
Paralepoderma cloacicola (Lühe, 1909)	AF151910	*Natrix natrix* (L.) (Squamata)	Kiev region, Ukraine
Macroderoididae McMullen, 1937			
Alloglossidium corti (Lamont, 1921)	JF440783	*Ameiurus melas* Rafinesque (Siluriformes)	Pine Lake, USA

Table 1 continued

Species	GenBank acc. no.	Host species (Order)	Locality
Alloglossidium fonti Tkach & Mills, 2011	JF440763	*Ameiurus melas* Rafinesque (Siluriformes)	Mud Lake, USA
Alloglossidium geminum (Mueller, 1930)	JF440771	*Ameiurus melas* Rafinesque Siluriformes)	Red River, USA
Alloglossidium kenti Simer, 1929	JF440806	*Ictalurus punctatus* Rafinesque (Siluriformes)	Pascagoula River, USA
Alloglossidium floridense Kasl, Fayton, Font & Criscione, 2014	KC812276	*Noturus leptacanthus* Jordan (Siluriformes)	Santa Fe River, USA
Macroderoides flavus Van Cleave & Mueller, 1932	HQ680851	*Esox niger* Lesueur (Esociformes)	Connecticut, USA
Macroderoides minutus Tkach & Kinsella, 2011	HQ680850	*Lepisosteus platyrhincus* DeKay (Lepisosteiformes)	Orange Lake, USA
Macroderoides spiniferus Pearse, 1924	EU850400	*Lepisosteus platyrhincus* DeKay (Lepisosteiformes)	Nueces River, USA
Macroderoides texanus Tkach, Strand & Froese, 2008	EU850398	*Atractosteus spatula* Lacépède (Lepisosteiformes)	Nueces River, USA
Macroderoides trilobatus Tyler, 1978	EU850406	*Lepisosteus platyrhincus* Dekay (Lepisosteiformes)	Cross Creek, USA
Macroderoides typicus (Winfield, 1929)	HQ680846	*Lepisosteus platyrhincus* DeKay (Lepisosteiformes)	Orange Lake, USA
Magnivitellinum simplex Kloss, 1966	**KU535683 KU535684**	*Astyanax aeneus* (Günther) (Characiformes)	Metzabok Lake, Chiapas, Mexico
Magnivitellinum simplex Kloss, 1966	**KU535677** **KU535678**	*Astyanax. mexicanus* De Filippi (Characiformes)	Micos, Qeretaro Mexico
Magnivitellinum simplex Kloss, 1966	**KU535679**	*Astyanax. mexicanus* De Filippi (Characiformes)	Media Luna, San Luis Potosí, Mexico
Magnivitellinum simplex Kloss, 1966	**KU535682**	*Astyanax aeneus* (Günther) (Characiformes)	Pichucalco River, Tabasco Mexico
Magnivitellinum simplex Kloss, 1966	**KU535680** **KU535681**	*Astyanax aeneus* (Günther) (Characiformes)	Raudal River, Veracruz, Mexico
Paramacroderoides kinsellai Tkach, Pulis & Overstreet, 2010	HM137661	*Lepisosteus oculatus* Winchell (Lepisosteiformes)	Oxbow Lake, USA

Table 1 continued

Species	GenBank acc. no.	Host species (Order)	Locality
Perezitrema bychowskyi (Caballero & Caballero, 1975)	**KU535685 KU535688-KU535691**	*Atractosteus tropicus* Gill (Lepisosteiformes)	Emiliano Zapata, Pantanos de Centla, Campeche, Mexico
Perezitrema bychowskyi (Caballero & Caballero, 1975)	**KU535686-KU535687 KU535692-KU535694**	*Atractosteus tropicus* Gill (Lepisosteiformes)	San Pedro, Pantanos de Centla, Tabasco, Mexico
Rauschiella poncedeleoni Razo-Mendivil & León-Règagnon, 2001	AY875678	*Leptodactylus melanonotus* (Hallowell) (Anura)	San Pedro las Playas, Guerrero, Mexico
Rauschiella tineri (Babero, 1951)	AY875677	*Leptodactylus melanonotus* (Hallowell) (Anura)	San Pedro las Playas, Guerrero, Mexico
Mesocoeliidae Dollfus, 1929			
Mesocoelium sp.	AF433677	–	–
Omphalometridae Looss, 1899			
Neoglyphe sobolevi (Shaldybin, 1953)	AF300329	*Sorex araneus* L. (Soricomorpha)	Vilkovo, Ukraine
Omphalometra flexuosa Rudolphi, 1809	AF300333	*Talpa europaea* L. (Soricomorpha)	Kosewo GoÂrne, Poland
Rubenstrema exasperatum (Rudolphi, 1819)	AF300331	*Sorex araneus* L. (Soricomorpha)	Vilkovo, Ukraine
Plagiorchiidae Luhe, 1901			
Aptorchis aequalis Nicoll, 1914	EF014729	*Emydura krefftii* (Gray) (Testudines)	Ross River, Australia
Choledocystus hepatica (Lutz, 1928)	AY875679	*Rhinella marina* (L.) (Anura)	San Pedro las Playas, México
Haplometra cylindracea (Zeder, 1800)	AF151933	*Rana arvalis* Nilsson (Anura)	Carpathian Mountains, Ukraine
Lecithopyge rastellus Perkins, 1928	AF151932	*Bombina variegata* (L.) (Anura)	Carpathian Mountains, Ukraine
Plagiorchis elegans (Rudolphi, 1802)	AF151911	–	–
Skrjabinoeces similis (Looss, 1899)	AY222279	*Pelophylax ridibundus* (Pallas) (Anura)	Kokaljane, Bulgaria
Reniferidae Pratt, 1902			
Dasymetra nicolli Holl & Allison, 1935	AF433672	*Nerodia rhombifer* Hallowell (Squamata)	USA
Lechriorchis tygarti Talbot, 1933	JF820603	*Lithobates sylvaticus* Leconte (Anura)	North Dakota, USA

Table 1 continued

Species	GenBank acc. no.	Host species (Order)	Locality
Ochetosoma kansense (Crow, 1913)	AF433671	*Drymarchon corais* Holbrook (Squamata)	USA
Renifer aniarum (Leidy, 1890)	HQ665459	*Natrix natrix* (L.) (Squamata)	Calabria, Italy
Telorchiidae Looss, 1899			
Opisthioglyphe ranae (Frölich, 1791)	AF151929	*Rana arvalis* Nilsson (Anura)	Ivano-Frankivsk region, Ukraine
Telorchis assula (Dujardin, 1845)	AF151915	*Natrix natrix* (L.) (Squamata)	Kiev region, Ukraine

100 mM Na2 EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. Genomic DNA was extracted from the supernatant using the DNAzol (Molecular Research Center, Cincinnati, Ohio, USA). Partial fragments (domains D1-D3) of the large subunit of the nuclear ribosomal DNA (28S) were amplified using polymerase chain reaction (PCR), using the forward primer BD3 5′-GAA CAT CGA CAT CTT GAA CG-3′ and the reverse primer 536 5′-CAG CTA TCC TGA GGG AAA C-3′ (García-Varela & Nadler, 2005). PCR cycling parameters consisted of denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.30 min, followed by a post-amplification incubation at 72°C for 10 min. Sequencing reactions were performed using four internal primers: BD2 5′-TAT GCT TAA ATT CAG CGG GT-3′ (Luton et al., 1992); 502 5′-CAA GTA CCG TGA GGG AAA GTT GC-3′ (García-Varela & Nadler, 2005); 503 5′-CCT TGG TCC GTG TTT CAA GAC G-3′ (Stock et al., 2001); and 504 5′-CGT CTT GAA ACA CGG ACT AAG G-3′ (García-Varela & Nadler, 2005). Reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contiguous sequences were assembled using Geneious Pro 4.8.4 (Biomatters Ltd., Auckland, New Zealand). Sequences were deposited in the GenBank database (see accession numbers in Table 1).

Alignment and phylogenetic analyses

Sequences of the 28S rRNA gene of *Magnivitellinum simplex* and *Perezitrema bychowskyi* were aligned with sequences of species of other genera of the family Macroderoididae available in Genbank (*Alloglossidium*, *Macroderoides* and *Paramacroderides* Venard, 1941) for testing their phylogenetic relationships within the Macroderoididae. The monophyly of the Macroderoididae was also tested by including sequence data from other 13 families of the Plagiorchioidea (see Table 1). Sequences were aligned with the software ClustalW (Thompson et al., 1997), implemented in the web http://www.genome.jp/tools/clustalw/. No alignment positions were excluded prior to the analysis due to the fact that the 28S rRNA gene posseses conserved fragments and show a low substitution rate. Tree searches were conducted under Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference (BI). *Auriculostoma astyanace* Scholz, Aguirre-Macedo & Choudhury, 2004 (Allocreadidae) was used as the outgroup for rooting the trees.

Parsimony analyses were performed using New Technology Search with Ratchet, tree drifting and tree fusing algorithms with 100 repetitions using the TNT software v 1.1 (Goloboff et al., 2008). All characters in the analyses were given the same weight, and were treated as unordered, and non-additive. Gaps were treated as missing data. Branch support was estimated with 1,000 bootstrap replicates with ten random addition sequences and TBR branch swapping. jModeltest version 3.0 (Guindon & Gascuel, 2003; Darriba et al., 2012) was used for inferring the best nucleotide substitution model and parameter settings for the 28S dataset. The ML trees were inferred using RAxML 7.0.4 (Stamatakis, 2006). Analyses were executed with 100 replicates and GTR CAT+I model was used. Each analysis was initiated with a random starting tree, using the rapid hill-climbing algorithm (-x) (Stamatakis et al., 2007). Clade support was assessed through 10,000 bootstrap replicates, and the rapid-hill climbing algorithm was also implemented

(Stamatakis et al., 2008). The software MrBayes v. 3.2.1 (Ronquist et al., 2012) was used to perform the BI analysis. The settings were two simultaneous runs of the Markov chain Monte Carlo (MCMC) for 10 million generations, sampling every 200 generations, a heating parameter value of 0.2 and a 'burn-in' of 10%. A 50% majority-rule consensus tree representing the posterior probability distribution of clades was produced from 20,000 trees. All phylogenetic trees were visualised with the software FigTree version 1.4.2. Sequence divergences were estimated using uncorrected p-distance model with the program PAUP* 4.0b10 (Swofford, 2003).

Phylogenetic analyses

The final 28S rDNA data set consisted of 63 sequences (32 representing 16 species of the family Macroderoididae), and included 1,273 nt positions, of which 450 were parsimony-informative. MP analysis recovered 32 equally parsimonious trees with a C.I. = 0.4286, R.I. = 0.7950 and a length of 1,363 steps. ML yielded a single tree with -ln = 8687.369841. The MP strict consensus tree, the ML tree and the 50% majority-rule consensus tree of BI yielded similar topologies (Fig. 1), and some relationships were supported by relatively high bootstrap and posterior probability support values. The phylogenetic trees showed *Magnivitellinum simplex* nested as the sister taxon of the members of *Alloglossidium*, while *Perezitrema bychowskyi* nested as the sister taxon of three species of *Macroderoides* (*M. texanus* Tkach, Strand & Froese, 2008; *M. minutus* Tkach & Kinsella, 2011; and *M. spiniferus* Pearse, 1924), rendering *Macroderoides* as paraphyletic (Fig. 1). The phylogenetic trees also revealed that Macroderoididae, as currently conceived, is not monophyletic. The family is split in two large sister clades. One clade contains the monotypic *Auridistomum chelydrae* (Auridistomidae), as the sister taxon of a group containing three species of *Macroderoides* (*M. typicus* Winfield, 1929; *M. flavus* Van Cleave & Mueller 1932; and *M. trilobatus* Tyler, 1978) + *Paramacroderoides kinsellai* Tkach, Pulis & Overstreet, 2010 + other three species of *Macroderoides* (mentioned above) + *Perezitrema*. The second large clade contains the Leptophallidae, including *Leptophallus nigrovenosus* (Bellingham, 1844), *Macrodera longicollis* (Abildgaard, 1788), *Metaleptophallus gracillimus* (Lühe, 1909), and *Paralepoderma cloacicola* (Lühe, 1909), as the sister group of a clade formed by *Alloglossidium* (five species) + *Magnivitellinum* (see Fig. 1).

Discussion

The MP, ML an BI analyses of the 28S rDNA dataset conducted in this study consistently show that the family Macroderoididae is not monophyletic, since some of its members, at least the ones for which sequence data are available, are sister taxa to other families of plagiorchioid trematodes such as the Auridistomidae and the Leptophallidae. First, the type-genus of the family, i.e. *Macroderoides*, nests as the siter taxon of *Auridistomum chelydrae*, a parasite of freshwater turtles. In addition to that, our phylogenetic analysis also shows unequivocally that the genus *Macroderoides* is paraphyletic. Tkach et al. (2010) were the first to notice that at least one species of *Macroderoides* was genetically closer to *Paramacroderoides* than to other *Macroderoides* spp. However, a year later, Tkach & Kinsella (2011) used *Paramacroderoides* as an outgroup in a phylogenetic analysis of *Macroderoides* based on partial sequences of the 18S and 28S rRNA genes and the complete ITS region, and these results supported the monophyly of *Macroderoides*, with two distinct clades for the North American species, and *Paramacroderoides* as their sister taxon. Although we only used partial 28S rDNA sequences, the inclusion of more members of the Plagiorchioidea resulted in the placement of *Paramacroderoides* as the sister taxon of *Perezitrema* and three species of *Macroderoides*, *M. spiniferus* and *M. texanus* + *M. minutus* (Fig. 1), providing further support for the paraphyly of *Macroderoides*.

The taxonomic history of *Perezitrema* has been controversial. The genus was originally included in the Acanthostomidae by Baruš & Moravec (1967) and subsequently assigned to the Opisthorchiidae Looss, 1899 (see Yamaguti, 1971), the Plagiorchiidae (see Brooks, 1980), and the Macroderoididae (see Moravec & Salgado-Maldonado, 2002). Our study provides further molecular evidence to demonstrate that *Perezitrema* does belong in the Macroderoididae, and its taxonomic position is now settled.

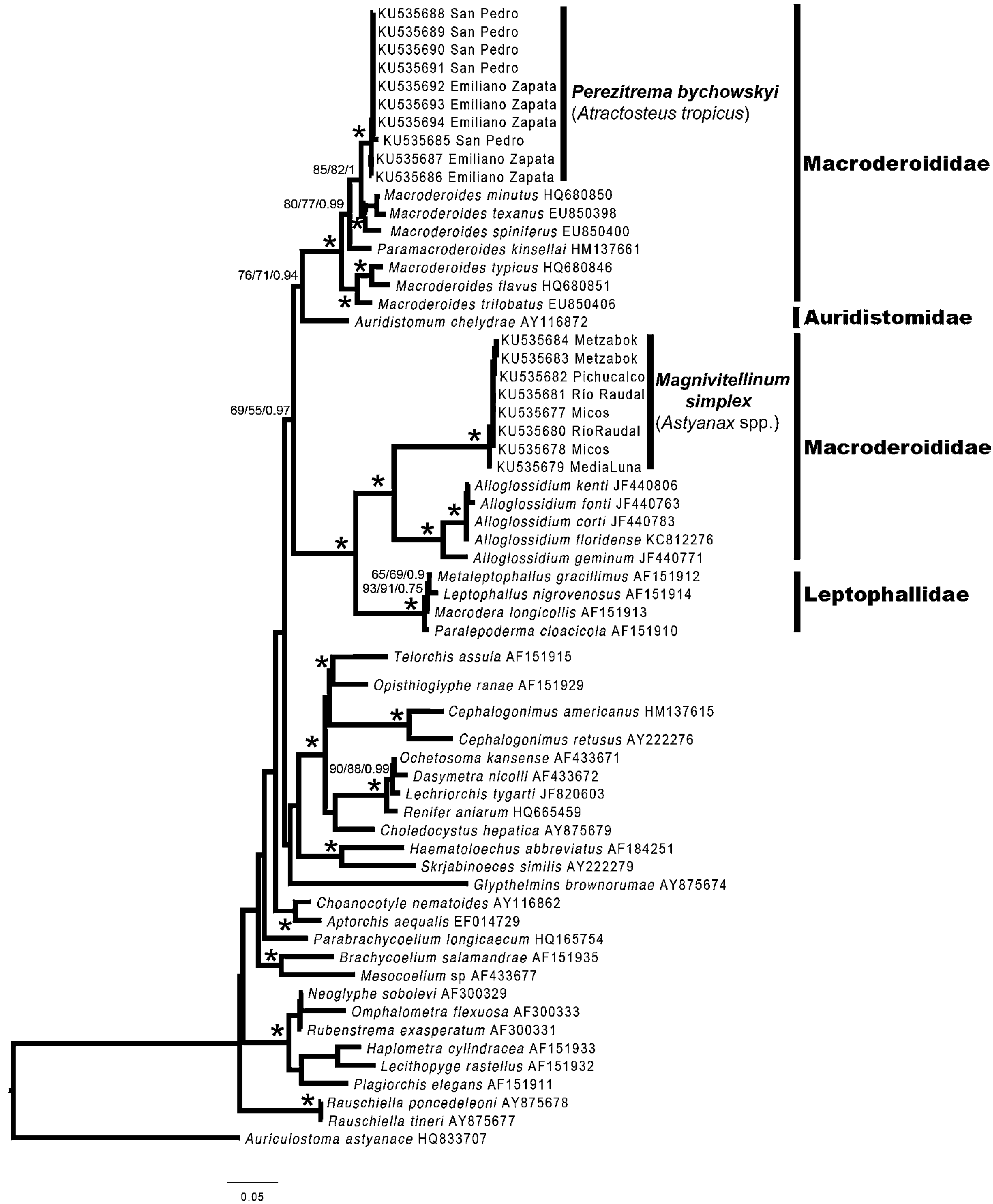


Fig. 1 Phylogenetic tree inferred through maximum likelihood of the 28S rDNA dataset. Numbers near internal nodes show MP/ML bootstrap clade frequencies and posterior probability clade frequencies (*= values above 90% for both ML and MP, and above 0.9 for BI)

Macroderoides is the type-genus of Macroderoididae, and the family is characterised by containing trematodes with the following characters: body fusiform to elongate, typically bearing tegumental spines, ventral sucker relatively small in the anterior end of body, cirrus-sac claviform, containing a bipartite seminal vesicle, ejaculatory duct unarmed, uterine seminal vesicle present, follicular vitellarium forming symmetrical lateral fields of variable extent, and I-shaped excretory vesicle. In their taxonomic revision of the family, Font & Lotz (2008a) modified the diagnosis to include characters exhibited by *Perezitrema*, i.e. a cyclocoel and a funnel-shaped oral sucker, characteristic of the genus (Moravec & Salgado-Maldonado, 2002). The presence of a cyclocoel in *Perezitrema* might be regarded as a morphological autapomorphy of the genus, in the same way *Paramacroderoides* is diagnosed by having a slender and elongate body with an oral sucker armed with two circular rows of large spines. Since both genera are valid and possess unique characters to diagnose them, then a new genus will be needed to accommodate the aforementioned three species of *Macroderoides*; however this requires further molecular phylogenetic analyses and a detailed assessment of their morphology.

A second result of our molecular phylogenetic analysis reveals that *Alloglossidium* and *Magnivitellinum* are sister taxa, and that these two genera are the sister group of members of the family Leptophallidae (Fig. 1). The latter comprise exclusively parasites of reptiles and possess a unique morphological characteristic uniting all four genera, i.e. the presence of an external seminal vesicle (Tkach et al., 1999). The position of this clade within the phylogeny of the plagiorchioid trematodes provides evidence that the current circumscription of the Macroderoididae is not satisfactory, since the family is not monophyletic. This is not the first time that the relationship between *Alloglossidium* and *Macroderoides* has been questioned. Based on a personal communication from V. V. Tkach, Bray & Hendrix (2007) (p. 861) mentioned that these genera do not cluster together and therefore, that the former although a plagiorchioid was not a macroderoidid. The results of our study provide the molecular evidence in support of this observation. Based on the new evidence, a new family has to be established to accommodate these two genera because clearly, just by host association, distribution, and phylogenetic interrelationships, they are not members of the Leptophallidae. Alloglossidiidae n. fam. is therefore proposed with the following diagnosis.

Alloglossidiidae n. fam.

Diagnosis: Body elongate. Tegument bearing spines, dense in the anterior extremity, decreasing in number at mid-level of hindbody. Oral sucker round, subterminal. Ventral sucker round, typically in anterior half of body. Prepharynx short or long. Pharynx well developed. Oesophagus distinct. Intestinal bifurcation about halfway between pharynx and ventral sucker. Caeca elongate, usually terminating between posterior testis and posterior extremity of body. Testes two, tandem or diagonal, typically entire, in hindbody. Cirrus-sac straight or curved, usually extending posteriorly beyond ventral sucker, contains bipartite seminal vesicle and coiled cirrus. Genital pore median or submedian, immediately anterior to ventral sucker. Ovary median or submedian, between ventral sucker and testes, sometimes close to or overlapping ventral sucker, spherical to oval, entire. Uterine seminal receptacle present. Uterus extends to posterior extremity of body, completely filling postcaecal space, with ascending and descending coils passing between testes; transverse uterine loops overlapping caeca or expanding into extracaecal space. Metraterm poorly differentiated. Eggs numerous, oval, operculate. Vitellarium consists of large follicles forming lateral fields; anterior margin of vitelline fields at different levels between ventral sucker and pharynx; posteriorly vitelline follicles usually extend to intertesticular area, confluent or not. Excretory vesicle I-shaped, pore terminal. In the intestine of freshwater fish (Ictaluridae, Characidae) or in freshwater crustaceans and leeches as progenetic metacercariae. Nearctic and Neotropical regions. Type-genus *Alloglossidium* Simer, 1929.

Taxonomic remarks

Molecular phylogenetic analyses have provided a framework to discuss the interrelationships among members of different trematode groups, and have challenged the traditional classification schemes based on morphological grounds. For instance, a recent molecular phylogeny of the Echinostomatoidea Looss, 1899 (Tkach et al., 2016) resulted in a new phylogeny-based classification of the group, where a number of systematic and nomenclatural changes were required. Our study follows that direction, although it was conducted at a lower scale across the hierarchy of the trematode classification, and a new family is described based on the molecular phylogenetic analysis, and the evaluation of some morphological traits.

The new family can be readily distinguished from members included traditionally within the Macroderoididae by the combination of the following characters: tegumental spines are very dense at the anterior extremity and the number of spines decreases at the mid-level of hindbody; in macroderoidids the entire tegument is covered with spines. Herein we provide evidence of the external surface of *M. simplex* through SEM for the first time, showing that no spines are found on the tegument in the posterior third of the body (Fig. 2). This character seems to be controversial since Smythe & Font (2001) used three character states, i.e. body fully spined, spines extending from anterior extremity of worms to mid-body and spines restricted to anterior extremity. However, the most recent descriptions of congeneric species (see Tkach & Mills, 2011; Tkach et al., 2013; Kasl et al., 2014) clearly show that spines become sparser toward posterior extremity of body and descriptions actually indicate that spine number and size decrease towards the posterior extremity of the body. While describing *A. demshini* Tkach, Greiman & Steffes, 2013 the authors pointed out that spines were not observed posterior to the level of pharynx under light microscope, although under SEM they are seen to reach the level of ventral sucker (Tkach et al., 2013). Based on the aforementioned arguments we suggest that other species of *Alloglossidium* require SEM studies to verify the spine distribution, and be used as a reliable character to separate species. Also, in the new family, the vitelline follicles extend from the level between the pharynx and the anterior end of the ventral sucker, and the intertesticular area, not surpassing the posterior testis; in macroderoidids, the vitellarium extends from the posterior margin of ventral sucker to the posterior extremity of the body, surpassing the level of posterior testis. The post-testicular space is wide in all members of Alloglossidiidae n. fam., and no members of the family possess a funnel-shaped oral sucker, or a cyclocoel, as some members of the Macroderoididae.

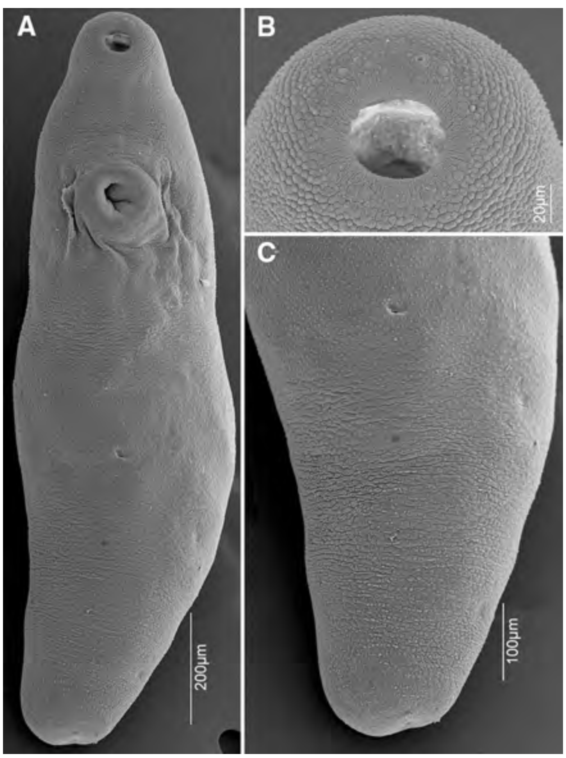


Fig. 2 Scanning electron images of *Magnivitellinum simplex*. A, Total view; B, Oral sucker region; C, Posterior half of the body showing the lack of spines on the body surface in the posterior third of the body

The inclusion of other genera traditionally allocated in the Macroderoididae into the new family, such as *Gauhatiana*, *Cirkennedya*, and *Malawitrema* (see Font & Lotz, 2008a), as well as those considered *incertae sedis* by Pojmańska et al. (2008), i.e. *Vietosoma* and *Pseudomagnivitellinum*, require further verification. No sequence data are available for these genera yet, rendering their inclusion into one of these families problematic. All of the species in these genera possess a spined tegument and in general show some morphological resemblance with members of both, the Macroderoididae or the Alloglossidiidae n. fam., except for species of *Vietosoma* Van Cleave & Mueller, 1932 and *Malawitrema*, which possess symmetrical testes and a different extent of the vitellarium. *Vietosoma parvum* Van Cleave & Mueller, 1932 possesses follicles distributed from the level of oral sucker to the posterior extremity of the body, whereas *Malawitrema stauferi* Bray & Hendrix, 2007 possesses restricted vitellarium, between the testes and ovary region. However, considering the aspect of host association, we believe that they could be clustered within the new family erected herein: both species are parasites of siluriform fishes, with ictalurid catfishes in North America (the channel catfish *Ictalurus punctatus* Rafinesque) as the type-host of *V. parvum*, and the African siluriforms *Clarias gariepinus* (Burchell) and *Bagrus meridionalis* Günther, as hosts of *M. staufferi*. Two additional species are parasites of siluriforms, *Gauthiana batrachii* Gupta, 1955 in the catfish *Clarias batrachus* (Linnaeus) in India and Philippines (Gupta, 1955; Arthur & Lumanlan-Mayo, 1997), and *Pseudomagnivitellinum ictalurum* in the black bullhead *Ictalurus melas* Rafinesque in North America (Dronen & Underwood, 1980). The validity of *Pseudomagnivitellinum* has been recently questioned (Pojmanska, 2008), while *Gauthiana* is accepted as a member of the Macroderoididae by Font & Lotz (2008a). Based on their morphological traits *G. batrachii* and *P. ictalurum* seem to be, in our opinion, valid species; however their taxonomic validity and possible inclusion into the family is pending new sequence data for a molecular phylogenetic analysis. If valid, we also believe that, by host associations and by sharing some morphological traits, both genera will be also members of Alloglossidiidae n. fam.

Host associations and biogeographical considerations

The two genera analysed in this study exhibit a different pattern of historical biogeography and the fact that the families to which they belong, the Macroderoididae (containing *Perezitrema*) and the Alloglossidiidae n. fam. (containing *Magnivitellinum*) include species parasitic in freshwater fish, with the sister taxa for each of them parasitising reptiles, poses an interesting host association and biogeographical scenario, where the two families seem to follow independent colonisation events *via* host-switching from ancestors occurring in other aquatic ectotherm vertebrates, such as amphibians and reptiles, to freshwater fish, considering also that the sister taxa of both families such as telorchids, cephalogonimids, ochetosomatids, etc., are all parasites of amphibians or reptiles (see Fig. 1). In the first case, *Auridistomum chelydrae* is found in freshwater turtles in North America (Yamaguti, 1971; Font & Lotz, 2008b), whereas six of the eight species of *Macroderoides* (as currently conceived) are mostly parasites of gars (*Atractosteus* Rafinesque and *Lepisosteus* Gill), bowfin (*Amia calva* Linnaeus) and pickerel (*Esox* Linnaeus) in the USA. Also the three North American species of the genus *Paramacroderoides* are found in gars (Tkach et al., 2010). Interestingly, the two valid species of *Perezitrema* are common parasites of gars, *P. bychowskyi* in the tropical gar (*A. tropicus* Gill) along the Gulf of Mexico and Atlantic slopes of Mexico and Nicaragua (Moravec & Salgado-Maldonado, 2002), and *P. viguerasi* in the Cuban gar *Atractosteous tristoechus* in Cuba (Baruš & Moravec, 1967).

In the second case, the Leptophallidae, formed by species that infect the digestive tract and lungs of snakes in Europe, Asia and North Africa (Tkach, 2008) is the sister group of the new family whose members are mainly parasitic in freshwater fish. In addition, the two genera currently included in the new family exhibit a particular host association and distribution pattern; *Alloglossidium* is found in ictalurid catfishes (a Nearctic freshwater fish group), or in crustaceans and leeches in North America (see Kasl et al., 2014). The five species for which sequences are available in the GenBank database are non-precocious and are parasites of ictalurids across Canada, USA and Mexico (see Gibson, 1996; Pérez-Ponce de León et al, 2007b; Kasl et al., 2014), exhibiting a remarkable host lineage specificity (see Choudhury et al., 2016). *Magnivitellinum*, the sister taxon of *Alloglossidium* has a Neotropical affinity since its species are found in characids and siluriform catfishes; this genus currently contains two species, *M. corvitellinum* Lacerda, Takemoto & Pavanelli, 2009 from a siluriform catfish in Brazil (Lacerda et al., 2009), and *M. simplex*, occurring across a wide geographic range from Argentina to northern Mexico, in close association with characids (Kohn et al., 2007; Pérez-Ponce de León et al., 2007b). Unfortunately, the origin and interrelationships among the genera allocated into the new family are more difficult to explain. The geographical disjunction between the Nearctic and Neotropical biogeographical regions, and the fact that these three fish groups are not closely related, challenges a plausible explanation of the phylogenetic affinities herein uncovered.

Notwithstanding, other trematodes exhibit a similar pattern of host association and geographical distribution. Tkach & Curran (2015) conducted a molecular phylogenetic analysis for the species of *Prosthenhystera* Travassos, 1922 and discovered that *P. caballeroi* Jiménez, 1973, a parasite of *Astyanax* spp. in Middle America, is the sister species of *P. obesa* (Diesing, 1850), a parasite of characids (and apparently in other Neotropical fish groups) in Brazil (see Kohn et al., 2007), and *P. oonastica* Tkach & Curran, 2015, a parasite of ictalurids in the USA. Although the relationships between sister trematode taxa among Nearctic ictalurid and Neotropical siluriforms and characids cannot be explained, the results of our study set the grounds to keep collecting more data to unravel this complex host-parasite association, and to incorporate other lines of evidence to explain this host and biogeographical relationships. Choudhury et al. (2016) discussed that along with exploratory and opportunistic surveys aimed at describing new taxa, we should consider more strategic hypothesis-driven sampling which can yield data for uncovering phylogenetic relationships of widely distributed taxa such as *Magnivitellinum simplex*, as well as patterns and processes of diversification across and within drainages and biogeographical regions.

Acknowledgements This study represents a partial fulfillment of DIHM to obtain a PhD in the Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México. DIHM also thanks the Consejo Nacional de Ciencia y Tecnología (CONACyT) for scholarship No. 245193. We thank Brenda Solorzano, Carlos Mendoza and Carlos Pedraza for their help during field work.

Funding This project was funded by the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT)-UNAM IN204514 to GPPL. This paper was written during the sabbatical leave of GPPL to the University of Otago, New Zealand. Thanks are due to Professor Robert Poulin for facilities provided in his laboratory, and to DGAPA-UNAM and CONACyT for their support through their sabbatical programs.

Compliance with ethical standards

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

Ethical approval All applicable institutional, national and international guidelines for the care and use of animals were followed. Hosts were collected under the Cartilla Nacional de Colector Científico de Flora y Fauna Silvestre FAUT-0057 issued to GPPL by the Secretaria del Medio Ambiente y Recursos Naturales.

References

Aguirre-Macedo, M. L., Scholz, T., González-Solís, G., Vidal-Martínez, V. M., Posel, P., Arjona-Torres, G., Dumailo S., & Siu-Estrada, E. (2001). Some adult endohelminths parasitizing freshwater fishes from the Atlantic drainages of Nicaragua. *Comparative Parasitology, 68*, 190–195.

Arthur, J. R., & Lumanlan-Mayo, S. (1997). *Checklist of the parasites of fishes of the Philippines*. Fisheries Technical Paper 369, FAO, 102 pp.

Baruš, V., & Moravec, F. (1967). Systematic studies of parasitic worms, found in the hosts *Lepisosteus tristoechus* (Ginglimodi, Lepisosteidae) and *Hyla insulsa* (Ecaudata, Hylidae) from Cuba. *Acta Societatis Zoologicae Bohemoslovacae, 31*, 1–14.

Brooks, D. R. (1980). Revision of the Acanthostominae Poche, 1926 (Digenea: Cryptogonimidae). *Zoological Journal of the Linnean Society, 70*, 313–382.

Choudhury, A., Aguirre-Macedo, L., Curran, S. S., Ostrowski de Núñez, M., Overstreet, R. M., Pérez-Ponce de León, G., & Portes-Santos, C. (2016). Trematodes of freshwater fishes of the Globe II: 'New World'. *Systematic Parasitology, 93*, 271–282.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods, 9*, 772.

Dronen, N. O., & Underwood, H. T. (1980). *Pseudomagnivitellinum ictalurum* gen. et sp. n. (Digenea: Macroderoididae) from the Black Bullhead of South-central Texas. *Proceedings of the Helminthological Society of Washington, 47*, 52–54.

Font, W. F., & Lotz, J. M. (2008a). Family Macroderoididae McMullen, 1937. In: Bray, R. A., Gibson, D. I. & Jones, A. (Eds). *Keys to the Trematoda, volume 3*. Wallingford: CABI Publishing and the Natural History Museum, pp. 373–380.

Font, W. F., & Lotz, J. M. (2008b). Family Auridistomidae Stunkard, 1924. In: Bray, R. A., Gibson, D. I. & Jones, A. (Eds). *Keys to the Trematoda, volume 3*. Wallingford: CABI Publishing and the Natural History Museum, pp. 326–329.

García-Varela, M., & Nadler, S. A. (2005). Phylogenetic relationships of Palaeacanthocephala (Acanthocephala) inferred from SSU and LSU rDNA gene sequences. *Journal of Parasitology, 91*, 1401–1409.

Gibson, D. I. (1996). Trematoda. In: Margolis, L. & Kabata, Z. (Eds). *Guide to the parasites of fishes of Canada, part IV. Trematoda*. Ottawa: NRC Research Press, 373 pp.

Guindon, S., & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology, 52*, 696–704.

Goloboff, P. A., Farris, J. S., & Nixon, K. C. (2008). TNT a free program for phylogenetic analysis. *Cladistics, 24*, 774–786.

Gupta, S. P. (1955). Trematode parasites of fresh-water fishes. *Indian Journal of Helminthology, 5*, 1–80.

Hoffman, G. L. (1999). *Parasites of North American freshwater fishes*. Ithaca, NY: Cornell University Press, 539 pp.

Kasl, E. L., Fayton, T. J., Font, W. F., & Criscione, C. D. (2014). *Alloglossidium floridense* n. sp. (Digenea:

Macroderoididae) From a Spring Run in North Central Florida. *Journal of Parasitology, 100*, 121–126.
Khalil, L. F. (1972). *Afroinacroderoides lazerae* gen et sp. nov. (Allocreadiidae: Walliniinea) a new digenean from the African freshwater fish *Clarias lazera*. *Journal of Helminthology, 46*, 341–344.
Kohn, A., Fernandes, B. M. M., & Cohen, S. C. (2007). *South American trematodes parasites of fishes*. Rio de Janeiro: Imprinta Express Ltda, 318 pp.
Kostadinova, A., & Pérez-del-Olmo, A. (2014). The systematics of the Trematoda. In: Toldedo, R. & Fried, B. (Eds). *Digenetic trematodes. Advances in Experimental Medicine and Biology*. New York: Springer, pp. 21–44.
Lacerda, A. C. F., Takemoto, R. M., & Pavanelli, G. C. (2009). A new trematode species parasitizing the catfish *Hoplosternum littorale* (Osteichthyes, Callichthyidae) from Paraná River, Brazil, with an emendation of the diagnosis of *Magnivitellinum* (Trematoda, Macroderoididae). *Acta Parasitologica, 54*, 37–40.
Luton, K., Walker, D., & Blair, D. (1992). Comparisons of ribosomal internal transcribed spacers from two congeneric species of flukes (Platyhelminthes: Trematoda: Digenea). *Molecular & Biochemical Parasitology, 56*, 323–327.
Moravec, F., & Salgado-Maldonado, G. (2002). Redescription of *Perezitrema bychowskyi* (Caballero & Caballero, 1975) (Trematoda:Macroderoididae), with remarks on the systematic status of *Perezitrema* Baruš & Moravec, 1996. *Systematic Parasitology, 53*, 199–206.
Pérez-Ponce de León, G., Choudhury, A., Rosas-Valdez, R., & Mejía-Madrid, H. (2007a). The systematic position of *Wallinia* spp. and *Margotrema* spp. (Digenea), parasites of Middle-American and Neotropical freshwater fishes, based on the 28S ribosomal RNA gene. *Systematic Parasitology, 68*, 49–55.
Pérez-Ponce de León, G., García-Prieto, L., & Mendoza-Garfias, B. (2007b). Trematode parasites (Platyhelminthes) of wildlife vertebrates in Mexico. *Zootaxa, 1534*, 1–247.
Pojmańska, T., Tkach, V. V., & Gibson, D. I. (2008). Genera *incertae sedis*, genera *inquirenda*, *nomina nuda*, larval or collective names and recently erected genera. In: Bray, R. A., Gibson, D. I. & Jones, A. (Eds). *Keys to the Trematoda, volume 3*. Wallingford: CABI Publishing and the Natural History Museum, pp. 735–755.
Razo-Mendivil, U., León-Régagnon, V., & Pérez-Ponce de León, G. (2006). Monophyly and systematic position of *Glypthelmins* (Digenea), based on partial lsrDNA sequences and morphological evidence. *Organisms, Diversity & Evolution, 6*, 308–320.
Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology, 61*, 539–542.
Smythe, A. B., & Font, W. F. (2001). Phylogenetic analysis of *Alloglossidium* (Digenea: Macroderoididae) and related genera: Life-cycle evolution and taxonomic revision. *Journal of Parasitology, 87*, 386–391.
Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics, 22*, 2688–2690.
Stamatakis, A., Blagojevic, F., Nikolopoulos, D., & Antonopoulos, C. (2007). Exploring new search algorithms and hardware for phylogenetics: RAxML meets the IBMcell. *Journal of VLSI Signal Processing, 48*, 271–286.
Stamatakis, A., Hoover, P., & Rougemont, J. (2008). A rapid bootstrap algorithm for the RAxML web servers. *Systematic Biology, 57*, 758–771.
Stock, S. P., Campbell, J. F., & Nadler, S. A. (2001). Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology, 87*, 877–889.
Swofford, D. L. (2003*). PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b10*. Sunderland, MA: Sinauer Associates.
Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*, 4876–4882.
Tkach, V. V. (2008). Family Leptophallidae Dayal, 1938. In: Bray, R. A., Gibson, D. I. & Jones, A. (Eds). *Keys to the Trematoda, volume 3*. Wallingford: CABI Publishing and the Natural History Museum, pp. 367–372.
Tkach, V. V., & Curran, S. S. (2015). *Prosthenhystera oonastica* n. sp. (Digenea: Callodistomidae) from ictalurid catfishes in southeastern United States and molecular evidence differentiating species in the genus across Americas. *Systematic Parasitology, 90*, 39–51.
Tkach, V. V., Grabda-Kazubska, B., Pawlowski, J., & Swiderski, Z. (1999). Molecular and morphological evidences for close phylogenetic affinities of the genera *Macrodera*, *Leptophallus*, *Metaleptophallus* and *Paralepoderma* (Digenea, Plagiorchioidea). *Acta Parasitologica, 44*, 170–179.
Tkach, V. V., Greiman, S. E., & Steffes, K. R. (2013). *Alloglossidium demshini* sp. nov. (Digenea: Macroderoididae) from leeches in Minnesota. *Acta Parasitologica, 58*, 434–440.
Tkach, V. V., & Kinsella, J. M. (2011). New *Macroderoides* (Digenea: Macroderoididae) from Florida gar, with molecular phylogeny of the genus. *Journal of Parasitology, 97*, 920–923.
Tkach, V. V., Kudlai, O., & Kostadinova, A. (2016). Molecular phylogeny and systematics of the Echinostomatoidea Looss, 1899 (Platyhelminthes: Digenea). *International Journal for Parasitology, 46*, 171–185.
Tkach, V. V., & Mills, A. M. (2011). *Alloglossidium fonti* sp. nov. (Digenea, Macroderoididae) from black bullheads in Minnesota with molecular differentiation from congeners and resurrection of *Alloglossidium kenti*. *Acta Parasitologica, 56*, 154–162.
Tkach, V. V., Pulis, E. E., & Overstreet, R. M. (2010). A new *Paramacroderoides* species (Digenea: Macroderoididae) from two species of gar in the southeastern United States. *Journal of Parasitology, 96*, 1002–1006.
Tkach, V. V., Snyder, S. D., & Swiderski, Z. (2001). On the phylogenetic relationships of some members of Macroderoididae and Ochetosomatidae (Digenea, Plagiorchioidea). *Acta Parasitologica, 46*, 267–275.

Tkach, V. V., Strand, J., & Froese, L. (2008). *Macroderoides texanus* n. sp. (Digenea: Macroderoididae) from alligator gar, *Atractosteus spatula* in Texas. *Parasitology Research, 104*, 27–33.

Yamaguti, S. (1971). *Synopsis of digenetic trematodes of vertebrates*. Tokyo: Keigaku Publishing Co., Parts I & II. 1074 pp. + 349 pl.

5. CAPÍTULO III

5. CAPÍTULO III. RELACIONES FILOGENÉTICAS DENTRO DE LA SUPERFAMILIA DIPLOSTOMOIDEA

En este tercer capítulo se presentan análisis filogenéticos que tratan de esclarecer con mayor detalle las relaciones de parentesco entre representantes de distintas familias que tradicionalmente se han agrupado dentro de la superfamilia Diplostomoidea. Con este propósito, se obtuvieron secuencias de los genes nucleares 18S y 28S, y del gen mitocondrial *cox* 1 de *Crocodilicola pseudostoma* de la familia Proterodiplostomidae, familia que hasta ese entonces carecía de representatividad en el Genbank, por lo que su posición en filogenias moleculares era aun incierta. *C. pseudostoma* es un parásito de cocodrilos pero tiene la capacidad de que el estadio larvario, conocido como metacercaria, madure dentro del pez siluriforme *Rhamdia guatemalensis*. Es importante recalcar que en este capítulo también se retoma uno de los objetivos particulares de la presente tesis, el cual es poner a prueba la eficiencia del gen *cox* 1 para la resolución de relaciones filogenéticas en niveles supraespecíficos. La información es presentada en el artículo titulado "**Filling the gaps in the classification of the Digenea Carus, 1863: systematic position of the Proterodiplostomidae Dubois, 1936 within the superfamily Diplostomoidea Poirier, 1886, inferred from nuclear and mitochondrial DNA sequences**", el cual fue publicado en la revista Systematic Parasitology.

Syst Parasitol (2017) 94:833–848
DOI 10.1007/s11230-017-9745-1

Filling the gaps in the classification of the Digenea Carus, 1863: systematic position of the Proterodiplostomidae Dubois, 1936 within the superfamily Diplostomoidea Poirier, 1886, inferred from nuclear and mitochondrial DNA sequences

David Iván Hernández-Mena · Martín García-Varela · Gerardo Pérez-Ponce de León

Received: 18 May 2017 / Accepted: 30 July 2017 / Published online: 18 August 2017

Abstract The Diplostomida Olson, Cribb, Tkach, Bray & Littlewood, 2003 is the less diverse order of the two orders within the subclass Digenea Carus, 1863 and is currently classified into three superfamilies, i.e. Brachylaimoidea Joyeux & Foley, 1930, Diplostomoidea Poirier, 1886, and Schistosomatoidea Stiles & Hassall, 1898. Although the suprageneric-level relationships have been elucidated with the use of molecular markers, the lack of representation of some groups obscure the phylogenetic relationships among families, rendering the classification unstable. Here, we tested the phylogenetic position of the family Proterodiplostomidae Dubois, 1936 based on partial 28S rDNA and complete 18S rDNA sequences for *Crocodilicola pseudostoma* (Willemoes-Suhm, 1870), a crocodile parasite that has been found as a progenetic metacercaria parasitising the pale catfish *Rhamdia guatemalensis* (Günther) in Mexico and in other siluruforms in the Neotropics. We augmented the representation of the species, genera and families within the Diplostomida, including mostly representatives of the superfamily Diplostomoidea, and assembled a dataset that contains 49 species for the 28S rRNA gene, and 45 species for the 18S rRNA gene. Additionally, we explored the phylogenetic signal of the mitochondrial gene *cox*1 in reconstructing the phylogenetic relationships of selected members of the superfamily. Our analyses showed that the family Proterodiplostomidae is the sister taxon to the paraphyletic Diplostomidae Poirier, 1886 and Strigeidae Railliet, 1919, with Cyathocotylidae Mühling, 1898 + Brauninidae Wolf, 1903 as their sister group. Analysis of concatenated 18S + 28S sequences revealed the Liolopidae Odhner, 1912 as the basal group of the superfamily Diplostomoidea, although analyses of independent datasets showed that the position of this family remains uncertain. Analysis based on *cox*1 unequivocally resolved the Proterodiplostomidae as the sister taxon to the Diplostomidae and Strigeidae, although the Cyathocotylidae was nested in a different clade, along with brachylaimoids and schistosomatoids.

This article is part of the Topical Collection Digenea.

Electronic supplementary material The online version of this article (doi:10.1007/s11230-017-9745-1) contains supplementary material, which is available to authorized users.

D. I. Hernández-Mena · M. García-Varela · G. Pérez-Ponce de León (✉)
Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico, D.F., Mexico
e-mail: ppdleon@ib.unam.mx

D. I. Hernández-Mena
Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Mexico, D.F., Mexico

Introduction

The order Diplostomida Olson, Cribb, Tkach, Bray & Littlewood, 2003 is the less diverse of the two orders of the subclass Digenea Carus, 1863, representing just

12.7% of all the digenean families, and containing approximately 1,477 species included in 210 genera and 19 families (Littlewood et al., 2015). Species included in the order are parasites of tetrapod vertebrates as definitive hosts, with the exception of the fish blood-flukes belonging to the family Aporocotylidae Odhner, 1912 (see Kostadinova & Pérez-del-Olmo, 2014; Littlewood et al., 2015). Species of the Diplostomida are currently classified into three superfamilies, i.e. the Diplostomoidea Poirier, 1886, Schistosomatoidea Stiles & Hassall, 1898 and Brachylaimoidea Joyeux & Foley, 1930 (see Olson et al., 2003). The Diplostomoidea is the most diverse group of the order, with 797 species parasitising mammals, birds and reptiles (Littlewood et al., 2015). Species of this superfamily are easily differentiated from other groups of digeneans in possessing a unique holdfast organ and a cirrus-sac and cirrus replaced by an atypical copulatory apparatus and terminal genitalia (except for the Cyathocotylidae Mühling, 1898) (Niewiadomska, 2002a). Six families containing 97 genera are currently recognised within the superfamily: the monotypic Brauninidae Wolf, 1903 and Bolbocephalodidae Strand, 1935, and the species-rich Strigeidae Railliet, 1919, Diplostomidae Poirier, 1886, Cyathocotylidae and Proterodiplostomidae Dubois, 1936 (see Niewiadomska, 2002a, b, c, d, e, f, g). Only two of the six families and five out of the 97 genera currently known for the superfamily were represented in the comprehensive phylogenetic analysis of the Digenea conducted by Olson et al. (2003). Kostadinova & Pérez-del-Olmo (2014) marked as important omissions the families Cyathocotylidae and Proterodiplostomidae and concluded that the assessment of the relationships within the superfamily required further exploration based on a wider array of taxa, including the type-genus of the family Strigeidae, *Strigea* Abildgaard, 1790.

The recent expansion of the genetic library of various molecular markers for several groups of trematodes has enhanced our capacity to reconstruct the phylogenetic relationships at different levels of the taxonomic hierarchy of the group. Particularly for diplostomoids, a large number of DNA sequences has been obtained, especially for clinostomids, strigeids and diplostomids, in studies on the phylogenetic relationships within species of a genus (e.g. Bell et al., 2001), studies of species delimitation (e.g. Galazzo et al., 2002; Dzikowski et al., 2003; Locke et al., 2010, 2015; Georgieva et al., 2013; Hernández-Mena et al., 2014; García-Varela et al., 2016a; Pérez-Ponce de León et al., 2016), and studies that link the larval stages in their intermediate fish or amphibian hosts with the adults in fish-eating birds (e.g. Locke et al., 2011; Blasco-Costa et al., 2016a; García-Varela et al., 2016b). In these studies, sequences of the nuclear ITS1-5.8-ITS2 and mitochondrial *cox*1 genes were primarily obtained. However, some studies have also provided 18S and 28S rDNA sequences (e.g. Pulis et al., 2013; Patrelle et al., 2015; Blasco-Costa et al., 2016a). Interestingly, 18S and/or 28S rDNA sequences of several diplostomoids have been added to the GenBank since the publication by Olson et al. (2003), including the cyathocotylid *Holostephanus dubinini* Vojtek & Vojtkova, 1968, from the great cormorant (Dzikowski et al., 2004); *Braunina cordiformis* Wolf, 1903, the only member of the family Brauninidae, a parasite of short beaked common dolphin (Fraija-Fernández et al., 2015); and *Strigea* sp. (mesocercariae), from brown frogs and water frogs (Patrelle et al., 2015).

Here, we contribute to the classification of the Digenea by presenting an updated phylogeny of the group, with a particular focus on the relationships within the superfamily Diplostomoidea. We present a molecular phylogenetic analysis using sequences of the 18S and 28S rRNA genes obtained from GenBank and newly generated sequences for a broad diversity of taxa belonging to the superfamily to investigate the systematic position of the Proterodiplostomidae. We expand the information on both nuclear genes and explore the phylogenetic signal of one additional gene for a higher-level classification, the cytochrome *c* oxidase subunit 1 (*cox*1).

Materials and methods

Specimen collection and identification

Adult specimens of various species belonging to five families (Clinostomidae Lühe, 1901, Cyathocotylidae, Diplostomidae, Proterodiplostomidae, and Strigeidae) were collected in different species of fish-eating birds or freshwater fishes from 13 localities in Mexico between 2010 and 2015 (Table 1). Birds were collected with a shotgun under permission FAUT 0202 issued by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) of Mexico to

MGV, and fish were captured using seine nets. The intestines of each individual host were removed and placed in Petri dishes with 0.75% or 0.65% saline solution and observed under a stereomicroscope. For fishes, the body cavity of each individual was also examined. Some of the specimens from each host were fixed in 4% hot formalin for morphological study, and other specimens were placed in vials with 100% ethanol for molecular analysis. For taxonomic identification, specimens were stained with Mayer's paracarmine and mounted on permanent slides with Canada balsam. Vouchers of some of the specimens collected for this study were deposited at the Colección Nacional de Helmintos (CNHE), Biology Institute, National Autonomous University of México, Mexico City, Mexico (Table 1). Host taxonomy follows Froese & Pauly (2017) for fish, Avibase (https://avibase.bsc-eoc.org/) for birds, and Integrated Taxonomic Information System, ITIS (https://www.itis.gov) for all other hosts.

DNA extraction, amplification and sequencing

Each specimen was digested and its genomic DNA extracted using the REDExtract-N-Amp Tissue PCR kit (Sigma, St. Louis, USA) following the manufacturer's instructions. The domains D1-D3 of the 28S rDNA and the entire 18S rDNA from nuclear ribosomal DNA were amplified using polymerase chain reaction (PCR). Additionally, the cytochrome *c* oxidase subunit 1 mitochondrial gene (*cox*1) was amplified. For 28S, the primers 139 and 536 were used for amplification. For 18S, two overlapping fragments were amplified with the primers 18S1A plus 32 and 652 plus 28 (Table 2). For *cox*1, we used the primers M13 and BarCoxR (Table 2). PCR reactions were performed with 2 µl of DNA extraction supernatant, 1 µl of each PCR primer (10 µM), 2.5 µl of 10× buffer, 1.5 mM of $MgCl_2$, 0.5 of dNTPs (10 mM), 16 µl of water and 1 U of Taq DNA polymerase. The following thermo-cycling profile was utilized for the three molecular markers: denaturation of DNA (95°C for 5 min); 36 cycles of amplification (94°C for 1 min, 50°C for 1 min and 72°C for 1 min); and a post-amplification incubation at 72°C for 10 min. Sequencing reactions were performed with ABI Big Dye terminator sequencing chemistry (Applied Biosystems, Boston, Massachusetts, USA). To sequence 18S amplicons, we used the four PCR primers; for 28S, we used the two PCR primers plus two internal primers, 503 and 504; and for *cox*1, we used the PCR primers (Table 2). Reaction products were separated and read on an ABI 3730xl Genetic Analyser (Applied Biosystems). The resulting contiguous sequences were assembled in Geneious Pro 4.8.4® (Biomatters Ltd., Auckland, New Zealand) to generate consensus sequences. The new consensus sequences of each molecular marker belonging to the different species included in this study were deposited in the GenBank under the accession numbers MF398316–MF398319 (*cox*1), MF398320–MF398348 (28S), and MF398349–MF398365 (18S) (see Supplementary Table S1 for details).

Alignment and phylogenetic analyses

Sequences for 18S, 28S and *cox*1 were generated for the following species: *Clinostomum marginatum* (Rudolphi, 1819), *C. tataxumui* Sereno-Uribe, Pinacho-Pinacho, García-Varela & Pérez-Ponce de León, 2013 (Clinostomidae), *Mesostephanus microbursa* Caballero, Grocott & Zerecero, 1953 (Cyathocotylidae), *Posthodiplostomum* sp., *Uvulifer* sp., *Hysteromorpha triloba* (Rudolphi, 1819), *Tylodelphys aztecae* García-Varela, Sereno-Uribe, Pinacho-Pinacho, Hernández-Cruz & Pérez-Ponce de León, 2015, *Austrodiplostomum ostrowskiae* Dronen, 2009 (Diplostomidae), *Crocodilicola pseudostoma* (Proterodiplostomidae), *Cardiocephaloides* sp., *Cotylurus gallinulae* (Lutz, 1928), *Australapatemon burti* (Miller, 1923), *Apharyngostrigea cornu* (Zeder, 1800), *Parastrigea plataleae* Hernández-Mena, García-Prieto & García-Varela, 2014, *P. cincta* Brandes, 1888, *P. diovadena* Dubois & Macko, 1972 and *Strigea* sp. (Strigeidae) (Table 1). The newly generated sequences were aligned with other sequences of 18S, 28S and *cox*1, available in the GenBank database, including those for species of the following families of the Diplostomida: Aporocotylidae, Brachylaimidae Joyeux & Foley, 1930, Brauninidae, Clinostomidae, Cyathocotylidae, Diplostomidae, Leucochloridiidae Poche, 1907, Liolopidae Odhner, 1912, Schistosomatidae Stiles & Hassall, 1898 and Spirochiidae Stunkard, 1921 (see Supplementary Table S1). Sequences for *Fasciola hepatica* Linnaeus, 1758 (Plagiorchiida La Rue, 1957) were employed in each dataset to root the phylogenetic trees.

Each dataset (18S, 28S and *cox*1) was individually aligned in the SATé software under the default setting SATé-II-fast (Liu et al., 2009, 2012), implementing

Table 1 Specimens of diplostomoids collected in birds and fish in different areas of Mexico used for sequencing and molecular phylogenetic analyses

Trematode species	Host species	Locality	CNHE numbers
Clinostomidae Lühe, 1901			
Clinostomum marginatum (Rudolphi, 1819)	*Ardea alba* Linnaeus	Ocotes, Oaxaca (16°36′57″N, 96°43′13″W)	8345
Clinostomum tataxumui Sereno-Uribe, Pinacho-Pinacho, García-Varela & Pérez-Ponce de León, 2013	*Ardea alba* Linnaeus	Tlacotalpan, Veracruz (18°36′00″N 95°39′00″W)	8338
Cyathocotylidae Mühling, 1898			
Mesostephanus microbursa Caballero, Grocott & Zerecero, 1953	*Sula nebouxii* Milne-Edwards	Isla Isabel, Nayarit (21°52′00″N, 105°54′00″W)	7286
Diplostomidae Poirier, 1886			
Austrodiplostomum ostrowskiae Dronen, 2009	*Phalacrocorax brasilianus* (Gmelin)	Presa La Angostura, Chiapas (16°11′31″N, 92°59′52″W)	9753
Hysteromorpha triloba (Rudolphi, 1819)	*Phalacrocorax brasilianus* (Gmelin)	Tlacotalpan, Veracruz (18°36′N, 95°39′W)	–
Posthodiplostomum sp.	*Phalacrocorax brasilianus* (Gmelin)	El Espino, Tabasco (18°14′47″N, 92°49′57″W)	–
Tylodelphys aztecae García-Varela, Sereno-Uribe, Pinacho-Pinacho, Hernández-Cruz & Pérez-Ponce de León, 2015	*Podilymbus podiceps* (Linnaeus)	Tlahúac, CDMX (19°15′58″N, 99°00′24″W)	9777
Uvulifer sp.	*Megaceryle alcyon* (Linnaeus)	Ocotes, Oaxaca (16°36′57″N, 96°43′13″W)	–
Proterodiplostomidae Dubois, 1936			
Crocodilicola pseudostoma (Willemoes-Suhm, 1870)	*Rhamdia guatemalensis* (Günther)	Catemaco Lake, Veracruz (18°24′46.7″N, 95°06′33.8″W)	10423
Strigeidae Railliet, 1919			
Apharyngostrigea cornu (Zeder, 1800)	*Nycticorax nycticorax* (Linnaeus)	El Huizache, Sinaloa (23°05′28″N, 106°15′57″W)	–
Apharyngostrigea cornu (Zeder, 1800)	*Ardea alba* Linnaeus	Tlacotalpan, Veracruz (18°36′00″N, 95°39′00″W)	10424
Australapatemon burti (Miller, 1923)	*Anas diazi* Ridgway	Chicnahuapan, Estado de Mexico (19°10′N, 99°29′W)	7176
Cardiocephaloides sp.	*Larus occidentalis* Audubon	Guerrero Negro, Baja California Sur (27°57′32″N, 114°03′22″W)	7175
Cotylurus gallinulae (Lutz, 1928)	*Aythya affinis* (Eyton)	La Esperanza, Sonora	7173
Parastrigea cincta Brandes, 1888	*Eudocimus albus* (Linnaeus)	Caimanero, Sinaloa (25°36′30″N, 108°26′25″W)	10425
Parastrigea diovadena Dubois & Macko, 1972	*Eudocimus albus* (Linnaeus)	Pijijiapan, Chiapas (15°31′54″N, 93°09′39″W)	10426
Parastrigea plataleae Hernández-Mena, García-Prieto & García-Varela, 2014	*Platalea ajaja* (Linnaeus)	El Huizache, Sinaloa (23°05′28″N, 106°15′57″W)	8258
Strigea sp.	*Caracara cheriway* (von Jacquin)	Presa La Angostura, Chiapas (16°11′31″N, 92°59′52″W)	10427

Table 2 Primers used for amplification and sequencing of ribosomal and mitochondrial DNA of the digenean species used in this study

Gene	Primer	Primer sequence (5′-3′)	Direction	Application	References
Ribosomal					
18S	18S1A	GGCGATCGAAAAGATTAAGCCATGCA	Forward	Amplification and sequencing	Nadler et al. (2000)
18S	32	CGAAGTCCTATTCCATTATTC	Reverse	Amplification and sequencing	This study
18S	652	GCAGCCGCGGTAATTCCAGCTC	Forward	Amplification and sequencing	Nadler et al. (2000)
18S	28	AGCGACGGGCGGTGTGT	Reverse	Amplification and sequencing	This study
28S	391	AGCGGAGGAAAAGAAACTAA	Forward	Amplification and sequencing	Nadler & Hudspeth (1998)
28S	536	CAGCTATCCTGAGGGAAAC	Reverse	Amplification and sequencing	García-Varela & Nadler (2005)
28S	503	CCTTGGTCCGTGTTTCAAGACG	Reverse	Amplification	Stock et al. (2001)
28S	504	CGTCTTGAAACACGGACTAAGG	Forward	Amplification	García-Varela & Nadler (2005)
Mitochondrial					
*cox*1	M13F	TGTAAAACGACGGCCAGT	Forward	Amplification and sequencing	Messing (1983)
*cox*1	BarCoxR	ATAAACCTCAGGATGCCCAAAAAA	Reverse	Amplification and sequencing	Razo-Mendivil (pers. com.)

100 iterations for each dataset. A concatenated dataset of 18S + 28S was also assembled. For the concatenated alignment, only the taxa with sequences available for both molecular markers were considered. All phylogenetic analyses were run under Maximum Likelihood (ML) and Bayesian inference (BI), employing the substitution model GTR + Γ for 18S and 28S and GTR + Γ + I for *cox*1. The models of nucleotide evolution were estimated in jModelTest v2 (Darriba et al., 2012). ML inference (100 replicates), model parameters and bootstrap support (1,000 replicates) were estimated with RAxML v. 7.0.4 (Stamatakis, 2006). MrBayes v. 3.2.1 (Ronquist et al., 2012) was used to perform BI analysis, running four independent MCMC runs of four chains each run (a heating parameter value of 0.5) for 20 million generations and sampling tree topologies every 1,000 generations (printfreq = 1,000; samplefreq = 1,000; diagnfreq = 10,000). 'Burn-in' periods were set to the first 1,500 generations. A 50% majority-rule consensus tree and nodal support estimated as posterior probability values were calculated from the remaining trees. The phylogenetic trees obtained from both analyses were visualized in FigTree v.1.4.3. (http://tree.bio.ed.ac.uk/software/figtree/).

Results

28S and 18S rDNA datasets

In total, 29 new partial 28S rDNA sequences were generated for 17 species of diplostomoids (Table 1). The alignment of the 28S rRNA gene sequences was 1,437 bp long and consisted of 64 sequences representing 49 species of digeneans, of which 31 were members of the Diplostomoidea. The nucleotide frequencies were as follows: A = 0.204, C = 0.217, G = 0.319, T = 0.259. The phylogenetic tree obtained from the ML analysis had a log-likelihood of -13,971.057555. The ML and BI trees yielded similar topologies (Fig 1A). These trees unequivocally resolved the Proterodiplostomidae as the sister group of the Strigeidae and Diplostomidae, a relationship supported by high bootstrap and posterior probability values.

Eighteen new sequences of the 18S rRNA gene were generated for 15 species of diplostomoids

(Table 1). The 18S dataset was 2,022 bp long and consisted of 50 sequences of 45 species, of which 13 were species of the superfamily Diplostomoidea. The nucleotide frequencies were as follows: A = 0.239, C = 0.220, G = 0.286, T = 0.256. The ML analysis yielded a single tree with a log-likelihood of -11,315.496983. The phylogenetic trees of ML and the consensus of the BI resulted in similar topologies (Fig. 1B). These trees showed the Proterodiplostomidae as the sister group of the Strigeidae and Diplostomidae. These relationships were also supported by high bootstrap and posterior probability values.

The resulting phylogenetic hypotheses of both nuclear genes analysed independently showed very similar topologies regarding the relationships among families (and superfamilies) of the order Diplostomida (Fig. 1A, B). Relationships were confirmed through an ML and BI analysis of the concatenated dataset of 18S + 28S (Fig. 2). For instance, all trees showed that the Diplostomoidea is monophyletic, with high bootstrap and posterior probability support values. Within diplostomoids, two clades were formed, one containing the Brauninidae (*B. cordiformis*) as the sister group of the Cyathocotylidae and a second clade containing the newly generated sequences for the Proterodiplostomidae (*Crocodilicola pseudostoma*) as the sister group of the Diplostomidae and Strigeidae; both clades were highly supported by bootstrap support and posterior probability values. Furthermore, the Diplostomidae and Strigeidae were paraphyletic, and relationships among them remain unclear. However, the Strigeidae was clearly divided into two

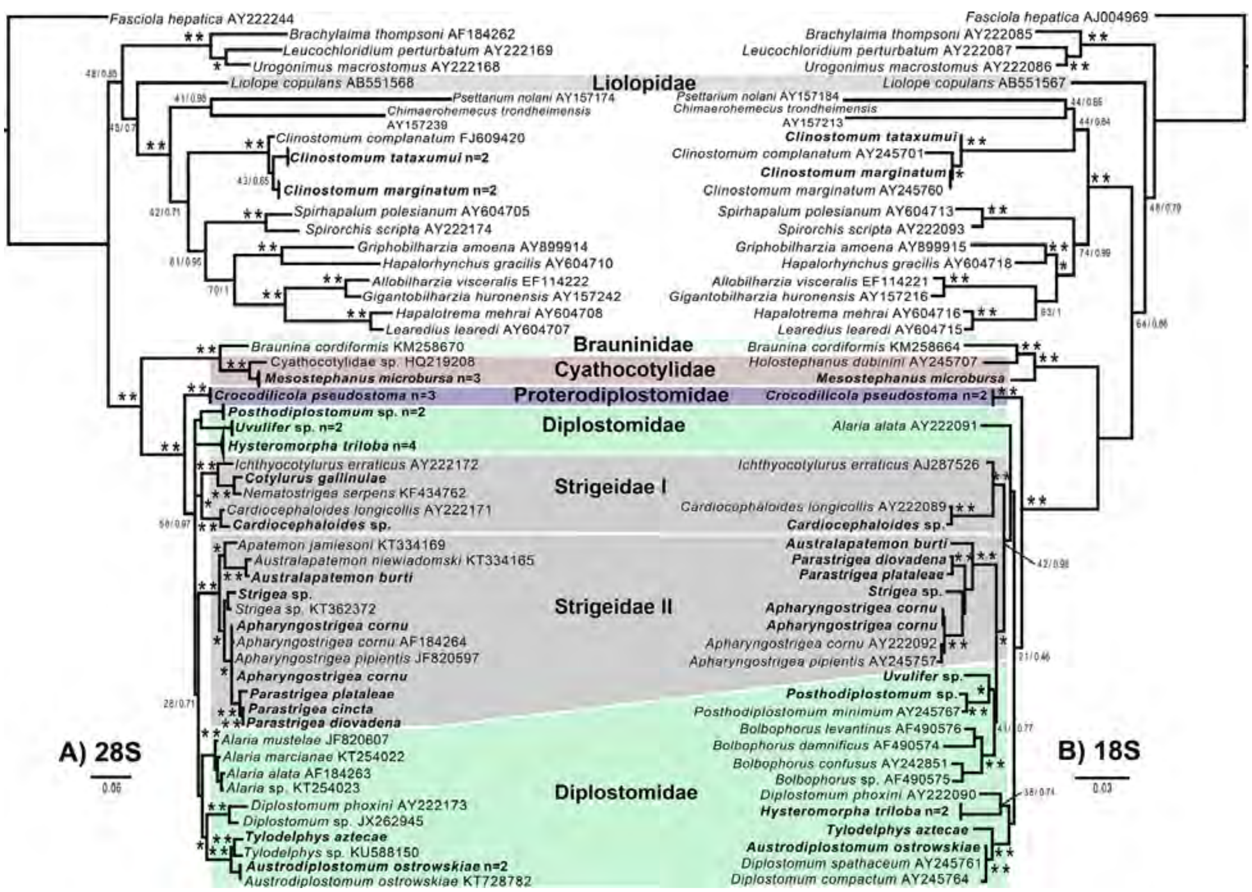


Fig. 1 Phylogenetic relationships among the taxa of the Diplostomoidea resulting from Maximum Likelihood and Bayesian inference analyses based on the partial sequences of the 28S rRNA gene (A) and the 18S rRNA gene (B). Bootstrap support values and posterior probability values are shown near the nodes. *Key*: **, bootstrap support of 80–100% and posterior probabilities of 0.80–1.00; *, bootstrap support of 60–79% and posterior probabilities of 0.60–0.79. The newly generated sequences are highlighted in bold and are presented without GenBank accession numbers. For some of the species analyses in this study more than one specimen was sequenced; the "n" in parentheses indicates the number of isolates obtained for each species. The scale-bar indicates the number of substitutions per site

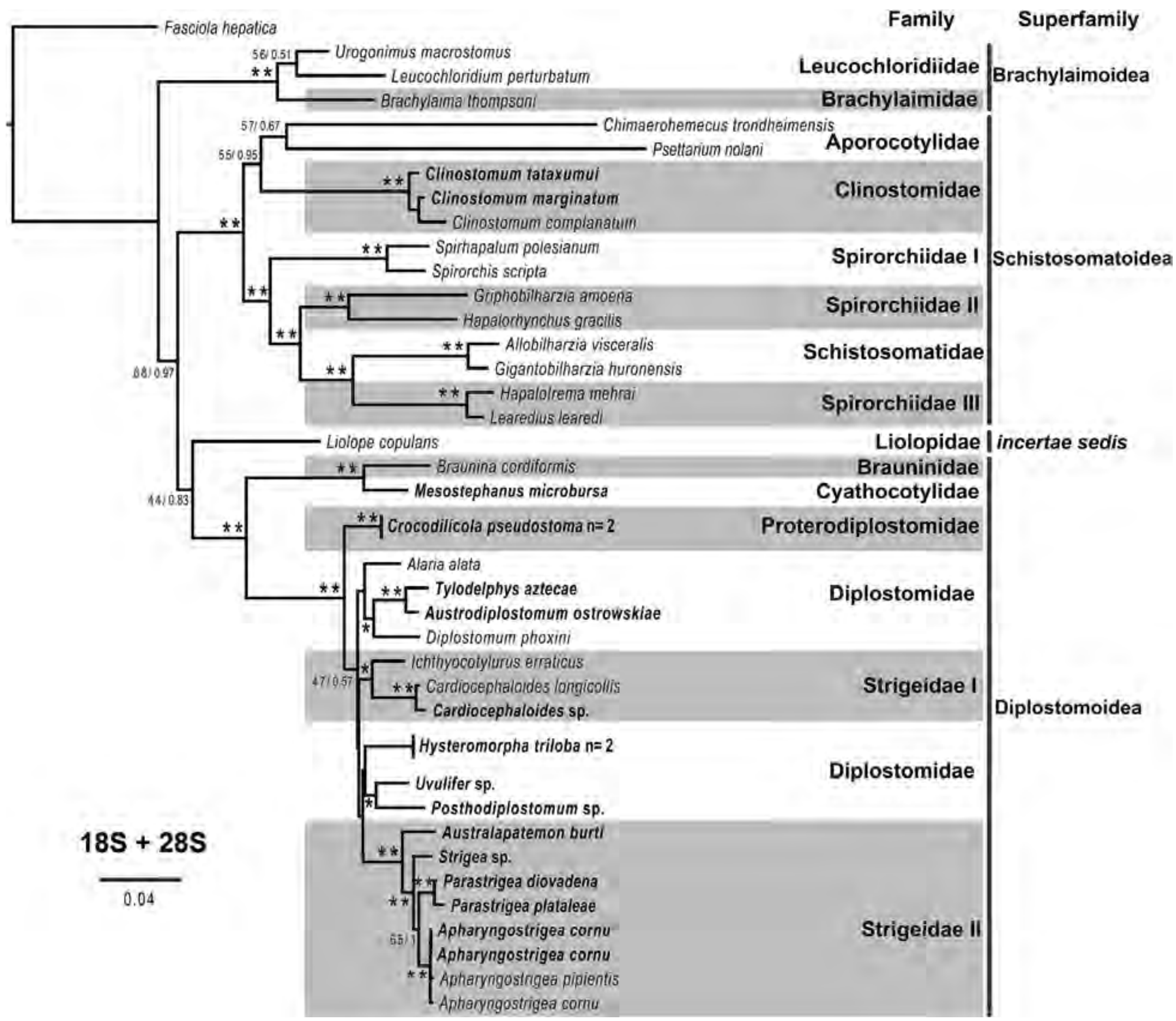


Fig. 2 Phylogenetic tree inferred with the combined nuclear datasets (18S + 28S), using Maximum Likelihood and Bayesian inference analyses. Bootstrap values and posterior probability values are shown near the nodes. *Key*: **, bootstrap support of 80–100% and posterior probabilities of 0.80–1.00; *, bootstrap support of 60–79% and posterior probabilities of 0.60–0.79. Labels along the vertical bars indicate the classification of the species at the level of family and superfamily

clades, one including the genera *Cardiocephaloides* Sudarikov, 1959, *Ichthyocotylurus* Odening, 1969, *Cotylurus* Szidat, 1928, and *Nematostrigea* Sandground, 1924 (the last two only present in the 28S dataset), and a second clade comprising the genera *Apatemon* Szidat, 1928 (only present in the 28S dataset), *Australapatemon* Sudarikov, 1959, *Apharyngostrigea* Ciurea, 1927, *Parastrigea* Szidat, 1928, and *Strigea* (Figs. 1A, B, 2). However, for diplostomids, even though two clades were formed, the relationships among the genera were inconsistent, and different topologies were obtained in the analysis of the concatenated 18S and 28S data. The systematic position of the Liolopidae was also inconsistent. *Liolope copulans* Cohn, 1902 was nested as the sister taxon of the Schistosomatoidea in both nuclear datasets analysed separately. However, in the concatenated dataset, *L. copulans* was nested as the basal member of the Diplostomoidea, although the relationships were supported by a very low bootstrap support value (44%) and a moderate posterior probability support value (0.83) (Fig. 2).

*cox*1 dataset

We obtained *cox*1 sequences for three species of the Diplostomoidea, i.e. *Mesostephanus microbursa* Caballero, Grocott & Zerecero, 1953 (n = 1), *Crocodilicola pseudostoma* (n = 2), and *Strigea* sp. (n = 1). The *cox*1 dataset contained 684 bp and comprised 80 sequences, of which 64 were for species of the Diplostomoidea. The nucleotide frequencies were as follows: A = 0.190, C = 0.127, G = 0.206, T = 0.477. The ML analysis exhibited a single tree with a log-likelihood of -16,187.659230. The phylogenetic trees from ML and BI analyses showed a similar topology (Fig 3). The Proterodiplostomidae was unequivocally nested as the sister group of the clade formed by the Diplostomidae and Strigeidae, with high bootstrap (95%) and posterior probability (1) support values. In the *cox*1 tree, the family Strigeidae was recovered as monophyletic, albeit with very low bootstrap and posterior probability support values (18% and 0.65, respectively). Instead, the Diplostomidae was paraphyletic, because the Strigeidae was included in the same clade. Within the Strigeidae, as in the 18S and 28S trees, the same two clades were formed, one with *Cardiocephaloides*, *Cotylurus* and *Ichthyocotylurus* (bootstrap: 67%; posterior probability: 0.98) and a second clade containing *Apatemon*, *Australapatemon*, *Apharyngostrigea*, *Parastrigea* and *Strigea* (bootstrap: 94%; posterior probability: 1). No *cox*1 sequences are available for *B. cordiformis* or *L. copulans*, and, interestingly, the *cox*1 dataset showed an unexpected relationship for the Cyathocotylidae because the family was not closely related to the proterodiplostomids, diplostomids or strigeids but was apparently nested within the Schistosomatoidea.

Discussion

The results of the phylogenetic analyses based on sequences of the nuclear genes 18S and 28S, the mitochondrial *cox*1 gene, and the concatenated dataset for the two nuclear markers unequivocally showed that the Proterodiplostomidae is the sister group of the Diplostomidae and Strigeidae, within the superfamily Diplostomoidea. Our study demonstrates the value of steadily adding new sequence data to a growing genetic library of several molecular markers for digeneans, searching for a stable and useful classification that represents an accurate account of interrelationships among digenean taxa. Littlewood (2008) noted that 18S and 28S rRNA genes provide the foundation of molecular systematics for the parasitic platyhelminths, having been used extensively for revealing interrelationships within and between families and across the phylum (e.g. Cribb et al., 2001; Olson et al., 2003). Additionally, the use of both nuclear genes in combined analyses may provide better resolution than each of the genes analysed separately, when they do not provide stability of taxa across the trees (see Lockyer et al., 2003; Waeschenbach et al., 2007; Littlewood, 2008). Our study further corroborates Littlewood's (2008) contention, since both 18S and 28S trees are congruent relative the position of the Proterodiplostomidae as the sister group of the Strigeidae and Diplostomidae, and this topology is also recovered by the combined phylogenetic analysis of both nuclear genes. Actually, in this study we explored the phylogenetic signal of the *cox*1 mitochondrial gene at deeper levels of the hierarchy of the digenean classification, and we found that the position of Proterodiplostomidae is also congruent with the topology of the trees recovered by the nuclear genes. Mitochondrial genes are not commonly used to reconstruct phylogenetic relationships at deeper levels because they evolve considerably faster than nuclear markers. For this reason, some authors have advocated that these genes would be better used to resolve younger clades resulting from recent radiations, although the analysis of multiple mitochondrial genes has demonstrated usefulness to resolve deeper level phylogenies in other platyhelminths (see Littlewood, 2008 and references therein; Littlewood et al., 2015).

The current tendency actually shows that the complete mitochondrial genomes of digeneans provide a phylogenetic signal because they offer a wealth of homologous markers. Therefore, complete mitochondrial genomes have been used to resolve deeper-level phylogenetic relationships of digeneans (e.g. Littlewood et al., 2006; Webster & Littlewood, 2012; Briscoe et al., 2016). In this study, we followed Littlewood's (2008) suggestion to conduct empirical tests of the performance of some popular mitochondrial genes, in this case, *cox*1, to reconstruct the phylogenetic relationships between major trematode lineages. Littlewood et al. (2015) and Blasco-Costa et al. (2016b) argued that the application of next-

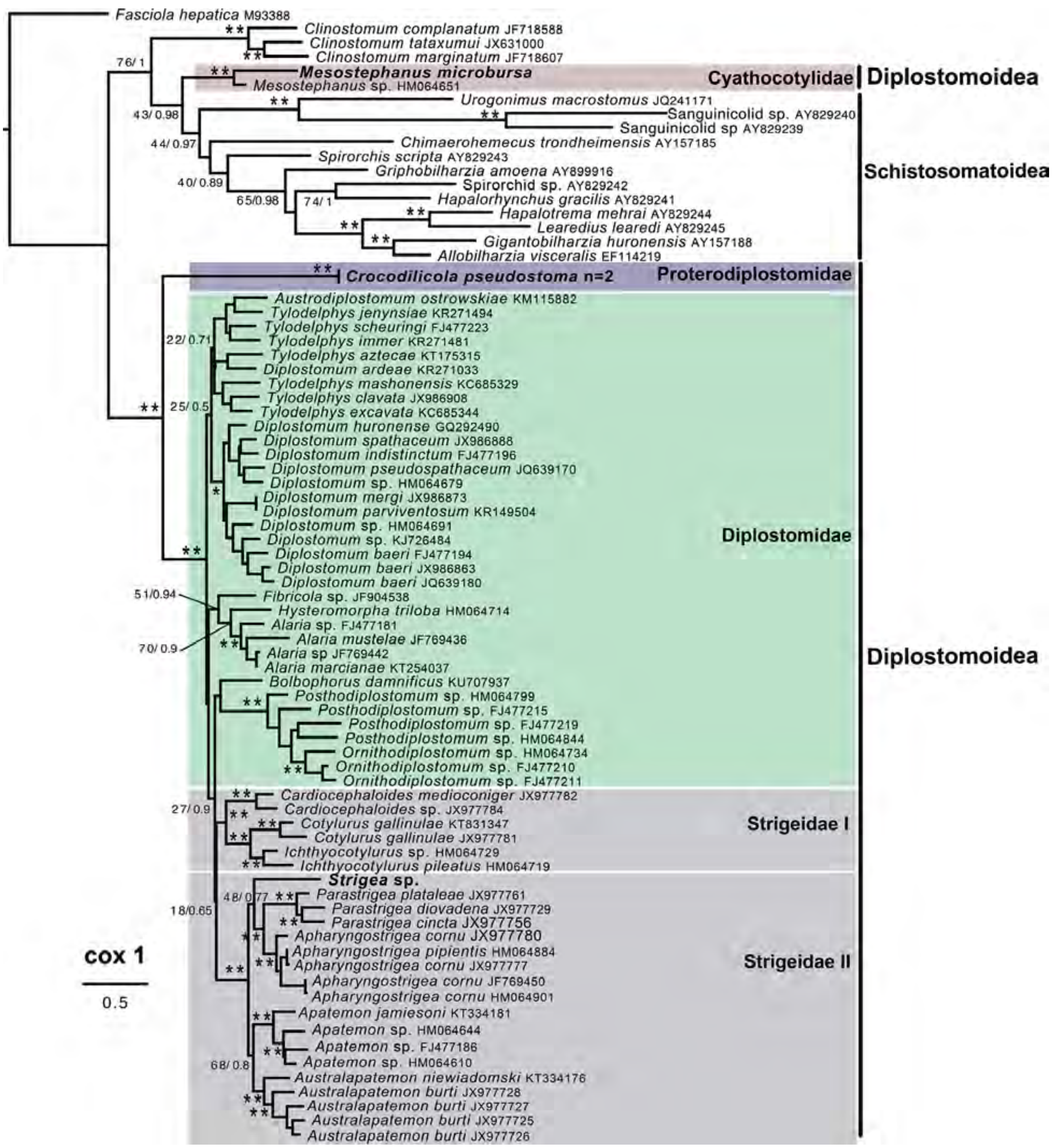


Fig. 3 Phylogenetic tree inferred from *cox* 1 dataset with Maximum Likelihood and Bayesian inference analyses. Bootstrap values and posterior probability values are shown near the nodes; **, bootstrap support of 80–100% and posterior probabilities of 0.80–1.00. The newly generated sequences are highlighted in bold and without GenBank accession numbers. Labels along the vertical bars indicate the classification of the species at the level of family and superfamily

generation sequencing and comparative mitogenomics will offer an unprecedented opportunity to provide better nodal support for phylogenetic relationship inference and that sequencing complete mitochondrial genomes has the potential to provide new insights into the systematics across Trematoda as a whole.

Phylogenetic position of the Proterodiplostomidae

The Proterodiplostomidae is a relatively small group of diplostomoids found exclusively in reptiles and not in birds and mammals, as are the other diplostomoids. The following is a diagnosis of the family according to Niewiadomska (2002e): "Body more or less distinctly bipartite; forebody flattened, with or without pseudo-suckers; hindbody cylindrical, claviform or conical, sometimes with thick walled capsule or series of suckers. Holdfast organ variable in size, may be provided with papillae. Parasites exclusively in reptiles. *Type-genus*: *Proterodiplostomum* Dubois, 1936". Members of the family are mainly characterised by the presence of a paraprostatic gland or paraprostate, an independent organ with the shape of a thin- or thick-walled tubule or pouch, surrounded by gland-cells; species are included in 17 genera (Niewiadomska, 2002e). The classification of the Proterodiplostomidae was first elaborated by Dubois (1936) and is mainly based on host-specificity, holdfast organ shape and size, the presence or absence of papillae, and the distribution of the vitellarium. Apparently, the classification of the family remained unchanged until 1970, when Dubois presented a taxonomic revision (Dubois, 1970); two main groups were separated, with species parasitising crocodiles and chelonians in one and those in snakes in the other. The first attempt to establish the systematic position of the family in reference to a phylogeny-based classification was made by Brooks et al. (1985) through a morphological cladistic analysis of the Digenea, where the so-called "Superfamily Strigeoidea Railliet, 1919" was comprised of the Liolopidae, Cyathocotylidae (including Brauninidae), Diplostomidae (including Proterodiplostomidae) and Strigeidae. Based on this classification scheme, Shoop (1989) used Proterodiplostomidae as the outgroup in a systematic analysis of the Diplostomidae and Strigeidae. Brooks et al. (1992) proposed a cladistic classification of the genera of the Proterodiplostomidae based on the arrangement of the terminal genitalia, particularly the way the uterus and paraprostate open into the genital pore, confirming the previously recognised division into two groups.

The proterodiplostomids are morphologically very similar and share several morphological synapomorphies with strigeids and diplostomids. As a result, their phylogenetic position as the sister clade of these two groups of diplostomoids, as discovered in molecular phylogenetic analysis in our study, is not an unexpected result. Likewise, our molecular analyses, including DNA sequences for a proterodiplostomid for the first time, provide the empirical test of the position of the family within the phylogeny of the superfamily Diplostomoidea. The proterodiplostomids and the ancestor of diplostomids and strigeids experienced a diversification process in different groups of tetrapods, with the former diversifying in reptiles and the latter diversifying in birds and mammals. The presence of a paraprostate in species of the Proterodiplostomatidae can be then regarded as a morphological autapomorphy of the family (Shoop, 1989). Although the proterodiplostomid used in the phylogenetic analyses, *C. pseudostoma*, was found parasitising the body cavity of a siluriform freshwater fish, *Rhamdia guatemalensis* (Günther), in Catemaco Lake, Veracruz, Mexico, as a progenetic metacercaria (Pérez-Ponce de León et al., 1992), the first record of the species was made by Caballero (1948) as a parasite of the intestine of *Crocodylus moreletti* (Dumeril & Bibron) from the same locality. Actually, *C. pseudostoma* was originally recorded as a parasite of *Alligator mississippiensis* (Daudin) in the USA (Willemoes-Suhm, 1871). Species of *Crocodilicola*, like many other proterodiplostomids, are considered to represent parasites of the digestive tract of alligators and crocodiles in the Americas (Conroy, 1986). However, progenetic metacercariae of *C. pseudostoma* have also been recorded in another three species of siluriforms in Brazil: Conroy (1986) found *C. pseudostoma* in *Rhamdia quelen* (Quoy & Gaimard, 1824) [as *Rhamdia hilarii* (Valenciennes)] in the State of Sao Paulo; Guidelli et al. (2003) found it in *Hemisorubim platyrhynchus* (Valenciennes) in the upper Paraná River floodplain; and Ferrari-Hoeinghaus et al. (2007) found this species in *Loricariichthys platymetopon* Isbrücker & Nijssen, 1979, also in the Paraná River floodplain.

Phylogenetic relationships within the Diplostomoidea

Shoop (1989) conducted a phylogenetic analysis of the Diplostomidae and Strigeidae based on morphology and concluded that the classification of the Diplostomoidea at the time did not reflect the real evolutionary relationships and needs to be reconstructed with reference to an accumulation of data from different sources. More recently, Niewiadomska (2002a) pointed out that patterns of host specificity of adults and some morphological traits such as the structure and shape of the forebody and holdfast organ, the distribution of the vitellarium (in whole body, forebody or hindbody), the presence or absence of bi-segmentation of the body, the presence or absence of a cirrus-sac or paraprostate and the structure of the copulatory apparatus, are commonly used for the general division of the Diplostomoidea. Based on the topology of the phylogenetic trees from our analyses, we present further comments on five particular groups belonging to the order Diplostomida: Cyathocotylidae, Brauninidae, Liolopidae, Diplostomidae and Strigeidae.

Cyathocotylidae. Members of the Diplostomoidea possess an atypical copulatory apparatus and terminal genitalia instead of a cirrus-sac and cirrus (Niewiadomska, 2002a). However, members of the family Cyathocotylidae possess a body generally undivided and a cirrus-sac, but they also have a holdfast organ and a terminal genital pore (Niewiadomska, 2002d). Cyathocotylids (and brauninids) thus have characteristics both of diplostomoids and other digeneans (Niewiadomska, 2002c, d). As adults, cyathocotylids are parasites of reptiles, birds and mammals, while the metacercariae are found in fishes, amphibians and aquatic invertebrates (Niewiadomska, 2002d). In our phylogenetic analyses based on the nuclear genes analysed either separately or concatenated, the Cyathocotylidae and Brauninidae are nested together and represent the sister group of the remaining diplostomoids, i.e. the Proterodiplostomidae, Diplostomidae and Strigeidae (Figs. 1, 2). It is plausible, then, to postulate that the holdfast organ and the terminal genital pore evolved in the ancestor of all diplostomoids. This ancestor possessed cirrus-sac and an undivided body. Both, an atypical cirrus-sac, and body bi-segmentation appeared in the ancestor of the Proterodiplostomidae plus Strigeidae and Diplostomidae. Interestingly, the topology of the trees obtained in the *cox*1 analyses showed a different position of the sequenced cyathocotylids, i.e. *Mesostephanus microbursa* from the blue-footed booby *Sula neuboxii* Milne-Edwards from Mexico, and *Mesostephanus* sp. (metacercariae) from the pumpkinseed *Lepomis gibbosus* (L.) from Canada. In the *cox*1 analyses, the two cyathocotylids are closely related to the Schistosomatoidea and not to the Diplostomoidea, with which they share a close morphological resemblance. This unexpected relationship is probably due to insufficient sampling for that molecular marker, the high nucleotide substitution rate of *cox*1, and/or the lack of *cox*1 sequences for *Braunina cordiformis*. The fact that *cox*1 sequences may not resolve suprageneric relationships cannot be ruled out.

Brauninidae. The monotypic Brauninidae (type-species: *Braunina cordiformis*) is characterised by an uncommon host association, since the type-species is found in cetaceans in seas across the globe (Niewiadomska, 2002c). The taxonomic relationships of this species with other digeneans was first demonstrated by Fraija-Fernández et al. (2015) in a phylogenetic analysis of nuclear DNA sequences (18S and 28S), where samples of *B. cordiformis* from the short beaked common dolphin *Delphinus delphis* L. from Argentina were included in a phylogenetic analysis of 177 taxa representing the broad diversity of the subclass Digenea. Within the order Diplostomida, the species nested as the sister clade of the Strigeidae + Diplostomidae, and this relationship was highly supported by bootstrap and posterior probability values (Fraija-Fernández et al., 2015). However, although these authors included representative sequences of other members of the order Diplostomida, no sequences of cyathocotylids were considered in their analyses. Our phylogenetic trees generated from nuclear gene datasets showed that *B. cordiformis* is grouped with the Cyathocotylidae. Still, the host association (in cetaceans, fish-eating), the body shape (cordiform), the presence of a cirrus-sac but the lack of oral and ventral suckers are considered autapomorphies of the family.

Liolopidae. The taxonomic placement of the family Liolopidae has been controversial, and it has been assigned to the Clinostomidae, Brachylaimidae and Harmostomidae Braun, 1900 (see Niewiadomska, 2002g, and references therein). Brooks & Overstreet (1978) considered the family Liolopidae valid, with 12 species described, one parasite of amphibians and 11 parasitising reptiles, including crocodiles, freshwater and marine snakes, lizards and turtles. Kanev et al. (2002) considered the Liolopidae a valid family within the Clinostomoidea Lühe, 1901 on the basis of adult morphology only, but this superfamily was not accepted by Olson et al. (2003). The family Liolopidae currently comprises four genera with species parasitic in amphibians and reptiles, and they have, as primary morphological characteristics, an intertesticular ovary, a uterus mostly posterior to terminal genitalia, and a 'strigeid-like' excretory system (Niewiadomska, 2002g). Moreover, they actually possess a tegument covered with minute and slender spines (see redescription of *L. copulans* in Baba et al., 2011). Baba et al. (2011) studied the life-cycle of *L. copulans*, a parasite of the salamander *Andrias japonicus* (Temmink) in Japan, and provided the first molecular evidence, using sequences of the 18S and 28S rRNA genes, of the systematic position of the family within the order Diplostomida. Their molecular phylogenetic analyses suggested that *L. copulans* could be one of the basal members of the order, but they also found inconsistent results between the 18S and 28S trees. The topology of their trees based on 28S placed *L. copulans* as the sister group of Brachylaimoidea + Schistosomatoidea, with Diplostomoidea as their sister group. Instead, the topology of the 18S tree placed *L. copulans* as the basal group of all members of the order Diplostomida. Our phylogenetic analysis, based on an expanded dataset for both 18S and 28S rRNA genes, showed a concordant placement in the trees (Fig. 1A, B). In both cases, *L. copulans* was the basal group of all of the Schistosomatoidea (including the Aporocotylidae, Clinostomidae, Spirorchiidae and Schistosomatidae), and all of them appeared as the sister group of a clade formed by the Brachylaimoidea. Interestingly, the analysis of the concatenated 18S and 28S datasets yielded a topology that we cannot explain at the moment, where *L. copulans* appears as the basal member of the entire superfamily Diplostomoidea, albeit with very low bootstrap and posterior probability support values (Fig. 2). We tentatively consider Liolopidae as *incertae sedis* because tree topology shows they should be considered a member of the Diplostomoidea, but the relationships are unresolved. Baba et al. (2011) concluded that *L. copulans* belongs in the order Diplostomida, but its phylogenetic position remained unclear. Tentatively, these authors placed the family Liolopidae within the superfamily Diplostomoidea on morphological grounds. It is worth noting that no phylogenetic analysis has shown that the family Liolopidae is closely related to the Clinostomidae, as previously considered (see Kanev et al., 2002). Despite the morphological resemblance of these two families, particularly in the intertesticular position of the ovary and the fact that metacercariae are found in fishes, Baba et al. (2011) showed that cercarial morphology is very different. In *L. copulans*, the cercaria is of the non-oculate longifurcate pharyngeate type, while the cercaria of the species of the Clinostomatidae is of the oculate brevifurcate apharyngeate type. Baba et al. (2011) concluded that to treat both families as members of the same superfamily was untenable, and the results of our phylogenetic analyses provide further support to this idea. Further, these authors suggested that adults of *L. copulans* shared some morphological features with the members of the Cyathocotylidae, e.g. the body is not distinctly divided into two segments, the seminal vesicle is enclosed in a cirrus-sac, and the ovary is intertesticular. However, they also recognised several differences, such as the lack of a holdfast organ and the pretesticular position of the cirrus-sac in *L. copulans*. Additionally, the position of the ovary in Cyathocotylidae is quite variable, and in some species, the ovary is pretesticular (Niewiadomska, 2002d). DNA sequences of other species of liolopids are necessary to infer their true phylogenetic relationships with other diplostomoids.

Diplostomidae. The case of the paraphyly of the Strigeidae and Diplostomidae is noteworthy and requires further studies where a denser sampling is conducted before proposing the required nomenclatural changes. Shoop (1989) showed in his morphological phylogenetic analysis that the Diplostomidae was a paraphyletic assemblage and considered the Strigeidae as monophyletic. The paraphyletic relationships between these two families was also recognised by Olson et al. (2003) and Kostadinova & Pérez-del-Olmo (2014), since both families were represented by a single branch as Diplostomidae + Strigeidae. Our phylogenetic analyses using nuclear DNA sequences showed that at least two groups of apparently unrelated members of the Diplostomidae are formed, with some apparent subgroupings, but these groups are in conflict when each nuclear gene is analysed separately, probably because the same genera are not represented in both datasets. The following interpretation is then based on the analysis of concatenated data (Fig. 2). Of the two groups, one includes the genera *Tylodelphys* Diesing, 1850, *Diplostomum* Nordmann, 1832 and *Austrodiplostomum* Szidat & Nani, 1951, *Alaria* Schrank, 1788; the second group includes *Posthodiplostomum* Dubois, 1936 and

Uvulifer Yamaguti, 1934 + *Hysteromorpha* Lutz, 1931. Instead, the tree obtained with the *cox*1 sequences showed that three unrelated groups are formed (Fig. 3), one containing *Diplostomum, Tylodelphys* and *Austrodiplostomum*; one containing *Fibricola* Dubois, 1932, *Hysteromorpha* and *Alaria*; and the other containing *Posthodiplostomum* and *Ornithodiplostomum* Dubois, 1936 + *Bolbophorus* Dubois, 1935. The placement of these genera in the phylogenetic tree is in conflict with the traditional classification of the Diplostomidae, where two groups are recognised according to host associations: Alariinae Hall & Wigdor, 1918 in mammals and Diplostominae Poirier, 1886 in birds (Dubois, 1936). Niewiadomska (2002b) recognised the division of Diplostomidae according to host specificity and metacercarial types into four subfamilies: one in mammals, the Alariinae, and three in birds, the Diplostominae, Crassiphialinae Sudarikov, 1960 and Codonocephalinae Sudarikov, 1959. This classification is almost concordant with the ML and BI *cox*1 phylogenetic trees obtained in this study, except for the conflicting placement of *Hysteromorpha*. The monotypic Codonocephalinae, whose members are parasitic in ardeids in the Palaearctic, is not represented in the phylogenetic tree, since no sequences are yet available. If this phylogeny is accurate, pending the inclusion of other representatives of the family Diplostomidae, the subfamily Crassiphalinae could be elevated to family level to include *Posthodiplostomum*, *Uvulifer*, *Ornithodiplostomum* and *Bolbophorus* (and possibly at least 11 other genera possessing a ‘neascus’ type of metacercariae and the vitellarium restricted to the hindbody). This nomenclatural change necessarily requires the inclusion of sequences of the type-species of the type-genus, *Crassiphiala bulboglosa* Van Haitsma, 1925, which is found, as an adult, in the intestine of alcedines in North America (Yamaguti, 1971; Niewiadomska, 2002b). The Alariinae could be also elevated to the family level to include *Alaria* and *Fibricola*, but this also requires more sequence data. The inclusion of *Hysteromorpha triloba* in this family is in conflict with the fact that the species shares all characteristics of species of the Diplostomidae, such as vitellarium in both fore- and hindbody, pseudosuckers, and a ‘diplostomulum’ type of metacercaria. The position of this species is ambiguous in trees obtained with each dataset analysed separately. The concatenated tree of two nuclear genes (18S and 28S, Fig. 2) actually shows a conflicting position of *H. triloba*, since it was resolved as the sister taxon of either *Diplostomum*, *Posthodiplostomum* or *Uvulifer* + *Posthodiplostomum*. In this case, it is also premature to propose nomenclatural changes, since classification is still unstable.

Strigeidae. In the case of the Strigeidae, a family that includes a distinct group of species with a characteristic cup-shaped forebody and a holdfast organ in the form of two (ventral and dorsal) lobes (Niewiadomska, 2002f), two main groups were consistently formed, one including species of *Ichthyocotylurus*, *Cardiocephaloides* (but also *Cotylurus* and *Nematostrigea*), and a second group that includes representatives of *Apatemon* + *Australapatemon* and *Strigea* + *Parastrigea* + *Apharyngostrigea*. These groupings are congruent with those obtained in previous studies where mitochondrial and ribosomal markers were used (Hernández-Mena et al., 2014; Blasco-Costa et al., 2016a). In our molecular phylogenetic analyses based on the nuclear genes, these two groups were not closely related. However, in the *cox*1 analysis, the two clades of the Strigeidae are grouped together, albeit with very low bootstrap and posterior probability support values (18/0.65). Both clades are included within a paraphyletic Diplostomidae (Fig. 3). The original classification by Dubois (1938) separated the family Strigeidae into two subfamilies, the Strigeinae Railliet, 1919 and Duboisiellinae Baer, 1938, according to their host group, i.e. birds and mammals, respectively, with two tribes (Strigeini and Cotylurini) within the Strigeinae (see Niewiadomska, 2002f, for further details about the taxonomic history). No sequences are yet available for the monotypic *Duboisiella* Baer, 1938, occurring in marsupial mammals in the Neotropical region. Therefore, the traditional classification of the Strigeidae cannot be tested at present. Considering the conflict in the topology of the trees obtained in the present study, we conclude that it would be premature to propose any nomenclatural changes, because the phylogenetic position of dipolostomids and strigeids is still unclear. More sequencing work, and the inclusion of other taxa of the Diplostomidae and Strigeidae, will be necessary to accomplish this task and obtain a stable classification of both families within the superfamily Diplostomatoidea.

Final considerations

To conclude, Cribb et al. (2003) noted that the classification of Digenea was evolving quite rapidly. Interestingly, 15 years later, the classification is still evolving as a result of the development of molecular tools and methodological approaches, as well as the increase in taxon sampling, even though some conflict has been found between the molecular phylogenetic classifications and that followed in the two major taxonomic treatments of trematodes, i.e. *Synopsis of Digenetic Trematodes of Vertebrates* (Yamaguti, 1971) and the *Keys to the Trematoda* (Gibson et al., 2002; Jones et al., 2005; Bray et al., 2008). These bibliographical sources remain the cornerstones for trematode identification. Still, a thorough sampling is required to expand the representation of species belonging to different families and superfamilies. Strigeids and diplostomids are just one example of the lack of representation needed to reconstruct the phylogenetic relationships upon which a stable classification can be assessed.

Acknowledgements This study represent a partial fulfillment of DIHM for the requirements to obtain PhD degree in the Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México. DIHM also thanks the Consejo Nacional de Ciencia y Tecnología (CONACyT) for student grant 245193. We thank Dr. Miguel Rubio-Godoy for the donation of specimens of *Mesostephanus* for molecular work. We thank two anonymous reviewers for their comments on the manuscript.

Funding This project was funded by the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-UNAM) IN204514 and IN202617 to GPPL, and PAPIIT-UNAM IN206716 to M.G.V.

Compliance with ethical standards

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

Ethical approval All applicable institutional, national and international guidelines for the care and use of animals were followed. Hosts were collected under the Cartilla Nacional de Colector Científico de Flora y Fauna Silvestre FAUT-0057 and 0202 issued to GPPL and MGV, respectively, by the Secretaria del Medio Ambiente y Recursos Naturales.

References

Baba, T., Hosoi, M., Urabe, M., Shimazu, T., Tochimoto, T., & Hasegawa, H. (2011). *Liolope copulans* (Trematoda: Digenea: Liolopidae) parasitic in *Andrias japonicus* (Amphibia: Caudata: Cryptobranchidae) in Japan: Life cycle and systematic position inferred from morphological and molecular evidence. *Parasitology International, 60,* 181–192.

Bell, A. S., Sommerville, S., & Valtonen, E. T. (2001). A molecular phylogeny of the genus *Ichthyocotylurus* (Digenea, Strigeidae). *International Journal for Parasitology, 31,* 833–842.

Blasco-Costa, I., Poulin, R., & Presswell, B. (2016a). Species of *Apatemon* Szidat, 1928 and *Australapatemon* Sudarikov, 1959 (Trematoda: Strigeidae) from New Zealand: Linking and characterising life cycle stages with morphology and molecules. *Parasitology Research, 115,* 271–289.

Blasco-Costa, I., Cutmore, S. C., Miller, T. L., & Nolan, M. J. (2016b). Molecular approaches to trematode systematics: 'Best practice' and implications for future study. *Systematic Parasitology, 93,* 295–306.

Bray, R. A., Gibson, D. I., & Jones, A. (Eds) (2008). *Keys to the Trematoda. Volume 3*. Wallingford, UK: CAB International & The Natural History Museum, 824 pp.

Briscoe, A. G., Bray, R. A., Brabec, J., & Littlewood, D. T. J. (2016). The mitochondrial genome and ribosomal operon of *Brachycladium goliath* (Digenea: Brachycladiidae) recovered from a stranded minke whale. *Parasitology International, 65,* 271–275.

Brooks, D. R., & Overstreet, R. (1978). The family Liolopidae (Digenea) including a new genus and two new species from crocodilians. *International Journal for Parasitology, 8,* 267–273.

Brooks, D. R., Catto, J. B., & Amato, F. R. (1992). A new phylogenetic classification of the genera of the Proterodiplostomatidae Dubois, 1936 (Digenea: Strigeiformes). *Proceedings of the Biological Society of Washington, 105,* 143–147.

Brooks, D. R., O'Grady, T., & Glen, D. R. (1985). Phylogenetic analysis of the Digenea (Platyhelminthes: Cercomeria) with comments in their adaptive radiation. *Canadian Journal of Zoology, 63,* 873–883.

Caballero, E. (1948). Estudios helmintológicos de la Cuenca del río Papaloapan III. Strigeidos de los lagartos de México. 2. *Anales de la escuela Nacional de Ciencias Biológicas, 5,* 217–221.

Conroy, G. A. (1986). *Crocodilicola pseudostoma* (Willemoes-Suhm, 1870) Poche, 1925 (Trematoda: Proterodiplostomidae), endoparásito del bagre pimelódido *Rhamdia hilarii* Val., 1840 del Estado de São Paulo, Brasil. *Revista Ibérica de Parasitología, 46,* 35–38.

Cribb, T. H., Bray, R. A., Littlewood, D. T. J., Pichelin, S., & Herniou, E. A. (2001). The Digenea. In: Littlewood, D. T. J. & Bray, R. A. (Eds) *Interrelationships of the Platyhelminthes.* London: Taylor & Francis, pp. 168–185.

Cribb, T. H., Bray, R. A., Olson, P. D., & Littlewood, D. T. J. (2003). Life cycle evolution in the Digenea: A new perspective from phylogeny. *Advances in Parasitology, 54,* 197–254.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: More models, new heuristics and parallel computing. *Nature Methods, 9,* 772.

Dubois, G. (1936). Les Diplostomes de Reptiles (Trematoda: Proterodiplostomidae nov. fam.) du Musée de Vienne. *Bulletin de la Société Neuchateloise des Sciences Naturelles, 61,* 5–80.

Dubois, G. (1938). Monographie des Strigeida (Trematoda). *Mémoires de la Société Neuchateloise des Sciences Naturelles, 6,* 1–535.

Dubois, G. (1970). Revision des Proterodiplostomatidae Dubois, 1936 (Trematoda: Strigeta). *Anales del Instituto de Biología, Universidad Nacional Autónoma de México, Serie Zoología, 41,* 51–60.

Dzikowski, R., Levy, M., Poore, M. F., Flowers, J. R., & Paperna, I. (2003). Genetic and morphologic differentiation of *Bolbophorus confusus* and *B. levantinus* (Digenea: Diplostomatidae), based on rDNA SSU polymorphism and SEM. *Diseases of Aquatic Organisms, 57,* 231–235.

Dzikowski, R., Levy, M. G., Poore, M. F., Flowers, J. R., & Paperna, I. (2004). *Clinostomum complanatum* and *Clinostomum marginatum* (Rudolphi, 1819) (Digenea: Clinostomidae) are separate species based on differences in ribosomal DNA. *Journal of Parasitology, 90,* 413–414.

Ferrari-Hoeinghaus, A. P., Takemoto, R. M., & Pavanelli, G. C. (2007). Digenetic trematode parasites of *Loricariichthys platymetopon* (Loricariidae, Siluriformes) of the upper Paraná river floodplain, Brazil. *Acta Scientarumn Biological Sciences, 29,* 327–329.

Fraija-Fernández, N., Olson, P. D., Crespo, E. A., Raga, J. A., Aznar, F. J., & Fernández, M. (2015). Independent host switching events by digenean parasites of cetaceans inferred from ribosomal DNA. *International Journal for Parasitology, 45,* 167–173.

Froese, R., & Pauly, D. (Eds) (2017). *FishBase. World Wide Web electronic publication*. http://www.fishbase.org, version 06/2017.

Galazzo, D. E., Dayanandan, S., Marcogliese, D. J., & McLaughlin, J. D. (2002). Molecular systematics of some North American species of *Diplostomum* (Digenea) based on rDNA-sequence data and comparisons with European congeners. *Canadian Journal of Zoology, 30,* 2207–2217.

García-Varela, M., & Nadler, S. A. (2005). Phylogenetic relationships of Palaeacanthocephala (Acanthocephala) inferred from SSU and LSU rDNA gene sequences. *Journal of Parasitology, 91,* 1401–1409.

García-Varela, M., Sereno-Uribe, A. L., Pinacho-Pinacho, C. D., Domínguez- Domínguez, O., & Pérez-Ponce de León, G. (2016a). Molecular and morphological characterization of *Austrodiplostomum ostrowskiae* Dronen, (Digenea: Diplostomatidae), a parasite of cormorants in the Americas. *Journal of Helminthology, 90,* 174–185.

García-Varela, M., Sereno-Uribe, A. L., Pinacho-Pinacho, C. D., Hernández- Cruz, E., & Pérez-Ponce de León, G. (2016b). An integrative taxonomic study reveals a new species of *Tylodelphys* Diesing, 1950 (Digenea: Diplostomidae) in central and northern Mexico. *Journal of Helminthology, 90,* 668–679.

Georgieva, S., Soldánová, M., Pérez-del-Olmo, A., Dangel, D. R., Sitko, J., Sures, B., et al. (2013). Molecular prospecting for European *Diplostomum* (Digenea: Diplostomidae) reveals cryptic diversity. *International Journal for Parasitology, 43,* 57–72.

Gibson, D. I., Jones, A., & Bray, R. A. (Eds) (2002). *Keys to the Trematoda. Volume 1*. Wallingford, UK: CAB International & The Natural History Museum, 521 pp.

Guidelli, G. M., Isaac, A., Takemoto, R. M., & Pavanelli, G. C. (2003). Endoparasite infracommunities of *Hemisorubi platyrhynchos* (Valenciennes, 1840) (Pisces: Pimelodidae) of the Baía river, upper Paraná river floodplain, Brazil: Specific composition and ecological aspects. *Brazilian Journal of Biology, 63,* 261–268.

Hernández-Mena, D. I., García-Prieto, L., & García-Varela, M. (2014). Morphological and molecular differentiation of *Parastrigea* (Trematoda: Strigeidae) from Mexico, with the description of a new species. *Parasitology International, 63,* 315–323.

Jones, A., Bray, R. A., & Gibson, D. I. (Eds) (2005). *Keys to the Trematoda. Volume 2*. Wallingford, UK: CAB International & The Natural History Museum, 745 pp.

Kanev, I., Radev, V., & Fried, B. (2002). Superfamily Clinostomoidea Lühe, 1901. In: Gibson, D. I., Jones, A. & Bray, R. A. (Eds). *Keys to the Trematoda, Volume 1*. Wallingford, UK: CABI Publishing and The Natural History Museum, pp. 111–112.

Kostadinova, A., & Pérez-del Olmo, A. (2014). The systematics of the Trematoda. In: Toldedo, R. & Fried, B. (Eds). *Digenetic Trematodes. Advances in Experimental Medicine and Biology*. New York: Springer, pp. 21–44.

Littlewood, D. T. J. (2008). Platyhelminth systematics and the emergence of new characters. *Parasite, 15,* 333–341.

Littlewood, D. T. J., Bray, R. A., & Waeschenbach, A. (2015). Phylogenetic patterns of diversity in the cestodes and trematodes. In: Morand, S., Krasnov, B. R. & Littlewood, D. T. J. (Eds) *Parasite diversity and diversification: Evolutionary ecology meets phylogenetics*. Cambridge: Cambridge University Press, pp. 304–319.

Littlewood, D. T., Lockyer, A. E., Webster, B. L., Johnston, D. A., & Le, T. H. (2006). The complete mitochondrial genomes of *Schistosoma haematobium* and *Schistosoma spindale* and the evolutionary history of mitochondrial genome changes among parasitic flatworms. *Molecular Phylogeneics and Evolution, 39,* 452–467.

Liu, K., Raghavan, S., Nelesen, S., Linder, C. R., & Warnow, T. (2009). Rapid and accurate large-scale coestimation of sequence alignments and phylogenetic trees. *Science, 324,* 1561–1564.

Liu, K., Warnow, T., Holder, M., Nelesen, S., Yu, J., Stamatakis, P., & Linder, R. (2012). SATé-II: Very fast and accurate simultaneous estimation of multiple sequence alignments and phylogenetic trees. *Systematic Biology, 61,* 90–106.

Locke, S. A., Al-Nasiri, F. S., Caffara, M., Drago, F., Kalbe, M., Lapierre, A. R., et al. (2015). Diversity, specificity and speciation in larval Diplostomidae (Platyhelminthes: Digenea) in the eyes of freshwater fish, as revealed by DNA barcodes. *International Journal for Parasitology, 45,* 841–855.

Locke, S. A., McLaughlin, J. D., Dayanandan, S., & Marcogliese, D. J. (2010). Diversity and specificity in *Diplostomum* spp. metacercariae in freshwater fishes revealed by cytochrome c oxidase I and internal transcribed spacer sequences. *International Journal for Parasitology, 40,* 333–343.

Locke, S. A., McLaughlin, J. D., Lapierre, A. R., Johnson, P. T. J., & Marcogliese, D. J. (2011). Linking larvae and adults of *Apharyngostrigea cornu*, *Hysteromorpha triloba*, and *Alaria mustelae* (Diplostomoidea: Digenea) using molecular data. *Journal of Parasitology, 97,* 846–851.

Lockyer, A. E., Olson, P. D., & Littlewood, D. T. J. (2003). Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): Implications and a review of the cercomer theory. *Biological Journal of the Linnean Society, 78,* 155–171.

Messing, J. (1983). New M13 vectors for cloning. *Methods in Enzymology, 101,* 20–78.

Nadler, S. A., & Hudspeth, D. S. S. (1998). Ribosomal DNA and phylogeny of the Ascaridoidea (Nemata: Secernentea): Implications for morphological evolution and classification. *Molecular Phylogenetics and Evolution, 10,* 221–236.

Nadler, S. A., Carreno, R. A., Mejia-Madrid, H., Ullberg, J., Pagan, C., Houston, R., & Hugot, J. P. (2000). Molecular phylogeny of clade III nematodes reveals multiple origins of tissue parasitism. *Parasitology, 134,* 1421–1442.

Niewiadomska, K. (2002a). Superfamily Diplostomoidea Poirier, 1886. In: Gibson, D. I., Jones, A. & Bray, R. A. (Eds). *Keys to the Trematoda, Volume 1*. Wallingford, UK: CABI Publishing and The Natural History Museum, pp. 159–196.

Niewiadomska, K. (2002b). Family Diplostomidae Poirier, 1886. In: Gibson, D. I., Jones, A. & Bray, R. A. (Eds). *Keys to the Trematoda, Volume 1*. Wallingford: CABI Publishing and The Natural History Museum, pp. 167–166.

Niewiadomska, K. (2002c). Family Brauninidae Wolf, 1936. In: Gibson, D. I., Jones, A. & Bray, R. A. (Eds). *Keys to the Trematoda, Volume 1*. Wallingford, UK: CABI Publishing and The Natural History Museum, pp. 199–200.

Niewiadomska, K. (2002d). Family Cyathocotylidae Mühling, 1898. Gibson, D. I., Jones, A. & Bray, R. A. (Eds). *Keys to the Trematoda, Volume 1*. Wallingford, UK: CABI Publishing and The Natural History Museum, pp. 201–214.

Niewiadomska, K. (2002e). Family Proterodiplostomidae Dubois, 1936. In: Gibson, D. I., Jones, A., and Bray, R. A. (Eds). *Keys to the Trematoda, Volume 1*. Wallingford, UK: CABI Publishing and The Natural History Museum, pp. 215–230.

Niewiadomska, K. (2002f). Family Strigeidae Railliet, 1919. In: Gibson, D. I., Jones, A. & Bray, R. A. (Eds). *Keys to the Trematoda, Volume 1*. Wallingford, UK: CABI Publishing and The Natural History Museum, pp. 231–244.

Niewiadomska, K. (2002g). Family Liolopidae Odhner, 1912. In: Gibson, D. I., Jones, A. & Bray, R. A. (Eds). *Keys to the Trematoda, Volume 1*. Wallingford, UK: CABI Publishing and The Natural History Museum, pp. 121–125.

Olson, P. D., Cribb, T. H., Tkach, V. V., Bray, R. A., & Littlewood, D. T. J. (2003). Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal for Parasitology, 33,* 733–755.

Patrelle, C., Portier, J., Jouet, D., Delorme, D., & Ferté, H. (2015). Prevalence and intensity of *Alaria alata* (Goeze, 1792) in water frogs and brown frogs in natural conditions. *Parasitology Research, 114,* 4405–4412.

Pérez-Ponce de León, G., García-Prieto, L., & Osorio-Sarabia, D. (1992). Helmintofauna del "juile" *Rhamdia guatemalensis* (Pisces: Pimelodidae), del lago de Catemaco, Veracruz. *Revista de la Sociedad Mexicana de Historia Natural, 43,* 25–31.

Pérez-Ponce de León, G., García-Varela, M., Pinacho-Pinacho, C. D., Sereno-Uribe, A. L., & Poulin, R. (2016). Species delimitation in trematodes using DNA sequences: Middle-American *Clinostomum* as a case study. *Parasitology, 143,* 1773–1789.

Pulis, E. E., Fayton, T. J., Curran, S. S., & Overstreet, R. M. (2013). A new species of *Intromuguil* (Digenea: Haploporidae) and redescription of *Intromugul mugilicolus*. *Journal of Parasitology, 99,* 501–508.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., et al. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology, 61,* 539–542.

Shoop, W. L. (1989). Systematic analysis of the Diplostomidae and Strigeidae (Trematoda). *Journal of Parasitology, 75,* 21–32.

Stamatakis, A. (2006). RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics, 22,* 2688–2690.

Stock, S. P., Campbell, J. F., & Nadler, S. A. (2001). Phylogeny of *Steinerma* Travassos, 1927 (Cephalobina: Steinermatidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology, 87,* 877–899.

Waeschenbach, A., Webster, B. L., Bray, R. A., & Littlewood, D. T. J. (2007). Added resolution among ordinal level relationships of tapeworms (Platyhelminthes: Cestoda) with complete small and large subunit nuclear ribosomal RNA genes. *Molecular Phylogenetics and Evolution, 45,* 311–325.

Webster, B. L., & Littlewood, D. T. J. (2012). Mitochondrial gene order change in *Schistosoma* (Platyhelminthes: Digenea: Schistosomatidae). *International Journal for Parasitology, 42,* 313–321.

Willemoes-Suhm, R. (1871). Uber einige Trematoden u. Nemathelminthen. *Zeitschrift für wissenschaftliche Zoologie, 2,* 175–203.

Yamaguti, S. (1971). *Synopsis of digenetic trematodes of vertebrates*. Tokyo: Keigaku Publishing Co., Parts I & II. 1074 pp. + 349 pl.

6. CAPÍTULO IV

6. CAPÍTULO IV. RELACIONES FILOGENÉTICAS ENTRE LAS SUPERFAMILIAS DE LA SUBCLASE DIGENEA

En este último capítulo se presentan los resultados del análisis realizado con secuencias de ADN del 28S para obtener una hipótesis filogenética sobre las relaciones dentro de la subclase Digenea. Para este capitulo se utilizaron las secuencias de todas las especies de trematodos publicadas en Genbank hasta Diciembre del 2017 y adicionamos nuevas secuencias del 28S generadas en el presente trabajo. El análisis filogenético se realizó a través del método de Máxima Verosimilitud. En este estudio se discuten principalmente las relaciones entre los diferentes taxa a nivel de familias y superfamilias y se discute la estabilidad de algunos grupos supraespecíficos (superfamilias y ordenes) que fueron propuestos anteriormente en trabajos pioneros como Cribb et al. (2001), Olson et al. (2003) y Littlewood et al. (2015). También se señalan algunos problemas taxonómicos de varias familias que son obtenidas como parafiléticas. Los resultados son presentados en el artículo titulado "**Updating the molecular phylogenetic tree of the Digenea (Platyhelminthes: Trematoda) based on 28S rDNA sequences before moving into the mitogenomic era**", el cual será enviado para su publicación a la revista Journal of Helminthology.

Updating the molecular phylogenetic tree of the Digenea (Platyhelminthes: Trematoda) based on 28S rDNA sequences before moving into the mitogenomic era.

G. Pérez-Ponce de León[1], David Iván Hernández-Mena[1,2]

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

2 Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, México, D.F., Mexico

Abstract

Digenea Carus, 1863 is a highly diverse group of parasitic platyhelminths parasitizing all major vertebrate groups as definitive hosts. Even though morphological characters are the cornerstone of digenean systematics, molecular tools have been instrumental in the search of a stable classification system in this group of parasitic worms. The first comprehensive molecular phylogenetic tree of the Digenea published in 2003, was based on two ribosomal genes (18S and 28S) for 163 taxa, representing 77 nominal families. The main objective of this study is to re-examine the phylogenetic relationships among the Digenea through the 28S rRNA gene before jumping into revolutionary advancements of next generation sequencing (NGS) technologies; we use 28S rDNA sequences because the genetic library has increased considerably between 2003 and 2017; it possess strong phylogenetic signal to resolve sister group relationships among species, and for inferring the phylogenetic relationships at higher levels of the hierarchy (family, superfamily, and suborder). We retrieved all the 28S rDNA sequences from the GenBank up until December 2017, and we added newly generated sequences. The new dataset consists of 1,077 digenean taxa allocated in 106 nominal families; Maximum Likelihood analyses were performed on the new dataset. Overall, results are consistent with previous proposals in terms of the classification at higher-level categories, and most superfamilies and suborders are recovered as monophyletic assemblages. The newly generated trees are proposed as a framework upon which the hypotheses on the classification of Digenea derived from complete mitochondrial genomes can be tested.

Introduction

Digenea Carus, 1963 and Aspidogastrea Faust and Tang, 1936 are the two subclasses within the class Trematoda Rudolphi, 1808 (Cribb *et al*., 2001; Gibson *et al*., 2002; Kostadinova & Pérez del Olmo, 2014). Digeneans are probably the most diverse and numerically abundant group of parasitic metazoans, with approximately 18,000 nominal species included in *c.* 2,500-2,700 nominal genera (see Cribb *et al*., 2001; Pérez-Ponce de León, 2001; Gibson *et al*. 2002; Olson *et al*., 2003; Kostadinova & Pérez del Olmo, 2014; Littlewood *et al*., 2015). Brooks *et al*. (1985, 1989) and later Brooks & McLennan (1993) conducted cladistic phylogenetic analyses of the Digenea using morphological traits from all life-history stages. These studies represented a framework upon which the proposed phylogenetic classification of the Digenea was further tested, using morphological characters and a few years later, through phylogenetic analyses using nuclear molecular markers. Gibson & Bray (1994) suggested a classification of the Digenea divided in three orders, i.e., Strigeida, Echinostomida and Plagiorchiida; actually, these orders were used to organize three comprehensive volumes aimed at providing keys for the identification of adult trematodes (Gibson *et al*., 2002; Jones *et al*., 2005; Bray *et al.* 2008). Gibson & Bray (1994) also predicted the increase in our understanding of the diversity, systematics and evolution of digeneans through molecular-based studies. Much progress has been gained in the last 25 years regarding digenean classification; however, more studies are still necessary to produce a stable classification system in a highly diversified group like digeneans, and such studies most be coupled with the continuous advancement of analytical and methodological tools. Pioneering work by Cribb *et al*. (2001) used morphological and life-cycle characters, in combination with sequence data from the 18S rRNA gene of 75 species of digeneans, and three aspidogastreans to study the evolution of Digenea. This study marked the starting point of what we currently know about the molecular phylogeny of the Digenea. Olson *et al*. (2003) provided a comprehensive molecular phylogenetic tree of the Digenea by using the complete small subunit of the ribosomal RNA gene (ssrDNA = 18S rDNA) and partial (D1-D3) large subunit ribosomal gene (lsrDNA = 28S rDNA) of 163 digenean taxa representing 77 nominal families, and seven aspidogastreans as outgroups; these nuclear genes were analyzed independently and combined through Parsimony and Bayesian Inference methods. The analysis by Olson *et al*. (2003) was instrumental in proposing a phylogeny-based classification scheme of the subclass Digenea; Olson *et al*., (2003) analyses have also been widely used as a framework upon which the evolution of digeneans can be studied (e.g., Fraija-Fernández *et al*., 2015). The most recent molecular phylogenetic analysis of this parasitic group was conducted by Littlewood *et al*. (2015); these authors studied the diversification patterns of trematodes in reference to molecular phylogenies and definitive vertebrate host usage. A database was built by these authors based on the available data on GenBank (up to

January 2013) for 18S and 28S rRNA genes. Phylogenetic trees (not shown in the paper) were undertaken considering information for discrete species, including 202 and 556 sequences of 18S and 28S, respectively (Littlewood *et al*., 2015). Diversity and diversification patterns were then studied by reference to the phylogenetic analysis at the superfamily level (see figs 16.1 and 16.3 in Littlewood *et al.*, 2015).

The number of DNA sequences of nuclear and mitochondrial molecular markers of digeneans has increased in the last 15 years at a steady pace. Interestingly, studies aimed at establishing more robust species limits have determined the increase in the number of mitochondrial DNA sequences, particularly of the cytochrome *c* oxidase subunit 1 (*cox*1) (fig. 1); meanwhile, studies of species diagnostics and phylogenetic assessments have resulted in an increase of the taxon representation at species, genus and family levels, particularly for the 28S rRNA gene (see fig. 1). Even though 18S and 28S rRNA genes are the bedrock of platyhelminth molecular systematics (see Lockyer *et al*., 2003; Littlewood, 2008) and the combination of both genes provides added resolution for interrelationships, the genetic library for 28S in terms of representation of species, genera, and families has grown at a faster rate, while sequencing the 18S ribosomal gene has been mostly abandoned, making the combination of both markers practically impossible. Thus far, the 28S rRNA ribosomal gene represents the largest dataset of digenean sequence data (fig. 1). Even though molecular data derived from ribosomal genes consistently resolves the interrelationships among platyhelminths (see Littlewood, 2008), the use of next generation sequencing methods in the last decade has increased our ability to obtain large number of sequence data. As a result, the complete mitochondrial genome (mitogenome) of some digenean species is now available, and it has been used to propose novel phylogenetic analyses based on the premise that not just the number of sequence base pairs, but also gene order and genome arrangement may provide evidence of shared ancestry (see Littlewood *et al.*, 2006). Also, it has been argued that mitogenomes could be more reliable and informative for phylogenetic analysis while analyzing interrelationships among platyhelminths such as cestodes (Waeschenbach *et al.*, 2012). As pointed out by Blasco-Costa *et al.*, (2016): “sequencing whole mitochondrial genomes is possibly the next step beyond traditional DNA barcoding”, and studies involving the use of mitogenomes of closely related species encompassing *c.* 14,000 bp may provide not just better resolution of interrelationships, but also higher nodal support for phylogenetic relationship inference. For instance, several studies have analysed the phylogenetic relationships among species of digeneans using mitogenomes (e.g., Webster & Littlewood, 2012; Brabec *et al*., 2015; Briscoe *et al*., 2016; Chen *et al*., 2016). In this paper, we test the phylogenetic signal of partial sequences of the 28S rRNA gene (D1-D3 variable regions) in resolving phylogenetic interrelationships within the Digenea, through a more complete dataset that includes 1,077 taxa included 106 families (up until December, 2017); we analyze potential inconsistencies with previous molecular phylogenetic assessments in terms of the classification of digeneans at superfamily and suborder levels, and we discuss the stability of the classification by comparing it with the interrelationships generated through complete mitochondrial genomes.

Materials and methods

Sampling of sequences from the GenBank

An search was carried out in the GenBank to find all the available sequences of the 28S ribosomal gene belonging to the subclass Digenea for a period comprised between January, 1990 and December, 2017. First, we analysed the current trends on the use of molecular markers in the study of digenean taxonomy; we searched the GenBank database exhaustively for DNA sequences of different molecular markers. Two main criteria for the selection of taxa were followed: a) that the sequences were longer than 400 bp; and b) that the sequences had been obtained from adult specimens; this could guarantee the proper taxonomic identification. Some exceptions were made, however, to achieve the highest representation of families during the sequence selection. If a sequence of certain genus was the only representative of a family although it was not identified up to species level (e.g., *Acanthochasmus* sp., *Amphiorchis* sp., etc.), or the identification was based on larval forms (cercariae or metacercariae), then the sequence was included.

Only the most commonly used markers were considered to be searched in the GenBank database, i.e., the large subunit of the ribosomal 28S rRNA gene (LSU); the small subunit of the ribosomal 18S rRNA gene (SSU), the internal transcribed spacers (ITS), including the ITS1, 5.8S and ITS2; the cytochrome *c* oxidase subunit I (*cox*1). The search strategy included six combinations of terms to retrieve all the available information on sequences of trematodes:

a) 28S: (“trematoda” [Organism] AND (“28S" OR “large subunit ribosomal") AND “year" [PDAT]) NOT “ITS" NOT “Internal" NOT “5.8S" NOT “18S" NOT “genome".

b) 18S: ("trematoda" [Organism] AND ("18S" OR "small subunit ribosomal") AND "year" [PDAT]) NOT "ITS" NOT "Internal" NOT "5.8S" NOT "28S" NOT "genome".

c) Only ITS1: ("trematoda" [Organism] AND "year" [PDAT] AND ("Internal transcribed spacer 1" OR "ITS1")) NOT "28S" NOT "Internal transcribed spacer 2" NOT "Internal transcribed spacer II" NOT "ITS2" NOT "genome" NOT "NADH" NOT "cytochrome" NOT "NDH".

d) Only ITS2: ("trematoda" [Organism] AND "year" [PDAT] AND ("Internal transcribed spacer 2" OR "ITS2")) NOT "18S" NOT "Internal transcribed spacer 1" NOT "Internal transcribed spacer I" NOT "ITS1" NOT "genome" NOT "NADH" NOT "cytochrome" NOT "NDH".

e) ITS 1 plus ITS2: ("trematoda" [Organism] AND "year" [PDAT] AND ("Internal transcribed spacer 1" OR "Internal transcribed spacer I" OR "ITS1") AND ("Internal transcribed spacer 2" OR "Internal transcribed spacer II" OR "ITS2") AND "5.8S") NOT "genome" NOT "NADH" NOT "cytochrome" NOT "NDH" 6. cox 1: ("trematoda" [Organism] AND "cytochrome" AND "oxidase" AND "subunit".

f) *cox* 1: ("trematoda" [Organism] AND "cytochrome" AND "oxidase" AND "subunit" AND "2016" [PDAT]) NOT "ITS" NOT "Internal" NOT "5.8S" NOT "28S" NOT "genome" NOT "18S" NOT "subunit 3" NOT "subunit III" NOT "subunit 2" NOT "subunit II"

For all the sequences retrieved for each molecular marker we determined two parameters: the number of published sequences (individuals), and the number of species sequenced. Both parameters were organized by publication year, and accumulation curves were built to visualize the increase of both parameters through time (see fig. 1). The accumulation curve of the number of species per year only considered those identified up to species level; taxa identified to genus level and recorded in GenBank as ‘genus’ “sp.” (excepting a few cases as previously mentioned), or those whose terminals were labelled ‘genus “*c.f.*” species’, or ‘genus “*aff.*” species’ or those recorded as ‘genus’ “Lineage I or II, etc”, were not included.

Newly generated sequences: DNA extraction, amplification and sequencing

54 novel sequences were generated for specimens collected from different vertebrate species in localities across Mexico (table 1). Specimens sampled were processed either for morphological examination to achieve the species identification, and for DNA extraction. Paragenophores (sensu Pleijel *et al*., 2008) of some digeneans collected in this study were deposited at the Colección Nacional de Helmintos (CNHE), Biology Institute, National Autonomous University of Mexico, Mexico City, Mexico (table 1). Genomic DNA was extracted from each individual using the REDExtract-N-Amp Tissue PCR kit (Sigma, St. Louis, USA) following the manufacturer´s instructions. The domains D1, D2 and D3 of the 28S rRNA gene were amplified using polymerase chain reaction (PCR). The use of primers for PCR amplification, thermo-cycling profile, and sequencing followed protocols described in more detail in Hernández-Mena *et al*. (2017).

Alignment and phylogenetic analyses

Newly generated 28S DNA sequences of digeneans were aligned with other sequences available in GenBank, along with those of some aspidogastreans that were employed as outgroups for rooting the trees. A first alignment was built to discard taxa with sequences that were not homologous, or to delete duplicate sequences. This alignment was performed with ClustalW as implemented on the website of Cipres Science Gateway (https://www.phylo.org). The final alignment was performed with the software SATé, under the default setting SATé-II-fast (Aligner= MAFT, Merger= MUSCLE, Tree Estimator= RAXML, Model= GTRCAT; Liu *et al*., 2009, 2012), implementing 200 iterations. After the iterations, the best alignment was used for phylogenetic analyses. Nucleotide substitution was chosen with jModelTest v2 (Darriba *et al*., 2012).

Phylogenetic trees were performed under Maximum Likelihood (ML), and the model of nucleotide evolution GTR+GAMMA+I was employed. The ML analysis was performed in raxmlGUI v. 1.3 (Silvestro & Michalak, 2012); the tree with the best fit was obtained after 100 repetitions, and 10,000 bootstrap replicates were run to estimate nodal support. The phylogenetic tree was visualized in FigTree v.1.4.3. (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Phylogenetic analyses with 28S

The final alignment of the 28S dataset consisted of 1,077 terminals and was 1,983 bp long; nulceotide frequencies were: A=0.215, C=0.216, G=0.316 and T=0.253. The phylogenetic tree obtained from the ML analysis had a likelihood of –ln=198962.897393. Phylogenetic analyses inferred through ML yielded a tree where the subclass Aspidogastrea is recovered as the sister group of the Digenea (fig. 2). Supplementary material S2 depict the ML phylogenetic treee for individual sequences. Figure 2 depicts the condensed phylogenetic tree showing families as terminals, and the arrangement of these families in superfamilies and suborders following the proposal of Olson *et al*. (2003). Figure 2 shows that the two known and widely accepted orders within the Digenea, i.e., Diplostomida Olson, Cribb, Tkach, Bray and Littlewood, 2003 and Plagiorchiida La Rue, 1957, are recovered as monophyletic groupings with high bootstrap support values. The phylogenetic classification of the Digenea followed as a reference in our study (Olson *et al.*, 2003), was highly supported by the phylogenetic analysis herein presented at the levels of superfamily and suborder. However, two of the superfamilies (Gorgoderoidea Looss, 1901 and Allocreadioidea Looss, 1902) and two of the suborders (Bucephalata La Rue, 1926 and Xiphidiata Olson, Cribb, Tkach, Bray and Littlewood, 2003) as recognized by Olson *et al* (2003), resulted paraphyletic in our analysis. The interrelationships of the Diplostomida, the less diverse order of the Digenea, were very consistent, with three major groups comprising three superfamilies: 1) Diplostomoidea Poirier, 1886 (including the paraphyletic diplostomids + strigeids, in addition to proterodiplostomids, cyathocotylids and brauninids); our analyses suggested that liolopids could be considered as members of the Diplostomoidea. 2) Brachylaimoidea Joyeux and Foley, 1930 (including brachylaimoids, leucochloridiids, and *Zeylanurotrema* Crusz and Sanmugasunderam, 1973), and 3) Schistosomatoidea Stiles and Hassall, 1898, including the blood flukes (aporocotylids, spirorchiids, and schistosomatids) and clinostomids. Two families are paraphyletic, i.e., Aporocotylidae Odhner, 1912 and Spirorchiidae MacCallum, 1921. Instead, the interrelationships of the Plagiorchiida were less consistent as several groupings are supported by low nodal support values (see fig. 2). For instance, Bucephalidae Poche, 1907 was not recovered as the basal member of the group and their representatives were nested as the sister taxa of Haplosplanchnidae Poche, 1926. These two families were closely related to pronocephaloids and paramphistomoids. Within the Order Plagiorchiida, at least six additional families were recovered as paraphyletic, including Derogenidae Nicoll, 1910, Cladorchiidae Fischoeder, 1901, Acanthocoplidae Lühe, 1906, Enenteridae Yamaguti, 1958, Plagirochiidae Lühe, 1901, and Brachycoeliidae Losss, 1899 (fig. 2).

The phylogenetic tree of the Digenea inferred in this study included members of 106 families. Several groupings at family level showed that representative genera and species are intermingled, i.e., Diplostomidae Poirier, 1886 + Strigeidae Railliet, 1919; Hemiuridae Looss, 1899 + Lecithasteridae Odhner, 1905; Microscaphidiidae Loos, 1900 + Mesometridae Poche, 1926; Paramphistomidae Fischoeder, 1901 + Gastrothylacidae Stiles & Goldberger, 1910 + Gastrodiscidae Monticelli, 1892 + Olveriidae Yamaguti 1958; Monorchiidae Odhner, 1911 + Acanthocolpidae Lühe, 1906; Apocreadiidae Skrjabin, 1942 + Megaperidae Manter, 1934; Heterophyidae Leiper, 1909 + Cryptogonimidae Ward, 1917 + Opisthorchiidae Looss, 1899; Zoogonidae Odhner, 1902 + Faustulidae Poche, 1926; Brachycoeliidae Looss, 1899 + Mesocoeliidae Dollfus, 1929; and Plagiorchiidae Lühe, 1901 + Choanocotylidae Jue Sue & Platt, 1998. Furthermore, at least five additional families require to be erected to assign genera classified as *inserta sedis* after the phylogenetic analysis of the 28S r RNA gene: *Zeylanurotrema* (considered either as member of the Brachylaimidae Joyeux and Foley, 1930 or Urotrematidae Poche, 1926); *Biospeedotrema* Bray, Waeschenbach, Dyal, Littlewood & Morand, 2014 (considered as member of the Opecoelidae Ozaki, 1925); *Polylekithum* Arnold, 1934 and *Paracreptotrematina* Amin & Myer, 1982 (considered as members of the Alocreadiidae Looss, 1902), and *Rauschiella* Babero, 1951 (considered as member of the Plagiorchiidae). Within the Plagiorchiida two major clades were formed, one including members of five superfamilies, i.e., Bivesiculoidea Yamaguti, 1934, Transversotrematoidea Witenberg, 1944, Heronimoidea Ward, 1918, Azygioidea Lühe, 1909, and Hemiuroidea Looss, 1899. The other 14 superfamilies were included in the second major clade (Fig. 2). In our analysis, *Pachytrema calculus* Looss, 1907, a parasite of the marine bird *Tringa nebularia* Gunnerus from the Danube delta, Odessa region (GenBank accession AF151942, Olson *et al*., 2003) was recovered as the basal member of this large group of superfamilies, albeit with a moderate bootstrap support value (72%). Although the Xiphidiata were recovered as paraphyletic since haploporids and atractotrematids were not nested with other members of the Gorgoderoidea, most xiphidiatans conformed a highly supported monophyletic group (98% of bootstrap support), including representatives of four large superfamilies of digeneans, i.e., Allocreadioidea, Gorgoderoidea, Microphalloidea Ward, 1901 and Plagiorchioidea Lühe, 1901.

Discussion

The updated phylogenetic analysis consisted of 1,077 taxa allocated in 106 nominal families (included in 22 superfamilies), almost 900 more taxa and 29 more families than those considered in the molecular phylogenetic analyses by Olson *et al.* (2003). Even with the increase in the number of sequence data, our results are largely consistent with those obtained in the previous phylogenetic analyses. With the exception of the suborder Xiphidiata and Bucephalata, and the superfamilies Gorgoderoidea and Allocreadioidea, all major groupings are recovered as monophyletic in our analyses, indicating the robustness of the classification of the Digenea. Probably the major difference between our analysis and those previously published (Olson *et al.* 2003; Littlewood *et al*. 2015) is related with the classification of the Order Plagirchiida. In Olson´s *et al.* (2003) tree, members of the Bivesiculidae are the basal members of the entire order Plagiorchiida, comprising two large monophyletic groups. In the analysis conducted by Littlewood *et al*. (2015), all plagiorchiids are included in a monophyletic group, although members of the Bivesiculoidea are also basal to all the other plagiorchiids. In our analysis, the order Plagiorchiida comprised two well-defined monophyletic clades, although one of them, including species of Bivesiculoidea, Transversotrematoidea, Heronimoidea, Azygioidea, and Hemiuroidea is supported by a very low bootstrap value (27%). The other clade is the largest and contain most of the families included in the subclass

Digenea, with *c*. 87 families, and is supported by moderate bootstrap support (72%). Members of the genus *Pachytrema* Looss, 1907 are the basal members of this clade. *Pachytrema* allegedly belong to the family Opisthorchiidae (WoRMS, 2018), although the position of this genus within the Plagiorchiida requires further scrutiny because opistorchiids are recovered as paraphyletic (see fig. 2). The lack of statistical support highlights the need for a denser taxon sampling and shows that the classification of the Digenea within the superfamily level requires also further scrutiny. A comparison among phylogenetic trees at the family level reveal a larger number of inconsistencies, as more cases of non-monophyletic groupings are found. The branch topology at family level may be a reflection of the fact that more taxa representative from different families, and actually more families were included in the current phylogenetic analyses; it might be also a reflection of the dynamic progress made on the taxonomy of particular trematode groups through molecular phylogenetic analyses. For instance, Tkach *et al*. (2000) used 28S rDNA sequences to study the interrelationships of the suborder Plagiorchiata, one of the derived and most diverse groups of the Digenea. These authors determined that the suborder, as traditionally conceived at that moment was not monophyletic, and proposed that it consisted of eight families included in two superfamilies, Plagiorchioidea and Microphalloidea. Olson *et al*. (2003) included more representatives of the order Plagiorchiida and proposed a new suborder, Xiphidiata, to include superfamilies contained in Plagiorchiata (*sensu* Tkach *et al*. 2000) plus the superfamily Gorgoderoidea and Allocreadioidea. Our analyses are consistent with Olson *et al*. (2003) except by the fact that Xiphidiata is not recovered as monophyletic since members of the Family Atractotrematidae Yamaguti, 1939, Haploporidae Nicoll, 1914 and one representative of the family Enenteridae, i.e., *Cadenatella* Dollfus, 1946, are not included in the group. Actually, Bray *et al*. (2009, 2014) demonstrated with molecular evidence that *Cadenatella* belongs in the superfamily Haploporoidea and not in Enenteridae. Still, haploporids are not recovered as members of the Xiphidiata. Additionally, several new families, or the elevation of subfamilies to family level have resulted in changes on the classifications at superfamily level., e.g., hemiurids and related groups (Blair *et al*. 1998), allocreadiids (Curran *et al.,* 2006, 2011; Choudhury *et al*., 2006; Pérez-Ponce de León *et al.*, 2007), echinostomids and related groups (Tkach *et al*., 2016) macroderoidiids (Hernández-Mena *et al*., 2016), etc.

The molecular phylogenetic analysis herein presented represent and advancement in our understanding of the interrelationships of the Digenea, and even though it is based on sequence data of only one molecular marker (28S), the taxon sampling shows that most families and certainly almost all of the superfamilies considered valid in traditional classifications are represented in the analysis. New phylogenetic analyses of digeneans use a large dataset derived from the complete mitochondrial genome (mitogenomes), with *c* 14,000 bp. Thus far, mitogenomes of 40 species of digeneans are available in the GanBank; most of them were obtained from species with medical and veterinary importance, e.g., *Schistosoma* Wainland, 1858 (Littlewood *et al*., 2006), *Paramphistomum* Fischoeder, 1901 and *Fasciola* Linnaeus, 1758 (Yan *et al*., 2013; Liu *et al*., 2014). However, novel findings using mitogenomes of other digenean species (see Brabec *et al*., 2015; Chen *et* al., 2016) have uncovered striking results in terms of the classification of the Digenea, particularly the dichotomy highly supported with the subclass divided in two orders, Diplostomida and Plagiorchiida. Brabec *et al*. (2015) sequenced the mitogenome of two species of *Diplostomum* Nordmann, 1832, a digenean from the intestine of fish-eating birds, and these authors surprisingly discovered that the Diplostomidae is nested within the order Plagiorchiida, and not within the order Diplostomida albeit with a moderate bootstrap support value (77%); this contrasts with all previous phylogenetic analyses either using DNA sequences (see Olson *et al*., 2003; Littlewood *et al*., 2015, this study), and even morphology (see Brooks *et al*., 1985, 1989). Interestingly, the addition of the mitogenome of other species such as the brachycladid *Brachycladium goliath* (van Beneden, 1858) (see Briscoe *et al*., 2016) obtained the same results. Chen *et al*. (2016) obtained the mitogenome of *Clinostomum complanatum* (Rudolphi, 1814) Braun, 1899, a parasite of the buccal cavity of birds (and eventually of humans), and their phylogenetic analysis along with other 15 digenean species, shows that *C. complanatum* is nested within a clade formed by species included in the order Plagiorchiida, with the highest bootstrap support value. Unfortunately, these authors did not include the complete mitochondrial genome of the two species of *Diplostomum* made available in Brabec *et al*. (2015).

The unexpected results reported by the aforementioned studies encouraged us to conduct the present study. First, we wanted to test the interrelationships among digeneans and the classification derived from the phylogenetic analysis, by considering a larger taxon sampling where more species of digeneans were represented. Our analyses included almost twice the number species than those used by Littlewood *et al*. (2015). Second, the results of the new analysis would serve to contrast with the topology derived from the phylogenetic analyses that made use of the complete mitochondrial genomes available for a reduced number of species. We agree with Blasco-Costa *et al.* (2016) who predicted that “sequencing the mitochondrial genomes of key trematode taxa has the potential to provide new insights into taxonomy, systematics, and evolutionary history, not only within selected families but also across the Trematoda as a whole”. Certainly, mitogenomes obtained through NGS methods allow the identification of more informative genes; this relatively new source of information has the potential to generate phylogenetic analyses with better nodal support and, in that way, classification schemes resulting from phylogenetic inference could be more robust and stable. In digeneans, mitochondrial and nuclear rDNA data provide conflicting results with respect to the families supporting the two widely accepted orders Diplostomida and Plagiorchiida. This conflicting signal between molecular markers has also been found in certain parts of the cestodes tree (see Waeschenbach *et al.*, 2012). To the best of our knowledge, the database we assembled in this study is the largest that has been analyzed for any group of platyhelminth parasites; analysis required a large computational time to be performed. Still, the number of species included in the 28S phylogenetic analysis of digeneans represents approximately 6% of the estimated diversity of this group of parasitic platyhelminths (1,077 out of *c.* 18,000 species); in contrast, only 0.2% of the digenean actual diversity is available for conducting phylogenetic inferences using mitogenomes. Even though NGS methods represent the future of taxonomic research, an important increase in taxon sampling is needed to accomplish the goal of inferring phylogenetic relationships upon which a stable classification scheme for a group such as digeneans could be proposed.

Acknowledgements. We thank the following people who kindly donated specimens of digeneans for sequencing work: Miguel Rubio, Valeria Salinas, Jorge Lopez, Rogelio Aguilar, Jonathan Granados and Martín García. We also thank Laura Márquez for her support with the use of the automatic sequencer and Luis García for providing specimens from the Colección Nacional de Helmintos for morphological

comparison

Financial Support. This research was supported by grants from the Programa de Apoyo a Proyectos de Investigación e Inovación Tecnológica (PAPIIT-UNAM) IN202514 to GPPL. DIHM thanks the support of the Programa de Posgrado en Ciencias Biológicas, UNAM and CONACYT for the scholarship to complete his PhD program.

Conflict of interest. None.

Ethical standards. Specimens of digeneans collected in vertebrates of Mexico were obtained under the Cartilla Nacional de Colector Científico (FAUT 0057) issued by the Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT) to GPPL.

References

Blasco-Costa I, Cutmore SC, Miller TL and Nolan MJ (2016) Molecular approaches to trematode systematics: 'best practice' and implications for future study. *Systematic Parasitology* **93**, 295–306.

Blair D, Bray RA, and Barker SC (1998). Molecules and morphology in phylogenetic studies of the Hemiuroidea (Digenea: Trematoda: Platyhelminthes) *Molecular Phylogenetics and Evolution* **9**, 15–25.

Brabec, J, Kostadinova A, Scholz T and Littlewood DTJ (2015) Complete mitochondrial genomes and nuclear ribosomal RNA operons of two species of Diplostomum (Platyhelminthes:Trematoda): a molecular resource for taxonomy and molecular epidemiology of important fish pathogens. *Parasites & Vectors* **8**, 336

Bray RA, Gibson DI and Jones A (2008) *Keys to the Trematoda. Volume 3*. 824 pp. Wallingford, UK: CAB International and The Natural History Museum.

Bray RA, Waeschenbach A, Cribb TH, Weedall GD, Dyal P and Littlewood DTJ (2009) The phylogeny of the Lepocreadioidea (Platyhelminthes, Digenea) inferred from nuclear and mitochondrial genes: Implications for their systematics and evolution. *Acta Parasitologica* **54**, 310–329.

Bray RA, Cribb TH, Waeschenbach A and Littlewood DTJ (2014) Molecular evidence that the genus *Cadenatella* Dollfus, 1946 (Digenea: Plagiorchiida) belongs in the superfamily Haploporoidea Nicoll, 1914. *Systematic Parasitology* **89**,15–21.

Briscoe AG, Bray RA, Brabec J and Littlewood DTJ (2016) The mitochondrial genome and ribosomal operon of *Brachycladium goliath* (Digenea: Brachycladiidae) recovered from a stranded minke whale. *Parasitology International* **65**, 271–275.

Brooks DR, O'Grady and Glen R (1985) Phylogenetic analysis of the Digenea (Platyhelminthes: Cercomeria) with comments in their adaptive radiation. *Canadian Journal of Zoology* **63**, 873–883.

Brooks DR, Bandoni SM, MacDonald CA O'Grady RT (1989) Aspects of the phylogeny of the Trematoda Rudolphi, 1808 (Platyhelminthes: Cercomeria). *Canadian Journal of Zoology* **67**, 260–924.

Brooks DR and McLennan D (1993) *Parascript. Parasites and the language of evolution*. Washington: Smithsonian Institution Press.

Chen L, Feng Y, Chen HM, Wang LX, Feng HL, Yang X, Mughal MN and Fang

R (2016) Complete mitochondrial genome analysis of *Clinostomum complanatum* and its comparison with selected digeneans. *Parasitology Research* **115**, 3249–3256.

Choudhury A, Rosas-Valdez R, Johnson RC, Hoffmann B, and Pérez-Ponce de León G (2007). The phylogenetic position of Allocreadiidae (Trematoda: Digenea) from partial sequences of the 18S and 28S ribosomal RNA genes. *Journal of Parasitology* **93**, 192-196.

Cribb TH, Bray RA, Littlewood DTJ, Pichelin S and Herniou EA (2001) The Digenea. pp. 168–185 *in* Littlewood DTJ and Bray RA (*Eds*) *Interrelationships of the Platyhelminthes.* London, Taylor and Francis.

Curran SS, Tkach VV and Overstreet RM (2006). A review of *Polylekithum* Arnold, 1934 and its familial affinities using morphological and molecular data, with description of *Polylekithum catahoulensis* sp. nov. *Acta Parasitologica* **51**, 238–248.

Curran SS, Tkach VV and Overstreet RM (2011). Phylogenetic affinities of *Auriculostoma* (Digenea: Allocreadiidae), with descriptions of two new species from Peru. *Journal of Parasitology* **97**, 661–670.

Darriba D, Taboada GL, Doallo R and Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9**, 772.

Fraija-Fernández N, Olson PD, Crespo EA, Raga JA, Aznar FJ and Fernández M (2015) Independent host switching events by digenean parasites of cetaceans inferred from ribosomal DNA. *International Journal for Parasitology* **45**, 167–173.

Gibson DI, and Bray RA (1994). The evolutionary expansion and host-parasite relationships of the Digenea. *International Journal for Parasitology* **24**, 1213–

1226.

Gibson DI, Jones A and Bray RA (Eds) (2002). *Keys to the Trematoda. Volume 1*. Wallingford, UK: CAB International and The Natural History Museum, 521 pp.

Hernández-Mena DI, Mendoza-Garfias B, Ornelas-García CP, Pérez-Ponce de León G (2016). Phylogenetic position of *Magnivitellinum* Kloss, 1966 and *Perezitrema* Baruš & Moravec, 1967 (Trematoda: Plagiorchioidea: Macroderoididae) inferred from partial 28S rDNA sequences, with the establishment of Alloglossidiidae n. fam. *Systematic Parasitology* **93**, 525–38

Hernández-Mena DI, García-Varela M and Pérez-Ponce de León G (2017). Filling the gaps within the classification of the Digenea Carus, 1963: systematic position of the Proterodiplostomatidae Dubois, 1936 within the superfamily Diplostomoidea Poirier, 1886, inferred from nuclear and mitochondrial DNA sequences. *Systematic Parasitology* **94**, 833–848.

Jones A, Bray RA and Gibson DI (Eds) (2005). *Keys to the Trematoda. Volume 2*. Wallingford, UK: CAB International and The Natural History Museum, 745 pp.

Kostadinova A and Pérez-del Olmo A (2014) The systematics of the Trematoda. In: Toldedo, R. and Fried, B. (Eds). *Digenetic Trematodes. Advances in Experimental Medicine and Biology*. New York: Springer Science + Business Media, pp. 21–44.

Littlewood DTJ (2008) Platyhelminth systematics and the emergence of new characters. *Parasite* **15**, 333–341.

Littlewood DTJ, Lockyer AE, Webster BL, Johnston DA and Le TH (2006). The complete mitochondrial genomes of *Schistosoma haematobium* and

Schistosoma spindale and the evolutionary history of mitochondrial genome changes among parasitic flatworms. *Molecular Phylogeneics and Evolution* **39**, 452–467.

Littlewood DTJ, Bray RA and Waeschenbach A (2015). Phylogenetic patterns of diversity in the cestodes and trematodes. In: Morand, S., Krasnov, B. R. and Littlewood, D. T. J. (Eds) *Parasite Diversity and Diversification: Evolutionary Ecology Meets Phylogenetics*. Cambridge: Cambridge University Press, pp. 304–319.

Liu K, Raghavan S, Nelesen S, Linder CR and Warnow T (2009). Rapid and accurate large-scale coestimation of sequence alignments and phylogenetic trees. *Science* **324**, 1561–1564.

Liu K, Warnow T, Holder M, Nelesen S, Yu J, Stamatakis P and Linder R (2012). SATé-II: Very fast and accurate simultaneous estimation of multiple sequence alignments and phylogenetic trees. *Systematic Biology* **61**, 90–106.

Liu GH, Gasser RB, Young ND, Song HO, Ai L and Zhu HQ (2014). Complete mitochondrial genomes of the ‘intermediate form’ of *Fasciola* and *Fasciola gigantica*, and their comparison with *F. hepatica.* *Parasites & Vectors* **7**,150.

Lockyer AE, Olson PD and Littlewood DTJ (2003) Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. *Biological Journal of the Linnean Society* **78**, 155–171.

Olson PD, Cribb TH, Tkach VV, Bray RA and Littlewood DTJ (2003) Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal for Parasitology* **33**, 733–755.

Pérez-Ponce de León G (2001) The diversity of digeneans (Platyhelminthes:

Cercomeria: Trematoda) in vertebrates in Mexico. *Comparative Parasitology* **68**, 1–8.

Pérez-Ponce de León G, Choudhury A, Rosas-Valdez R and Mejia-Madrid H (2007). Systematic position of Wallinia spp. and Margotrema spp., parasites of middle-American and Neotropical freshwater fish based on 28S ribosomal RNA gene. *Systematic Parasitology* **68**, 49–55.

Pleijel F, Jondelius U, Norlinder E, Nygren A, Oxelman B, Schander C, Sundberg P, and Thollesson M (2008). Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. Molecular Phylogenetics and *Evolution* **48**, 369-371.

Silvestro D and Michalak I (2012). raxmlGUI: a graphical front-end for RaxML. *Organisms Diversity & Evolution* **12**, 335.

Tkach VV, Pawlowski J and J. Mariaux (2000). Phylogenetic analysis of the suborder Plagiorchiata (Platyhelminthes, Digenea) based on partial lsrDNA sequences. *International Journal for Parasitology* **30**, 83–93.

Tkach VV, Kudlai O and Kostadinova A (2016). Molecular phylogeny and systematics of the Echinostomatoidea Looss, 1899 (Platyhelminthes: Digenea). *International Journal for Parasitology* **46**, 171–185.

Waeschenbach A, Webster BL, Bray RA and Littlewood DTJ (2012). Added resolution among ordinal level relationships of tapeworms (Platyhelminthes: Cestoda) with complete small and large subunit nuclear ribosomal RNA genes. *Molecular Phylogenetics and Evolution* **45**, 311-325.

Webster BL and Littlewood DTJ (2012). Mitochondrial gene order change in *Schistosoma* (Platyhelminthes: Digenea: Schistosomatidae). *International Journal for Parasitology* **42**, 313–321.

WoRMS (2018). Opisthorchiidae Looss, 1899. Accessed at:

http://www.marinespecies.org/aphia.php? p=taxdetails&id=108442 on 2018-04-06

Yan HB, Wang XY, Lou ZZ, Li L, Blair D, Yin H (2013). The Mitochondrial Genome of *Paramphistomum cervi* (Digenea), the First Representative for the Family Paramphistomidae. *PLoS ONE* **8**, e71300

Figure Legends

Fig. 1. Number of sequences of Digeneans for nuclear and mitochondrial markers for the period comprised between January 1990 and December 2017.

Fig. 2 Phylogenetic relationships among the Digenea with families as terminals resulting from Maximum Likelihood analyses based on the partial sequences of the 28S rRNA gene. Family names follow those accepted in the Keys to the Trematoda (Gibson *et al*., 2002; Jones *et al*., 2005; Bray *et al*., 2008), and some from revisionary work conducted in the last decade, after the publication of the three volumes. Bootstrap support values are shown near the nodes. Superfamilies and suborders following Olson *et* al. (2003) are shown on the right side of the figure. The scale-bar indicates the number of substitutions per site.

Supplementary material

Supplementary Figure S1. Phylogenetic relationships among the Digenea with species as terminals resulting from Maximum Likelihood analyses based on the partial sequences of the 28S rRNA gene. Bootstrap support values are shown near the nodes. The scale-bar indicates the number of substitutions per site.

Table 1. Species and families of digeneans sequenced in the present study.

Species	Host	Locality	GenBank
Allocreadiidae			
Auriculostoma totonacapanensis	*Astyanax aeneus* (F)	Metzabok, Chiapas	
Apocreadiidae			
Crassicutis sp.	*Vieja synspila* (F)	Naha, Chiapas	
Homalometron sp. 1a	*Balistes vetula* (F)	Puerto Morelos, Quintana Roo	
Homalometron sp. 1b	*Balistes vetula* (F)	Puerto Morelos, Quintana Roo	
Bucephalidae			
Bucephalus sp.	*Caranx caballus* (F)	Chamela, Jalisco	
Rhipidocotyle sp.	*Kyphosus elegans* (F)	Chamela, Jalisco	
Cephalogonimidae			
Cephalogonimus sp.	*Lithobates* sp. (A)	Lago de Zacapu, Michoacán	
Cladorchiidae			
Megalodiscus sp. 1	*Lithobates* sp. (A)	San Pedro Tlaltizapán, Estado de México	
Megalodiscus sp. 2	*Lithobates brownorum* (A)	Pantanos de Centla, Tabasco	
Cryptogonimidae			
Acanthostomum minimum	*Rhamdia guatemalensis* (F)	Cobá, Quintana Roo	
Cryptogonimidae gen sp 1	*Centropomus undecimalis* (F)	Pantanos de Centla, Tabasco	
Cryptogonimidae gen sp 2	*Centropomus undecimalis* (F)	Pantanos de Centla, Tabasco	
Cryptogonimidae gen sp 3	*Centropomus undecimalis* (F)	Salsipuedes, Tabasco	
Tabascotrema verai	*Petenia splendida* (F)	Metzabok, Chiapas	
Derogenidae			
Genarchella sp. 1	*Herichthys labridens* (F)	Axtla de Terrazas, San Luis Potosí	
Genarchella sp. 2	*Astyanax aeneus* (F)	Cenote Noc ac, Yucatán	

Halipegus sp.	*Lithobates* sp. (A)	Atenquique, Jalisco
Dicrocoeliidae		
Parametadelphis sp.	*Pteronotus parnellii* (M)	Isla Don Panchito, Jalisco
Echinostomatidae		
Rhopalias macracantus	*Didelphis masurpialis* (M)	Tlacotalpan, Veracruz
Rhoplias coronatus	*Didelphis masurpialis* (M)	Tzucacab, Yucatan
Enenteridae		
Cadenatella sp.	*Kyphosus elegans* (F)	Chamela, Jalisco
Faustulidae		
Bacciger astyanactis	*Astyanax aeneus* (F)	Ejido Reforma Agricola, Chiapas
Gorgoderidae		
Xystretum solidum	*Balistes vetula* (F)	Puerto Morelos, Quintana Roo
Gyliauchenidae		
Cincinnogyliauchen sp.	Angel (F)	Chamela, Jalisco
Haploporidae		
Saccocoelioides sp.	*Dorosoma anale* (F)	Tenosique, Tabasco
Hemiuridae		
Hemiuridae gen. sp.	*Brycon guatemalensis* (F)	Tenosique, Tabasco
Lecithochirium sp.	*Trichiurus lepturus* (F)	Alvarado, Veracruz
Opisthadena sp.	*Ocyurus chrysurus* (F)	Chamela, Jalisco
Heterophyidae		
Galactosomum puffini a	*Sula nebouxii* (B)	Isla Isabel, Nayarit
Galactosomum puffini b	*Sula nebouxii* (B)	Isla Isabel, Nayarit
Haplorchis pumilo	*Caracara cheriway* (B)	Presa La Angostura, Chiapas
Opisthometra planicollis	*Sula nebouxii* (B)	Isla Isabel, Nayarit
Hirudinellidae		
Hirudinella ventricosa	*Euthynnus alletteratus* (F)	Alvarado, Veracruz
Lepocreadiidae		

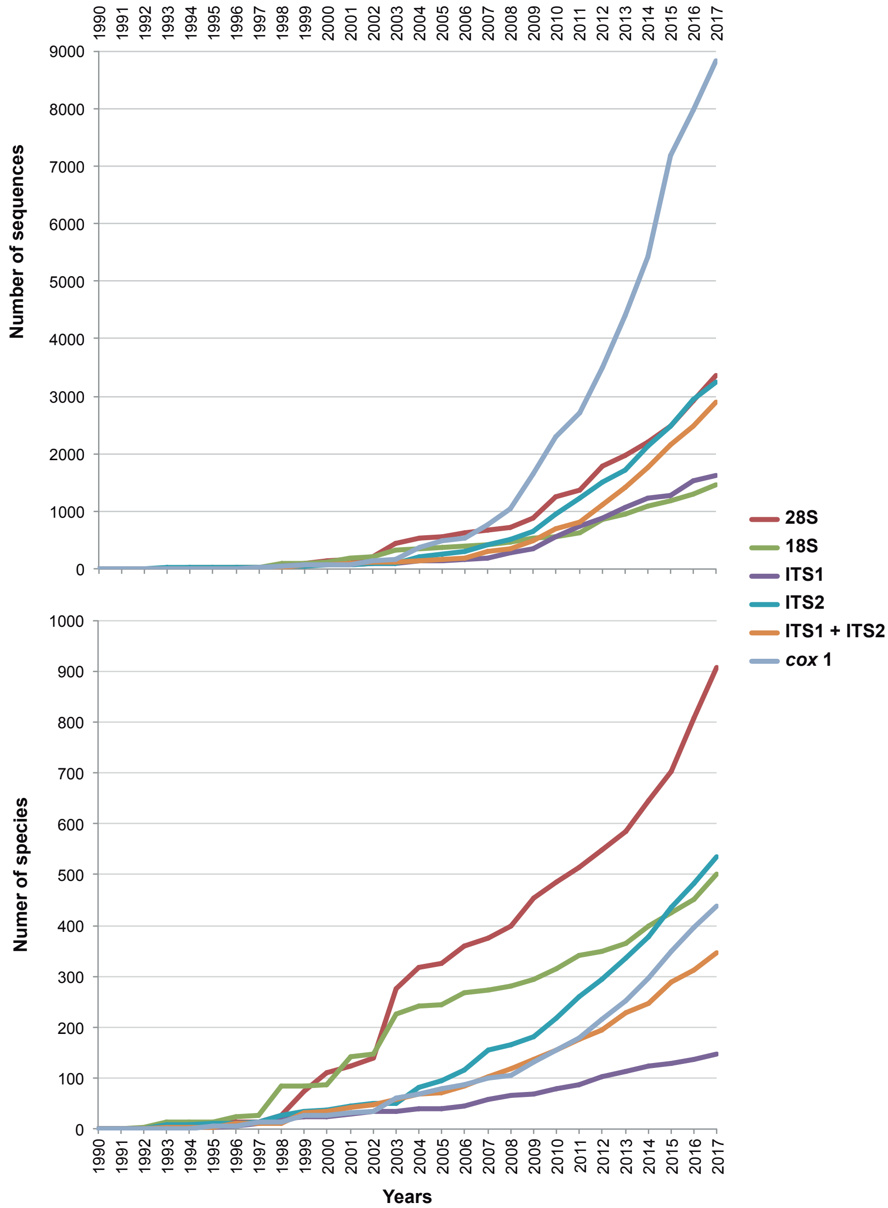
Hypocreadium sp. 1	*Balistes polypepis* (F)	Chamela, Jalisco
Hypocreadium sp. 2	*Balistes vetula* (F)	Puerto Morelos, Quintana Roo
Lepotrema sp.	*Balistes vetula* (F)	Puerto Morelos, Quintana Roo
Preptetos sp.	*Umbrina xanti* (F)	Chamela, Jalisco
Lecithasteridae		
Lecithasteridae gen sp	*Haemulon flaviguttatum* (F)	Chamela, Jalisco
Microscaphidiidae		
Octangioides ujati	*Vieja synspila* (F)	San Antonio del Río, Campeche
Monorchiidae		
Monorchiidae gen sp 1	*Haemulon flavolineatum* (F)	Puerto Morelos, Quintana Roo
Monorchiidae gen sp 2	*Haemulon flavolineatum* (F)	Puerto Morelos, Quintana Roo
Opecoelidae		
Cainocreadium sp. 1	Coconaco (F)	Chamela, Jalisco
Cainocreadium sp. 2	*Haemulon flavolineatum* (F)	Puerto Morelos, Quintana Roo
Helicometra sp. 1	*Haemulon flaviguttatum* (F)	Chamela, Jalisco
Helicometra sp. 2		
Opecoeloides sp. 1		Chamela, Jalisco
Opecoeloides sp. 2	*Umbrina xanti* (F)	Chamela, Jalisco
Opecoelidae gen. Sp. 1		Puerto Morelos, Quintana Roo
Opegaster sp.	*Tomicodon* sp. (F)	Barra de Cuatunalco, Oaxaca
Plagiorchiidae		
Choledocystus sp.	*Rhinella horribilis* (A)	Presa Temascal, Oaxaca
Renicolidae		
Renicola thapari	*Sula nebouxii* (B)	Isla Isabel, Nayarit
Telorchiidae		
Telorchis corti	*Thamnophis eques* (R)	Jarácuaro, Michoacán

A= amphibian, R= reptilian, B=bird, M=mammal, F=fish

Figure 1

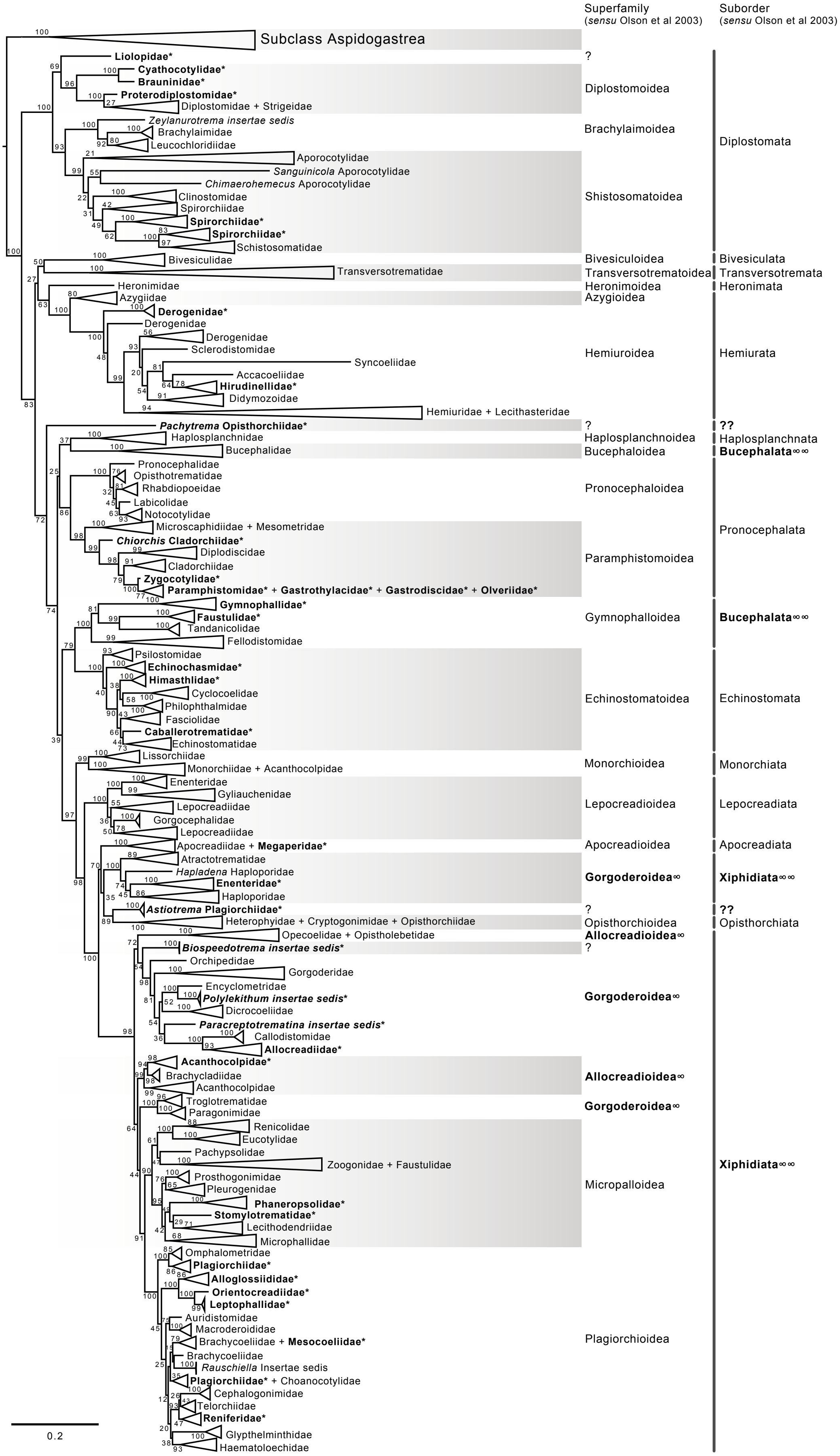
A)

B)



Number of sequences
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
1990 1991 1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017
28S
18S
ITS1
ITS2
ITS1 + ITS2
cox 1
Numer of species
1000
900
800
700
600
500
400
300
200
100
0
Years

Figure 2


Superfamily
(sensu Olson et al 2003)
Suborder
(sensu Olson et al 2003)
Subclass Aspidogastrea
Liolopidae*
Cyathocotylidae*
Brauninidae*
Proterodiplostomidae*
Diplostomidae + Strigeidae
Zeylanurotrema insertae sedis
Brachylaimidae
Leucochloridiidae
Aporocotylidae
Sanguinicola Aporocotylidae
Chimaerohemecus Aporocotylidae
Clinostomidae
Spirorchiidae
Spirorchiidae*
Spirorchiidae*
Schistosomatidae
Bivesiculidae
Transversotrematidae
Heronimidae
Azygiidae
Derogenidae*
Derogenidae
Derogenidae
Sclerodistomidae
Syncoeliidae
Accacoeliidae
Hirudinellidae*
Didymozoidae
Hemiuridae + Lecithasteridae
Pachytrema Opisthorchiidae*
Haplosplanchnidae
Bucephalidae
Pronocephalidae
Opisthotrematidae
Rhabdiopoeidae
Labicolidae
Notocotylidae
Microscaphidiidae + Mesometridae
Chiorchis Cladorchiidae*
Diplodiscidae
Cladorchiidae
Zygocotylidae*
Paramphistomidae* + Gastrothylacidae* + Gastrodiscidae* + Olveriidae*
Gymnophallidae*
Faustulidae*
Tandanicolidae
Fellodistomidae
Psilostomidae
Echinochasmidae*
Himasthlidae*
Cyclocoelidae
Philophthalmidae
Fasciolidae
Caballerotrematidae*
Echinostomatidae
Lissorchiidae
Monorchiidae + Acanthocolpidae
Enenteridae
Gyliauchenidae
Lepocreadiidae
Gorgocephalidae
Lepocreadiidae
Apocreadiidae + Megaperidae*
Atractotrematidae
Hapladena Haploporidae
Enenteridae*
Haploporidae
Astiotrema Plagiorchiidae*
Heterophyidae + Cryptogonimidae + Opisthorchiidae
Opecoelidae + Opistholebetidae
Biospeedotrema insertae sedis*
Orchipedidae
Gorgoderidae
Encyclometridae
Polylekithum insertae sedis*
Dicrocoeliidae
Paracreptotrematina insertae sedis*
Callodistomidae
Allocreadiidae*
Acanthocolpidae*
Brachycladiidae
Acanthocolpidae
Troglotrematidae
Paragonimidae
Renicolidae
Eucotylidae
Pachypsolidae
Zoogonidae + Faustulidae
Prosthogonimidae
Pleurogenidae
Phaneropsolidae*
Stomylotrematidae*
Lecithodendriidae
Microphallidae
Omphalometridae
Plagiorchiidae*
Alloglossiididae*
Orientocreadiidae*
Leptophallidae*
Auridistomidae
Macroderoididae
Brachycoeliidae + Mesocoeliidae*
Brachycoeliidae
Rauschiella Insertae sedis
Plagiorchiidae* + Choanocotylidae
Cephalogonimidae
Telorchiidae
Reniferidae*
Glypthelminthidae
Haematoloechidae
0.2
?
Diplostomoidea
Brachylaimoidea
Shistosomatoidea
Bivesiculoidea
Transversotrematoidea
Heronimoidea
Azygioidea
Hemiuroidea
?
Haplosplanchnoidea
Bucephaloidea
Pronocephaloidea
Paramphistomoidea
Gymnophalloidea
Echinostomatoidea
Monorchioidea
Lepocreadioidea
Apocreadioidea
Gorgoderoidea∞
?
Opisthorchioidea
Allocreadioidea∞
?
Gorgoderoidea∞
Allocreadioidea∞
Gorgoderoidea∞
Micropalloidea
Plagiorchioidea
Diplostomata
Bivesiculata
Transversotremata
Heronimata
Hemiurata
??
Haplosplanchnata
Bucephalata∞∞
Pronocephalata
Bucephalata∞∞
Echinostomata
Monorchiata
Lepocreadiata
Apocreadiata
Xiphidiata∞∞
??
Opisthorchiata
Xiphidiata∞∞


0.2

7. DISCUSIÓN GENERAL

7. DISCUSIÓN GENERAL

Considerando la diversidad de especies de digéneos que existen como parásitos de vertebrados, la cual se estima en alrededor de 18,000 especies (Cribb, 2001; Kostadinova y Pérez del Olmo, 2014; Littlewood et al., 2015), se cuenta con un número relativamente pequeño de especies secuenciadas publicadas en bases de datos internacionales, como el GenBank o Bold. Hasta el año 2017, para el marcador ribosomal 28S encontramos secuencias de aproximadamente 1,000 especies, lo que representa el 6% de la diversidad conocida hasta la fecha. Esta es probablemente la razón de que varios grupos de digéneos tengan una posición filogenética incierta, es decir, el muestreo de taxones está muy lejos de ser completado. Por ello, durante este proyecto de tesis se identificaron especies de trematodos que aún no tuviesen secuencias disponibles en el GenBank, y una vez colectados e identificados morfológicamente, se secuenciaron con el propósito de incrementar la librería genética y a su vez, ubicar su posición filogenética, y en algunos casos particulares, evaluar si la clasificación tradicional de dichas especies, basada en los caracteres morfológicos, era la correcta o si era necesario proponer re-arreglos taxonómicos.

En este trabajo se obtuvieron secuencias de ADN de diferentes marcadores nucleares y al menos de uno mitocondrial para proponer hipótesis filogenéticas a distintas escalas taxonómicas y evaluar la señal filogenética de cada uno de éstos. Se exploró la utilidad de los marcadores seleccionados dependiendo de la pregunta realizada para cada capítulo; la selección de cada marcador se vio influenciada por varios factores: el primero fue la disponibilidad de secuencias de cada marcador; en algunos grupos, por ejemplo en Macroderoiididae, solamente se contaban con secuencias de un solo marcador, por lo que para el análisis en este estudio se secuenció solo aquel que nos permitiera realizar las comparaciones y los análisis filogenéticos. El segundo factor se relacionó con la señal filogenética de los genes seleccionados y si éste o éstos eran útiles para la escala taxonómica de la pregunta que se quería contestar (Blasco-Costa et al., 2016); por ejemplo, para la descripción de especies nuevas se consideró que no era necesario secuenciar genes altamente conservados, como el 18S que básicamente no permitían discernir entre especies hermanas. Este último factor se relaciona con la tasa de sustitución de cada marcador.

El gen mitocondrial *cox* 1 tiene una tasa de sustitución relativamente alta, misma que permite observar variación inter e intraespecífica (Vilas et al., 2005). Esa misma variabilidad ha determinado que el gen sea utilizado en análisis filogenéticos que van desde el nivel de especies cercanamente emparentadas, hasta géneros de una misma familia, ya que las hipótesis filogenéticas obtenidas con este marcador han sido consistentes con respecto a las obtenidas por marcadores más conservados, como el 28S (Hernández-Mena et al., 2017). Los marcadores mitocondriales por lo general no han sido muy utilizados en filogenias moleculares de taxa supraespecíficos, porque de acuerdo con Hillis y Dixon (1991), las secuencias con tasa de sustitución más rápida no generan hipótesis robustas a esos niveles porque pueden tener altos niveles de homoplasia, que se incrementa si la probabilidad de cambio de las bases en cada posición también se incrementa, generando alineamientos poco consistentes. La homoplasia en caracteres moleculares no es fácilmente detectable por el problema de saturación de los estados de carácter en secuencias de ADN. Sin embargo, en trematodos pocas veces se ha explorado el alineamiento de las secuencias de ADN de este tipo de marcadores utilizando como referencia sus secuencias de aminoácidos. En el trabajo referido como capítulo III, precisamente se puso a prueba la eficiencia del fragmento de *cox* 1, usado recurrentemente en la iniciativa del Código de Barras de ADN, para establecer la posición filogenética de la familia Proterodiplostomidae; dicho estudio reveló que el marcador mitocondrial es útil aún para establecer las relaciones filogenéticas entre representantes de familias diferentes de digéneos, ya que dicho gen, al usar el alineamiento producto de su traducción a aminoácidos, generó una hipótesis filogenética congruente con aquella obtenida partir de los genes 18S y 28S, en términos de las relaciones entre las familias Proterodiplostomidae, Diplostomidae y Strigeidae. Por otra parte, resultó evidente que mientras más alejados filogenéticamente estaban los taxones, la resolución de la *cox* 1 resultaba no ser congruente con las otras hipótesis; nuestros resultados indican que no debe descartarse a priori su incorporación en estudios tendientes a determinar la posición de taxones entre familias cercanamente relacionadas.

Contrario a lo que ocurre con los marcadores mitocondriales, los marcadores nucleares han sido utilizados con mayor frecuencia para diferenciar no solo entre especies, si no también taxa supraespecíficos. Otros marcadores, como los espaciadores transcritos internos (ITS), también han sido utilizados para generar hipótesis de relaciones filogenéticas y más particularmente para establecer límites entre especies;

estos han sido analizado tanto de manera independiente (ITS1 ó ITS2) como en conjunto (ITS1+ITS2 incluyendo al 5.8S) (Nolan y Cribb, 2005). En general, se cuenta con un número mayor de especies secuenciadas para el ITS2, seguido por la combinación de ITS1+ITS2. Algunos estudios que han utilizado en particular el ITS1 en trematodos, mostraron que es más variable cerca de su extremo 5´, en donde puede presentar varios elementos repetidos en tandem, y que es más conservado cerca del 5.8S, por lo que la variabilidad de las tasas a lo largo de su longitud le ha conferido la posibilidad de utilizarse a distintos niveles taxonómicos (Nolan y Cribb, 2005), es decir, que la región variable le permitía diferenciar especies mientras que la región conservada podría ser útil para indicar sobre su posición filogenética (Schulenburg et al., 1999). Sin embargo, también se ha señalando que existen problemas para alinear adecuadamente las secuencias de ITS1 entre especies pertenecientes a familias diferentes, debido a la variabilidad de su extremo 5´ (Vilas et al., 2005). Por su parte, el 5.8S es una región muy corta y altamente conservada, y su uso para reconstrucciones filogenéticas ha sido descartado, ya que su efectividad para generar filogenias robustas es puesta en duda debido a su longitud y a su lenta tasa de sustitución (Hillis y Dixon, 1991; Hershkovitz y Lewis,1996; Coleman, 2003). Por su parte, el ITS2 generalmente es un buen marcador que puede ayudar a diferenciar especies ya que no es tan variable a niveles intraespecíficos (Nolan y Cribb, 2005; Vilas et al., 2005), no tiene regiones repetidas como el ITS1 y su longitud puede ser altamente variable dentro y entre familias, por lo que su uso ha sido limitado en la exploración de las relaciones entre de familias de digéneos.

Varios han sido los trabajos que han argumentado sobre la variabilidad de cada uno de los ITS. Por un lado algunos estudios consideran que el ITS1 es más variable que el ITS2 (Luton, et al., 1992; Morgan y Blair, 1995; van Herwerden et al. 1999; Tkach et al., 2000b; Galazzo et al., 2002; Nolan y Cribb 2005), y por el otro, Vilas et al. (2005) reportaron que no existen diferencias importantes entre las tasas de sustitución de ambas regiones. Con base en todo lo anterior y en virtud de que los alineamientos de secuencias de ITS para los análisis filogenéticos a niveles supraespecíficos han sido problemáticos, principalmente el alineamiento de ITS1, se ha preferido no utilizarlos en comparaciones entre familias o taxa superiores (Coleman, 2003); alternativamente, se ha sugerido que recurrir a la estructura secundaria de estas regiones puede ayudar a su correcto alineamiento (Michot et al., 1993; Morgan y Blair, 1995; Coleman, 2003), aunque esto ha sido relativamente poco explorado recientemente.

Por su parte, el gen 18S es el marcador ribosomal que tiene la tasa de cambio más lenta (Hillis y Davis, 1986), y por ello se ha utilizado casi exclusivamente para resolver relaciones filogenéticas a niveles altos de la clasificación, ya que se ha visto que es poco informativo para discernir adecuadamente entre especies hermanas y para proponer hipótesis filogenéticas robustas a ese nivel. Se ha observado que las regiones que están comprometidas con alguna función vital dentro del organismo (por ejemplo la traducción del ARN mensajero) acumulan menos mutaciones para preservar la supervivencia de los seres vivos (McManus y Bowles, 1996; Gasser, 2001). Aún cuando para este marcador se cuenta con alrededor de 460 especies secuenciadas, por su baja tasa de sustitución, es probablemente el marcador con el menor número de secuencias publicadas (individuos), ya que se ha preferido secuenciar un limitado número de ejemplares por cada especie de digéneo.

Este estudio reveló claramente que el gen 28S es el que ha sido secuenciado para un mayor número de especies de trematodos, además de ser uno de los que cuenta con más individuos secuenciados. Por ello, se propone como el marcador más óptimo para realizar análisis moleculares en el grupo de los trematodos, desde la diferenciación entre especies, hasta la realización de inferencias filogenéticas entre géneros, familias y superfamilias. Únicamente a nivel intraespecífico, este gen ribosomal cuenta con menor resolución que los genes mitocondriales, como el *cox* 1, por lo cual, no se recomienda su uso en estudios de variación genética poblacional o de filogeografía en digéneos. El gen 28S cuenta con regiones conservadas que tienen tasas de sustitución lentas y con regiones intermedias que tienen tasas de cambió más rápidas (Hillis y Dixon, 1991), por lo que, la heterogeneidad de las tasas de las regiones que conforman el 28S ha favorecido su popularidad para ser seleccionado como el marcador por excelencia en digéneos. Un gran número de publicaciones han utilizado a este gen indistintamente para contestar preguntas desde el nivel de especie hasta el nivel que incluye taxa supraespecíficos de la jerarquía taxonómica. Los diferentes estudios que integran el presente trabajo son un reflejo de esta tendencia. Desde las descripciones de especies presentadas en el capítulo I, hasta la inferencia filogenética de la subclase Digenea presentada en el capítulo IV, hicieron uso de este marcador ribosomal y, en todos los casos resultó ser de gran utilidad para abordar las preguntas que se habían generado. Las principales razones por las que el

28S se constituye como el marcador de gran utilidad en digéneos se pueden resumir en que 1) se compone de regiones que tienen distintas tasas de sustitución; 2) es generalmente muy efectivo para delimitar y reconocer especies, así como también para resolver hipótesis de relaciones filogenéticas en diferentes niveles taxonómicos; 3) es relativamente sencillo de alinear por las regiones altamente conservadas que presenta; y 4) es el marcador que tiene el mayor número de especies secuenciadas, con mayor representatividad de géneros y de familias. Este último aspecto es muy importante pues los trabajos de investigación referentes a la inferencia de tasas de diversificación, edades de divergencia o bien aquellos dirigidos a reconstruir estados de carácter ancestrales, requieren una mayor representatividad, un muestreo taxonómico mas amplio, para lograr conclusiones robustas. Como ejemplo puede referirse el trabajo de Littlewood et al. (2015), en el cual, utilizando una hipótesis filogenética obtenida a partir de secuencias ADN de 18Sy 28S publicadas en GenBank tratan de describir en términos generales los patrones de diversidad en los digéneos, y señalan como dicha diversidad esta distribuida de manera muy dispar entre los dos ordenes que conforman la subclase Digenea, ya que Diplostomida contiene el 12.3% de las especies, mientras que Plagiorchiida tiene el 87.7% de especies. Estos autores señalan como dentro de las distintas superfamilias se pueden encontrar grupos de especies que son parasitas exclusivamente de peces o que son parasitas de tetrápodos, por lo cual, el tener una mayor representatividad de especies de digéneos en las filogenias moleculares puede ayudar a comprender como han sido las transiciones entre los diferentes grupos de huéspedes..

A pesar del reconocimiento de la importancia del uso de marcadores moleculares en el estudio de la diversidad, sistemática y evolución de los digéneos, aún no existe consenso sobre la utilización de determinados marcadores para responder preguntas específicas a distintos niveles taxonómicos (Blasco-Costa et al., 2016), ya que por ejemplo, para la delimitación de especies se han publicado trabajos en donde se ha utilizado *cox* 1, ITS ó 28S de manera independiente, ó bien, se han combinado los tres marcadores ó dos de ellos. De acuerdo con Blasco-Costa et al. (2016), la mejor práctica para el buen desempeño del trabajo taxonómico en digéneos debe incluir la obtención de al menos dos genes que evolucionen de manera independiente, como un nuclear y un mitocondrial; asimismo, estos autores refirieron la necesidad de generar secuencias, de tantas combinaciones de especie de parásito/especies de huéspedes o bien localidades geográficas, como fuera posible. Desafortunadamente, la heterogeneidad en el uso de marcadores en este grupo de parásitos ha ocasionado que no se puedan añadir todas las especies secuenciadas que potencialmente son útiles, porque no se tienen disponibles el mismo número de marcadores o las mismas regiones homólogas para cada una de las especies disponibles en GenBank, para hacer estudios multigenes. Por ejemplo, en el capítulo I donde se presentan descripciones de especies, en uno de ellos solo fue posible utilizar secuencias del gen 28S puesto que para las especies congénericas con las cuales se estaba estableciendo la diferenciación, solo se contaba con datos para ese marcador; en el segundo trabajo donde se describen dos especies, si se lograron utilizar dos marcadores ribosomales en los análisis filogenéticos para establecer la monofilia y el nivel de divergencia genética. En ninguno de los dos casos fue posible utilizar secuencias de genes mitocondriales que hubiesen resultado ideales, dado que no se cuenta aún con secuencias de ese marcador para otras especies congenéricas. La homogeneización de los marcadores moleculares que son secuenciados permitiría que los estudios comparativos puedan ser más completos al contar con una mayor disponibilidad de taxa que se requieren para formular hipótesis de relaciones filogenéticas más robustas.

Finalmente, el uso de un mayor número de loci se vuelve cada vez más importante para analizar a las especies desde una perspectiva evolutiva; por ejemplo, la utilización de un solo locus para los análisis filogenéticos, sobre todo a nivel interespecífico, puede resultar en interpretaciones erróneas sobre las hipótesis filogenéticas obtenidas, dado que pueden existir polimorfismos dentro de la especie (Templeton, 2001; Domínguez y Vázquez 2009), ó también puede haber problemas de homoplasia en loci con una alta tasa de sustitución (Hillis y Davis, 1991). Esto nos indica que la adición de más marcadores moleculares podría ayudar a mejorar la evaluación de cada uno de ellos de manera recíproca con respecto a las reconstrucciones filogenéticas de los parásitos que se generan a distintos niveles taxonómicos. Al menos dos marcadores deberían ser utilizados tal y como lo propusieron Blasco-Costa et al. (2016).

8. CONCLUSIONES GENERALES

8. CONCLUSIONES GENERALES

1. Se describen tres nuevas especies de digéneos parásitos de peces de agua dulce pertenecientes a la familia Allocreadiidae (Orden Plagiorchiida), de las cuales dos parasitan a peces Characiformes en México y Guatemala, y una mas parasita a una especie del orden Gymnotiformes en Costa Rica. Estas nuevas especies son *Auriculostoma lobata*, *A. tica* y *Wallinia cryptica.*
2. Las nuevas especies se describieron utilizando una aproximación de taxonomía integrativa en donde se utilizaron caracteres morfológicos (incluyendo microscopía electrónica de barrido) en combinación con caracteres moleculares y con datos sobre la asociación hospedadora y la distribución geográfica.
3. Se describe una nueva familia de digéneos, Alloglossidiidae, dentro de la superfamilia Plagiorchioidea. Esta nueva familia incluye hasta el momento a dos géneros, *Alloglossidium* y *Magnivitellinum*, los cuales son parásitos de peces dulceacuícolas en el Continente Americano. Esta descripción se basa en el resultado de análisis filogenéticos empleando el gen ribosomal 28S y una revisión de la morfología de los géneros representativos.
4. Por poseer regiones tanto conservadas como variables, el gen ribosomal del ADN nuclear 28S es un marcador molecular idóneo para realizar la diferenciación entre especies de digéneos, así como también para analizar la posición filogenética de taxa supraespecíficos.
5. El gen mitocondrial *cox* 1 es altamente variable por lo que es muy útil para discernir entre especies hermanas y para desarrollar estudios a nivel poblacional, lo que incluye el reconocimiento de especies crípticas, sin embargo, también resulta útil, en algunas instancias, para determinar las interrelaciones entre géneros pertenecientes a una misma familia o bien, entre géneros de familias cercanamente relacionadas.
6. La familia Proterodiplostomidae, que incluye especies que parasitan exclusivamente a reptiles, representa el grupo hermano de las especies que se incluyen en las familias Diplostomidae y Strigeidae que como adultos parasitan principalmente a aves ictiófagas.
7. La hipótesis que obtuvimos sobre las relaciones filogenéticas de los taxa mayores dentro de la subclase Digenea usando secuencias del 28S y añadiendo el mayor número posible de las especies secuenciadas hasta el 2017, en general

fue consistente con las hipótesis propuestas por Olson et al. (2003) y Littlewood et al. (2015), quienes consideraron menos especies y familias.

8. Las relaciones filogenéticas que obtuvimos en nuestro análisis demostraron que las superfamilias Allocreadioidea y Gorgoderoidea, así como también los ordenes Bucephalata y Xiphidiata (sensu Olson et al (2003) son grupos parafileticos.
9. Nuestra hipótesis sobre las relaciones filogenéticas entre las familias consideradas en el presente estudio, confirmó la consistencia de las 24 superfamilias que ya habían sido reconocidas por Littlewood et al. (2015).
10. Los arreglos filogenéticos obtenidos en el nuevo análisis mostraron que se necesitan realizar estudios filogenéticos y morfológicos mas detallados en algunas familias que resultaron ser parafileticas y en aquellos géneros considerados como *incertae sedis* que no se agruparon dentro de alguna familia en particular, ya que estos potencialmente pueden representar nuevas familias que aun no se han descrito. Se recobraron como parafiléticas 33 familias y los géneros que no se agruparon dentro de alguna familia son: *Zeylanurotrema*, *Biospeedotrema*, *Polylekithum*, *Rauschiella*, *Pachytrema*, *Chiorchis*, *Hapladena*, *Astiotrema* y *Paracreptotrematina.*

9. BIBLIOGRAFÍA

9. BIBLIOGRAFÍA

Adlard, R.D., S.C. Barker, D. Blair y T. H. Cribb. 1993. Comparison of the second internal transcribed spacer (ribosomal DNA) from populations and species of Fasciolidae (Digenea). *International Journal for Parasitology*, 23: 423–425.

Bartoli, P. y O. Jousson. 2003. The life-cycle of Podocotyle scorpaenae (Opecoelidae: Digenea) demonstrated by comparative analysis of ribosomal DNA sequences. In: Taxonomie, ecologie et evolution des metazoaires parasites. Taxonomy, ecology and evolution of metazoan parasites (Livre hommage a` Louis Euzet) (C. Combes and J. Jourdane, eds), pp. 17–28. Perpignan: Presses Universitaires de Perpignan.

Baversrock, P. R., R. Fielke, A. M. Johnson, R. A. Bray y I. Beveridge. 1991. Conflicting phylogenetic hypotheses for the parasitic platyhelminths tested by partial sequencing of 18S ribosomal RNA. *International Journal for Parasitology*, 21:329–339.

Bell, A., C. Sommerville y E. Valtonen. 2001. A molecular phylogeny of the genus *Ichthyocothylurus* (Digenea, Strigeidae). *International Journal for Parasitology*, 31: 833–842.

Blair, D. 1993. The phylogenetic position of the Aspidobothrea within the parasitic flatworms inferred from ribosomal RNA sequence data. *International Journal for Parasitology*, 23:169–178.

Blair, D. y S. C. Barker. 1993. Affinities of the Gyliauchcnidae: utility of the 18S rDNA gene for phylogenetic inference in the Digenea (Platyhelminthes). *International Journal for Parasitology*, 23:527–532.

Blasco-Costa, I., S. C. Cutmore, T. L. Miller y M. J. Nolan. 2016. Molecular approaches to trematode systematics: 'best practice' and implications for future study. *Systematic Parasitology*, 93:295–306.

Bray, R. A., D. Gibson y A. Jones. 2008. *Keys to the Trematoda* Vol 3. CAB International and The Natural History Museum, London, pp. 824.

Brooks, D. R. y E. P. Hoberg. 2000. Triage for the Biosphere: The need and rationale for taxonomic inventories and phylogenetic studies of parasites. *Comparative Parasitology*, 67: 1–25.

Brooks, D.R., O'Grady, R.T. y Glen, D.R. 1985. Phylogenetic analysis of the Digenea (Platyhelminthes: Cercomeria) with comments on their adaptive radiation. *Canadian Journal of Zoology*, 63, 411–443.

Brusentsov, I. I., A. V. Katokhin, I. V. Brusentsova, S. V. Shekhovtsov, S. N. Borovikov, G. G. Goncharenko, L. A. Lider, B. V. Romashov, O. T. Rusinek, S. K. Shibitov, M. M. Suleymanov, A. V. Yevtushenko y V. Mordvinov. 2013. Low genetic diversity in wide-spread Eurasian liver fluke *Opisthorchis felineus* suggests special demographic history of this trematode species. *PLoS One*, 8: e62453.

Campos, A., M. P. Cummings, J. L. Reyes y J. P. Laclette. 1998. Phylogenetic relationships of Platyhelminthes based on 18S ribosomal gene sequences. *Molecular Phylogenetics and Evolution*, 10:1–10.

Cribb, T.H., R. A. Bray, D. T. J. Littlewood, S.P. Pichelin y E. A. Herniou. 2001. The Digenea. In: Littlewood, D.T.J., Bray, R.A. (Eds.) *Interrelationships of the Platyhelminthes.*, Taylor and Francis, London, pp. 168–185.

Cribb, T.H., R.A. Bray, P.D. Olson, y D.T.J. Littlewood. 2003. Life cycle evolution in the Digenea: a new perspective from phylogeny. *Advances in Parasitology*, 54: 197–254.

Criscione, C. D. y M. S. Blouin. 2004. Life cycles shape parasite evolution: comparative population genetics of salmon trematodes. *Evolution* 58, 198–202.

Coleman, A.W. 2003. ITS2 is a double-edged tool for eukaryote evolutionary comparisons. *Trends in Genetics*, 19: 370–375.

Ehlers U. 1985. Phylogenetic relationships within the Plathelminthes. In: S. C. Morris, J. D. George, R. Gibson, H. M. Platt, (Eds.). *The Origins and Relationships of Lower Invertebrates*. Clarendon Press, Oxford, pp. 143–158.

Ehlers, U. 1986. Comments on a phylogenetic system of the Platyhelminthes. *Hydrobiologia*, 132: 1–12.

Galazzo, D.E., S. Dayanandan, D.J. Marcogliese, y J.D. McLaughlin. 2002. Molecular systematics of some North American species of Diplostomum (Digenea) based on rDNA-sequence data and comparisons with European congeners. *Canadian Journal of Zoology*, 80: 2207–2217.

García-Varela, M., A.L. Sereno-Uribe, C.D. Pinacho-Pinacho, O. Domínguez-Domínguez y G. Pérez- Ponce de León. 2015. Molecular and morphological characterization of *Austrodiplostomum* ostrowskiae Dronen, (Digenea: Diplostomatidae), a parasite of cormorants in the Americas. *Journal of Helminthology*, 4: 1–12.

Gasser, R.B. 2001. Identification of parasitic nematodes and study of genetic variability using PCR approaches. In: *Parasitic nematodes: Molecular biology, biochemistry and immunology* (M. Kennedy and W. Harnett, eds). Oxon: CABI Press.

Gibson, D. I. 1987. Questions in digenean systematics and evolution. *Parasitology*, 95: 429–460.

Gibson, D.I., A. Jones y R. A. Bray. 2002. *Keys to the Trematoda*, vol. 1. CABI Publishing and The Natural History Museum, London, pp.

Goulding, T. y Cohen, S. 2014. Phylogeography of a marine acanthocephalan: lack of cryptic diversity in a cosmopolitan parasite of mole crabs. *Journal of Biogeography*, 41: 965–976.

Hernández-Mena, D. I., L. García-Prieto y M. García-Varela. 2014. Morphological and molecular differentiation of *Parastrigea* (Trematoda: Strigeidae) from Mexico, with the description of a new species. *Parasitology International*, 63: 315–323.

Hernández-Mena, D. I, M. García-Varela y Pérez-Ponce de León. 2017. Filling the gaps within the classification of the Digenea Carus, 1963: systematic position of the Proterodiplostomatidae Dubois, 1936 within the superfamily Diplostomoidea Poirier, 1886, inferred from nuclear and mitochondrial DNA sequences. *Systematic Parasitology*, 94: 833–848.

Hershkovitz, M.A. y L.A. Lewis. 1996. Deep-level diagnostic value of the rDNA-ITS region. *Molecular Biology and Evolution*, 13: 1276–1295.

Hillis, D.M. y S.K. Davis. 1986. Evolution of ribosomal DNA: fifty million years of recorded history in the frog genus Rana . *Evolution*, 40: 1275–1288.

Hillis, D.M. y M.T. Dixon. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Quarterly Review of Biology*, 66: 411–453.

Jones, A., R. A. Bray y D. Gibson. 2005. *Keys to the Trematoda* Vol 2. CAB

International and The Natural History Museum, London, 745 pp.

Jousson, O., P. Bartoli y J. Pawlowski. 1999. Molecular identification of developmental stages in Opecoelidae (Digenea). *International Journal for Parasitology,* 29: 1853–1858.

Jousson, O., P. Bartoli, L. Zaninetti y J. Pawlowski. 1998. Use of the ITS rDNA for elucidation of some life-cycles of Mesometridae (Trematoda, Digenea). *International Journal for Parasitology*, 28:1403–1411.

Kaukas, A. y D. Rollinson. 1997. Interspecific variation within the ‘hypervariable’ region of the 18S ribosomal RNA gene among species of *Schistosoma* Weinland, 1858 (Digenea). *Systematic Parasitology,* 36: 157–160.

Kostadinova, A. y A. Pérez-del Olmo. 2014. The systematics of the trematoda. *Advances in Experimental Medicine and Biology*, 766:21-44.

Králová-Hromadová, I., E. Bazsalovicsová, J. Štefka, M. Špakulová, S. Vávrová, T. Szemes, V. Tkach, A. Trudgett y M. Pybus. 2011. Multiple origins of European populations of the giant liver fluke *Fascioloides magna* (Trematoda: Fasciolidae), a liver parasite of ruminants. *International Journal for Parasitology* 41: 373–383.

La Rue, G.R., 1957. The classification of digenetic Trematoda: a review and a new system. *Experimental Parasitology*, 6: 306–344.

Leén-Régagnon, V. y C.E.L. Paredes. 2002. *Haematoloechus danbrooksi* n. sp. (Digenea: Plagiorchioidea) from *Rana vaillanti* from Los Tuxtlas, Veracruz, Mexico. *Journal of Parasitology,* 88: 1215–1221.

León-Régagnon, V. y D. R. Brooks. 2003. Molecular phylogeny of Haematoloechus Looss, 1899 (Digenea: Plagiorchiidae), whit emphasis on North American species. T*he Journal of Parasitology*, 89: 1206–1211.

León-Règagnon, V., D. R. Brooks y G. Pérez-Ponce de León. 1999. Differentiation of Mexican species of Haematoloechus Loss, 1899 (Digenea: Plagiorchiformes): Molecular and morphological evidence. *Journal of Parasitology*, 85:935–946.

Littlewood, D. T. J. y P. D. Olson. 2001. Small subunit rDNA and the Platyhelminthes: signal, noise, conflict and compromise. ln Littlewood, D. T. J. y R. A. Bray (Eds.). *Interrelationships of the Platyhelminthes*. Taylor & Francis, pp. 262- 278

Littlewood, D.T.J., K. Rohde, R. A. Bray y E. Herniou. 1999a. Phylogeny of the

Platyhelminthes and the evolution of parasitism. *Biological Journal of the Linnean Society*, 68: 257–287.

Littlewood, D.T.J., K. Rohde y K. A. Clough, K.A. 1999b. The interrelationships of all major groups of Platyhelminthes: phylogenetic evidence from morphology and molecules. *Biological Journal of the Linnean Society*, 66: 75–114.

Littlewood, D. T. J., M. J. Telford y R. A. Bray. 2004. Protostomes and Platyhelminthes. In Cracraft, J. y M. J. Donoghue (Eds.). *Assembling the Tree of Life*. Oxford University Press, pp. 209–223.

Littlewood, D. T. J., R. A. Bray y A. Waeschenbach. 2015. Phylogenetic patterns of diversity in cestodes and trematodes. In: Morand, S., B. Krasnov y D. T. J. Littlewood (Eds.). *Parasite Diversity and Diversification: Evolutionary Ecology meets Phylogenetics*. Cambridge University Press, pp. 304–319.

Litvaitis, M. K. y K. Rohde. 1999. A molecular test of platyhelminth phylogeny: inferences from partial 28s rDNA sequences. *Invertebrate Biology*, 118:42–56.

Liu, G. H.,B. Li, J. Y. Li, H. Q. Song, R. Q. Lin, X. Q. Cai, F. C. Zou, H. K. Yan, Z. G. Yuan, D. H. Zhou y X. Q. Zhu. 2012. Genetic variation among *Clonorchis sinensis* isolates from different geographic regions in China revealed by sequence analyses of four mitochondrial genes. *Journal of Helminthology*, 86: 479–484.

Lockyer, A.E., P.D. Olson y D. T. J. Littlewood. 2003. Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. *Biological Journal of the Linnean Society*, 78: 155–171.

Luton, K., D. Walker y D. Blair, 1992. Comparisons of ribosomal internal transcribed spacers from two congeneric species of flukes (Platyhelminthes: Trematoda: Digenea). *Molecular and Biochemical Parasitology*, 56: 323–328.

McManus, D. P. y J. Bowles. 1996. Molecular genetics approaches to parasite identification: their value in diagnostic parasitology and systematics. *International Journal for Parasitology*, 26: 687–704.

Michot, B., L. Despres, F. Bonhomme y J.P. Bachellerie. 1993. Conserved secondary structures in the ITS2 of trematode pre-rRNA. *Federation of European Biochemical Societies Letters*, 316: 247– 252.

Morgan, J.A.T. y D. Blair. 1995. Nuclear rDNA ITS sequence variation in the trematode genus *Echinostoma* : an aid to establishing relationships within the 37-collar-spine group. *Parasitology*, 111: 609–615.

Nadler, S. A. y G. Pérez-Ponce de León. 2011. Integrating molecular and morphological approaches for characterizing parasite cryptic species: Implications for parasitology. *Parasitology*, 138: 1688–1709.

Nolan, M. J. y T. H. Cribb. 2005. The use and implications of ribosomal DNA sequencing for the discrimination of digenean species. *Advances in Parasitology* 60:101–163.

Olson P. D. y V. V. Tkach. 2005. Invited Review: Advances and trends in the molecular systematics of the parasitic Platyhelminthes. *Advances in Parasitology*, 60: 165–243.

Olson P. D., T. H. Cribb, V. V. Tkach, R. A. Bray y D. T. J. Littlewood. 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal for Parasitology*, 33: 733–755.

Overstreet, R.M., S.S. Curran, L.M. Pote, D.T. King, C.K. Blend, y W.D. Grater. 2002. *Bolbophorus damnificus* n. sp. (Digenea: Bolbophoridae) from the channel catfish Ictalurus punctatus and American white pelican Pelecanus erythrorhynchos in the USA based on life-cycle and molecular data. *Systematic Parasitology*, 52: 81–96.

Park, J.K., Kim, K.H., Kang, S. et al. 2007. A common origin of complex life cycles in parasitic flatworms: evidence from the complete mitochondrial genome of *Microcotyle sebastis* (Monogenea: Platyhelmunthes). *BMC Evolutionary Biology*, 7, 11.

Pearson, J. C. 1992. On the position of the digenean family Heronimidae: an inquiry into a cladistic classificatjon of the Digenea. *Systematic Parasitology*, 21: 81–166.

Pérez-Ponce de León, G. y S. A. Nadler. 2010. What we don´t recognize can hurt us: A plea for awareness about cryptic species. *Journal of Parasitology*, 96: 453–464.

Pérez-Ponce de León, G., L. García-Prieto y B. Mendoza-Garfias. 2007. Trematode parasites (Platyhelminthes) of wildlife vertebrates in Mexico. *Zootaxa*, 1534, 247 pp.

Pérez-Ponce de León, U. Razo-Mendivil, R. Rosas-Valdez, B. Mendoza-Garfias y H.

Mejía-Madrid. 2008. Description of a new species of *Crassicutis* Manter, 1936, parasite of *Cichlasoma beani* Jordan (Osteichthyes: Cichlidae) in Mexico, based on morphology and sequences of the ITS1 and 28S ribosomal RNA genes. *The Journal of Parasitology*, 94: 257–263.

Perkins, E. M., S. C. Donnellan, T. Bertozzi y I. D. Whittington. 2010. Closing the mitochondrial circle on paraphyly of Monogenea (Platyhelminthes) infers evolution in the diet of parasitic fltatworms. *International Journal for Parasitology*, 40: 1237–1245.

Poulin, R. 1997. Species richness of parasite assemblages: evolution and patterns. *Anual Review in Ecology and Systematics*, 28: 341-358.

Poulin, R. y T. H. Cribb. 2002. Trematode life cycles: short is sweet?. *Trends in Parasitology*, 18, 176–183.

Razo-Mendivil,U., B. Mendoza-Garfias, G. Pérez-Ponce de León y M. Rubio-Godoy. 2014. A New Species of *Auriculostoma* (Digenea: Allocreadiidae) in the Mexican Tetra *Astyanax mexicanus* (Actinopterygii: Characidae) from Central Veracruz, Mexico, Described with the Use of Morphological and Molecular Data. *Journal of Parasitology*, 100: 331–337.

Rohde, K., 2001. The Aspidogastrea: an archaic group of Platyhelminthes. In: Littlewood, D.T.J., Bray, R.A. (Eds.), *Interrelationships of the Platyhelminthes*, Taylor and Francis, London, pp. 159–167.

Rohde, K., C. Hefford, J. T. Ellis, P. R. Y. Baverstock, A. M. Johnson, N. A. Y. Watson y S. Dittmann. 1993. Contributions to the phylogeny of Platyhelminthes based on partial sequencing of 18s ribosomal DNA. *International Journal for Parasitology*, 23: 705–724.

Rohde, K., A. M. Johnson, P. R. Y. Baverstock y N. A. Watson. 1995. Aspects of the phylogeny of Platyhelminthes based on 18s ribosomal DNA and protonephridial ultrastructure. *Hydobiologia* 305: 27-35.

Sereno-Uribe, A.L., C.D. Pinacho-Pinacho, M. García-Varela y G. Pérez-Ponce de León. 2013. Using mitochondrial and ribosomal DNA sequences to test the taxonomic validity of *Clinostomum complanatum* Rudolphi, 1814 in fish-eating birds and freshwater fishes in Mexico, with the description of a new species. *Parasitology*

Research, 112: 2855–2870.

Sereno-Uribe A.L., C.D. Pinacho-Pinacho, V. Sanchéz-Cordero y M. García-Varela. 2014. Morphological and molecular analyses of larval and adult stages of *Echinoparyphium recurvatum* von Linstow 1873 (Digenea: Echinostomatidae) from central Mexico. *Journal of Helminthology*, 89: 1–7

Schell, S. C. 1985. *Handbook of trematodes of North America North Mexico*. University Press of Idaho, Moscú, 263 pp.

Schulenburg, J.H.G. y J.W. Wagele. 1998. Molecular characterization of digenetic trematodes associated with *Cyathura carinata* (Crustacea: Isopoda) with a note on the utility of 18s ribosomal DNA for phylogenetic analysis in the Digenea (Platyhelminthes: Trematoda). *International Journal for Parasitology*, 28: 1425–1428.

Schulenburg, J.H.G., U. Englisch y J.W. Waegele. 1999. Evolution of ITS1 rDNA in the Digenea (Platyhelminthes: Trematoda): 3´ end sequence conservation and its phylogenetic utility. *Journal of Molecular Evolution*, 48: 2–12.

Shoop, W.L. 1988. Trematode transmission patterns. *Journal of Parasitology*, 74: 46–59.

Snyder, S.D. y V.V. Tkach. 2001. Phylogenetic and biogeographical relationships among some holarctic frog lung flukes (Digenea: Haematoloechidae). *Journal of Parasitology*, 87: 1433–1440.

Tkach, V., J. Pawlowski y J. Mariaux. 2000a. Phylogenetic analysis of the suborder Plagiorchiata (Platyhelminthes, Digenea) based on partial lsrDNA sequences. *International Journal for Parasitology*, 30, 83–93.

Tkach, V.V., J. Pawlowski y V. P. Sharpilo. 2000b. Molecular and morphological differentiation between species of the *Plagiorchis vespertilionis* group (Digenea, Plagiorchiidae) occurring in European bats, with a redescription of *P. vespertilionis* (Muller, 1780). *Systematic Parasitology*, 47: 9–22.

Tkach, V., J. Pawlowski, J. Mariaux y Z. Swiderski. 2001. Molecular phylogeny of the suborder Plagiorchiata and its position in the system of Digenea. In: Littlewood, D.T.J. & Bray, R.A. (Eds) *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, pp. 186–193.

Tkach V.V., Littlewood, D.T.J., Olson, P.D., Kinsella, J.M. y Świderski, Z. 2003.

Molecular phylogenetic analysis of the Microphalloidea Ward, 1901. *Systematic Parasitology*, 56: 1–15.

van Herwerden, L., D. Blair y T. Agatsuma. 1998. Intra- and interspecific variation in nuclear ribosomal internal transcribed spacer 1 of the *Schistosoma japonicum* species complex. *Parasitology*, 116: 311–317.

van Herwerden, L., D. Blair y T. Agatsuma. 1999. Intra- and interindividual variation in ITS1 of *Paragonimus westermani* (Trematoda: Digenea) and related species: implications for phylogenetic studies. *Molecular Phylogenetics and Evolution*, 12: 67–73.

Vilas, R., C. D. Criscione y M. S. Blouin. 2005. A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites. *Parasitology*, 131: 839-846.

Willems, W.R., A. Wallberg y U. Jondelius. 2005. Filling a gap in the phylogeny of flatworms: relationships within the Rhabdocoela (Platyhelminthes), inferred from 18S ribosomal DNA sequences. *Zoologica Scripta*, 35: 1–17.

Yamaguti, S. 1975. *A synoptical review of life histories of Digenetic Trematodes of Vertebatres*. Keigaku Publishing, Tokio: 590 pp.

10. APÉNDICES

Durante los cuatro años de duración del programa de Doctorado del Posgrado de Ciencias Biológicas, UNAM, se participó activamente en la elaboración de publicaciones científicas con varios enfoques y objetivos diferentes a la presente tesis. En estos trabajos se contribuyó ya sea en la colecta de ejemplares en campo, en la identificación de los organismos, en el procesamiento del material para análisis morfológicos y moleculares o bien en la realización de análisis filogenéticos moleculares, así como también, en la discusión de los resultados. De antemano, agradezco a todos los autores por la invitación para colaborar en los artículos publicados, los cuales a continuación se muestran como apéndices de esta tesis.

Available online at www.sciencedirect.com

Revista Mexicana de Biodiversidad

Revista Mexicana de Biodiversidad 88 (2017) 646–653

www.ib.unam.mx/revista/

Ecology

Seasonal variation of gastro-intestinal helminths of three bat species in the dry forest of western Mexico

Variación estacional de helmintos gastrointestinales en tres especies de murciélagos en el bosque tropical caducifolio del occidente de México

Valeria B. Salinas-Ramos [a,*], L. Gerardo Herrera [b], David I. Hernández-Mena [a], David Osorio-Sarabia [c], Virginia León-Règagnon [b]

[a] *Posgrado en Ciencias Biológicas, Instituto de Biología, Universidad Nacional Autónoma de México, Apartado postal 70-153, 04510 Mexico City, Mexico*
[b] *Estación de Biología Chamela, Instituto de Biología, Universidad Nacional Autónoma de México, Apartado postal 21, 48980 San Patricio, Jalisco, Mexico*
[c] *Departamento de Zoología, Laboratorio de Helmintología, Instituto de Biología, Universidad Nacional Autónoma de México, Apartado postal 70-153, 04510 Mexico City, Mexico*

Received 18 October 2016; accepted 24 April 2017
Available online 12 August 2017

Abstract

Studies on helminths of chiropterans are relatively uncommon compared to those of other animals, and seasonal changes in helminth load have been rarely examined. We characterized the gastro-intestinal helminth load of 3 bats species to test for the existence of seasonal changes in response to known seasonal environmental and bat prey fluctuations. We did not find seasonal variation in most of the cases. However, the prevalence of 4 endoparasite species was significantly higher during one of the seasons. The highest richness was registered in *Pteronotus parnellii* during the wet season. The effective number of species was higher during the dry season in the 3 species of *Pteronotus*. Diet seems to be an important driver of helminth infracommunity structure, but we found heterogeneous patterns in the relationship between diversity and load of helminths and seasonal patterns of bat's diets and abundance of potential intermediate hosts.

Keywords: Bat; Endoparasites; Interactions; *Pteronotus*; Seasonality

Resumen

Las investigaciones sobre los helmintos de quirópteros son relativamente escasas en comparación con las de otros vertebrados y los cambios estacionales en su carga han sido poco estudiados. En este estudio se caracterizó la carga de helmintos gastrointestinales de 3 especies de murciélagos para probar la existencia de cambios estacionales en respuesta a las fluctuaciones ambientales y de presas. No se encontró variación estacional en la mayoría de los casos. Sin embargo, la prevalencia de 4 especies de endoparásitos fue significativamente mayor durante una de las épocas. La mayor riqueza de especies se registró en *P. parnellii* durante la época de lluvias. El número efectivo de especies fue mayor durante estaciones secas en las 3 especies de *Pteronotus*. La dieta parece dirigir la estructura de las infracomunidades de helmintos, aunque se encontraron patrones heterogéneos en la relación entre la diversidad y la carga de helmintos y los patrones estacionales de la dieta y la abundancia de los posibles huéspedes intermediarios.

Palabras clave: Murciélago; Endoparásitos; Interacciones; *Pteronotus*; Estacionalidad

* Corresponding author.
E-mail address: airelav2@hotmail.com (V.B. Salinas-Ramos).
Peer Review under the responsibility of Universidad Nacional Autónoma de México.

http://dx.doi.org/10.1016/j.rmb.2017.07.007

Parasitology International 66 (2017) 152–162

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Parasciadicleithrum octofasciatum n. gen., n. sp. (Monogenoidea: Dactylogyridae), parasite of *Rocio octofasciata* (Regan) (Cichlidae: Perciformes) from Mexico characterised by morphological and molecular evidence

Carlos A. Mendoza-Palmero [a,*], Isabel Blasco-Costa [b], David Hernández-Mena [a], Gerardo Pérez-Ponce de León [a]

[a] Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Apartado Postal 70-153, Código Postal 04510, México, D. F., Mexico
[b] Natural History Museum of Geneva, P.O. Box 6134, CH-1211 Geneva, Switzerland

ARTICLE INFO

Article history:
Received 3 October 2016
Received in revised form 11 January 2017
Accepted 12 January 2017
Available online 13 January 2017

Keywords:
Neotropical cichlids
Phylogeny
Convergent evolution
Dactylogyridae
Mexico

ABSTRACT

Based on an integrative taxonomic approach, combining morphological characters and partial sequences of the 28S rRNA gene, a new genus and species, *Parasciadicleithrum octofasciatum*, is proposed to accommodate dactylogyrids infecting the gills of *Rocio octofasciata* (Cichlidae) from a tributary of the Lacantún River basin, Chiapas State, southern Mexico. Morphologically, the new genus resembles members of *Sciadicleithrum* (Dactylogyridae) in the presence of membranes on the anterior margins of both ventral and dorsal haptoral bars. However, *Parasciadicleithrum octofasciatum* n. sp. shows morphological differences with respect to a subset of species of *Sciadicleithrum* occurring in the same host group and geographic area, such as the body size and gonad dimensions (length and width of germarium and testis, respectively, smaller in the new genus). Phylogenetic analyses performed herein based on Maximum Likelihood and Bayesian Inference criteria, showed that *Parasciadicleithrum octofasciatum* n. sp. represents an undescribed taxon morphologically very similar to species of *Sciadicleithrum* from which it can be consistently distinguished on the basis of molecular data. *P. octofasciatum* n. sp. appeared genetically distant from *Sciadicleithrum* spp. and nested with dactylogyrid parasites of African cichlids. *Parasciadicleithrum* n. gen. showed divergence levels in the 28S rDNA sequences of 25–26% with respect to *Sciadicleithrum* spp. Therefore, on the basis of molecular evidence mainly, and morphological differences highlighted above, the erection of the new genus is proposed. The evolutionary and ecological factors that may have influenced the associations between Neotropical cichlids and their dactylogyrid parasites are briefly discussed.

1. Introduction

Neotropical fish of the family Cichlidae (Perciformes) are highly diverse with over 500 species distributed from northern Patagonia in Argentina up to northern Mexico [1]. Approximately 205 helminth species have been recorded from about 295 species of cichlids across the Americas with trematodes and monogenoids as the most species-rich groups [2]. American cichlids harbour a diverse monogenoidean fauna represented by 46 species, of which the genus *Sciadicleithrum* Kritsky, Thatcher et Boeger, 1989 is the most diverse with 26 species, followed by *Gussevia* Kritsky, Thatcher et Boeger, 1986 with 17 species, *Trinidactylus* Hanek, Molnar et Fernando, 1974 and *Tucunarella* Mendoza-Franco, Scholz et Rozkošná, 2010 with one species each (all belonging to the Dactylogyridae), and *Gyrodactylus geophagensis* Boeger et Popazoglo, 1995 (Gyrodactylidae) as the only gyrodactylid currently described on Neotropical cichlids [2,3–5] (Vanhove et al. [2] listed 49 species in their inventory including African monogenoids introduced to America with their hosts).

In Mexico, in the last two decades, 51 of 54 species of cichlids distributed in hydrological systems across the country (representing 94% of all cichlids registered in Mexico) have been studied with relative intensity for parasitic helminths [6,7]. However, only four species of the Dactylogyridae (formally described) and three unidentified gyrodactylids have been recorded in Mexican cichlids: *S. bravohollisae* Kritsky, Vidal-Martínez et Rodríguez-Canul, 1994 (infecting 16 fish species), *S. meekii* Mendoza-Franco, Scholz et Vidal-Martínez, 1997 (in five fish species), *S. mexicanum* Kritsky, Vidal-Martínez et Rodríguez-Canul, 1994 (in 12 fish species) and *S. splendidae* Kritsky, Vidal-Martínez et Rodríguez-Canul, 1994 (in five fish species). The species *S. bravohollisae*, *S. meekii* and *S. mexicanum* have been also reported from other areas in Middle-America [8–10].

* Corresponding author.
E-mail address: cmpamtheus@yahoo.es (C.A. Mendoza-Palmero).

http://dx.doi.org/10.1016/j.parint.2017.01.006
1383-5769/© 2017 Published by Elsevier Ireland Ltd.

Syst Parasitol (2017) 94:819–829
DOI 10.1007/s11230-017-9740-6

Morphological and molecular characterisation of *Aporocotyle margolisi* Smith, 1967 (Digenea: Aporocotylidae) from the North Pacific hake *Merluccius productus* (Ayres) (Gadiformes: Merlucciidae) off Oregon, USA

Jesus S. Hernández-Orts · David I. Hernández-Mena · Gema Alama-Bermejo · Roman Kuchta · Kym C. Jacobson

Received: 2 March 2017 / Accepted: 2 June 2017 / Published online: 6 July 2017

Abstract Aporocotylid blood flukes conspecific with *Aporocotyle margolisi* Smith, 1967 were collected from the bulbus arteriosus of the North Pacific hake *Merluccius productus* (Ayres). This study revisits the morphology of *A. margolisi*, including drawings, measurements and scanning electron microscopy images, and provides for the first time molecular data for the large subunit of the ribosomal RNA (28S rDNA) and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) genes for this species. A 28S rDNA phylogenetic study of *A. margolisi*, and all available *Aporocotyle* spp., was also performed. The distribution range of *A. margolisi* is extended to the Pacific coast of the USA. We provide a morphological comparison of *Aporocotyle* spp. from the Pacific coast in North America as well as other *Aporocotyle* spp. infecting hake. Comparisons with the original description revealed that the new specimens of *A. margolisi* were considerably larger with respect to all morphological features, except for shorter spines. Molecular results showed a close relationship between *A. margolisi* and *A. argentinensis* Smith, 1969 from the Argentine hake *Merluccius hubbsi* Marini. The phylogenetic relationships of *Aporocotyle* spp. point to a possible co-speciation of hakes species and these blood fluke parasites.

This article is part of the Topical Collection Digenea.

Electronic supplementary material The online version of this article (doi:10.1007/s11230-017-9740-6) contains supplementary material, which is available to authorized users.

Jesus S. Hernández-Orts and David I. Hernández-Mena have contributed equally to this work.

J. S. Hernández-Orts (✉) · G. Alama-Bermejo
Centro de Investigación Aplicada y Transferencia Tecnológica en Recursos Marinos Almirante Storni (CIMAS – CCT CONICET – CENPAT) y Escuela Superior de Ciencias Marinas (ESCiMar), Universidad Nacional del Comahue, Güemes 1030, 8520 San Antonio Oeste, Río Negro, Argentina
e-mail: jesus.hernandez.orts@gmail.com

D. I. Hernández-Mena
Laboratorio de Helmintología, Instituto de Biología, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Ciudad Universitaria, 04510 Mexico City, Mexico

D. I. Hernández-Mena
Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 Mexico City, Mexico

G. Alama-Bermejo · R. Kuchta
Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, 37005 České Budějovice, Czech Republic

G. Alama-Bermejo
Department of Microbiology, Oregon State University, Corvallis, OR 97331, USA

Revista Mexicana de Biodiversidad 89 (2018): 39-45

Rediscovery of the nemertean *Prostoma graecense* from Xochimilco, 75 years after its first and only record

Redescubrimiento del nemertino Prostoma graecense *de Xochimilco, 75 años después de su primer y único registro*

Taxonomy and Systematics

Omar Lagunas-Calvo[a], David Iván Hernández-Mena[b], Gerardo Rivas[a], Samantha Contreras-Mirón[b], Berenit Mendoza-Garfias[c], Olga M. Echeverría-Martínez[d], Alejandro Oceguera-Figueroa[b,]*

[a] *Departamento de Biología Comparada, Facultad de Ciencias, Universidad Nacional Autónoma de México, Apartado Postal 70-399, 04510 México City, Mexico*
[b] *Laboratorio de Helmintología, Instituto de Biología, Universidad Nacional Autónoma de México, Apartado postal 70-153, 04510 México City, Mexico*
[c] *Laboratorio de Microscopia Electrónica, Instituto de Biología, Universidad Nacional Autónoma de México, Apartado postal 70-153, 04510 México City, Mexico*
[d] *Laboratorio de Microscopia Electrónica, Facultad de Ciencias, Universidad Nacional Autónoma de México, Apartado Postal 70-181, 04510 México City, Mexico*

*Corresponding author. *E-mail-address:* aoceguera@ib.unam.mx (A. Oceguera-Figueroa).

Recibido: 23 enero 2017; aceptado: 27 septiembre 2017

Abstract

The identity of the freshwater nemertean *Prostoma graecense* (Böhming, 1892) from Lake Xochimilco is solved 75 years after its first recording. Identification of the specimens was accomplished via both morphological and molecular means, the latter employing the mitochondrial cytochrome *c* oxidase subunit I. Recent literature and our results suggest that this species has a cosmopolitan distribution and has, presumably, been recently introduced to areas well removed from its original distribution, which is unknown. The current study provides background for continuing the work with freshwater and marine nemerteans and underscores the importance of including molecular data in future studies of the group.

Keywords: Freshwater invertebrates; Nemertea; Tetrastemmatidae; Cox1; Taxonomy

Resumen

Se resuelve la identidad taxonómica del nemertino dulceacuícola *Prostoma graecense* (Böhming, 1892) del lago de Xochimilco 75 años después del primer registro. La identificación de los ejemplares se realizó con base en caracteres morfológicos y moleculares, esta última empleando el citocromo c oxidasa subunidad I mitocondrial. La literatura reciente y nuestros resultados sugieren que esta especie tiene una distribución cosmopolita y que ha sido introducida, presumiblemente, en áreas lejanas de su distribución original, la cual es desconocida. El presente estudio proporciona un contexto para continuar los estudios sobre nemertinos de agua marina y dulceacuícola y enfatiza la importancia de incluir datos moleculares en futuros estudios del grupo.

Palabras clave: Invertebrados dulceacuícolas; Nemertea; Tetrastemmatidae; Cox1, Taxonomía

ISSN versión impresa: 1870-3453; versión electrónica: 2007-8706; Universidad Nacional Autónoma de México, Instituto de Biología.

Journal of Helminthology

cambridge.org/jhl

Research Paper

Cite this article: Briosio-Aguilar R, García-Varela M, Hernández-Mena DI, Rubio-Godoy M, Pérez-Ponce de León G (2018). Morphological and molecular characterization of an enigmatic clinostomid trematode (Digenea: Clinostomidae) parasitic as metacercariae in the body cavity of freshwater fishes (Cichlidae) across Middle America. *Journal of Helminthology* 1–14. https://doi.org/10.1017/S0022149X18000445

Received: 8 March 2018
Accepted: 12 April 2018

Author for correspondence:
G. Pérez-Ponce de León
E-mail: ppdleon@ib.unam.mx

Morphological and molecular characterization of an enigmatic clinostomid trematode (Digenea: Clinostomidae) parasitic as metacercariae in the body cavity of freshwater fishes (Cichlidae) across Middle America

R. Briosio-Aguilar[1,2], M. García-Varela[1], D.I Hernández-Mena[1], M. Rubio-Godoy[3] and G. Pérez-Ponce de León[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]Posgrado en Ciencias Biológicas, UNAM and [3]Instituto de Ecología, A.C. Red de Biología Evolutiva, km 2.5 Ant. Carretera a Coatepec, Xalapa, 91070 Veracruz, Mexico

Abstract

The family Clinostomidae Lühe, 1901 contains 29 species allocated to seven genera, of which *Clinostomum* Leidy, 1856 is the most diverse, with *c.* 14 valid species. The diversity of *Clinostomum* has been assessed, combining morphological and molecular data. The genetic library for species in this genus has increased steadily, although there is little or no information for the other genera included in the family. Molecular phylogenetic relationships among the genera of clinostomids have not been assessed, and their classification is still based on morphological traits. The monotypic *Ithyoclinostomum* was described from a fish-eating bird in Brazil, and its metacercariae have been found in several locations in South America, parasitizing erythrinid freshwater fishes. We collected unusually large metacercariae from the body cavity of cichlids in several locations across Middle America. These metacercariae exhibited some resemblance to *Ithyoclinostomum*, although several differences prevent their inclusion in *Ithyoclinostomum dimorphum*, casting doubt on their taxonomic identification. The main objective of this paper was to characterize the metacercariae collected in cichlids using both morphology and molecular data from three molecular markers, and to assess the molecular phylogenetic relationships among the genera of Clinostomidae to establish the position of the newly generated sequences. We took a conservative position and tentatively placed the metacercariae as belonging to *Ithyoclinostomum*.

Introduction

Members of the cosmopolitan family Clinostomidae Lühe, 1901 are parasites of the buccal cavity, oesophagus or intestine of birds, reptiles and occasionally mammals (Ukoli, 1966; Kanev *et al.*, 2002). The taxonomic status and phylogenetic relationships among the genera allocated to this family have been uncertain. Currently, the family comprises seven genera in four subfamilies according to Kanev *et al.* (2002): Clinostominae Lühe, 1901 with the cosmopolitan genus *Clinostomum* Leidy, 1856 containing *c.* 14 species parasitizing fish-eating birds as definitive hosts (see Locke *et al.*, 2015; Pérez-Ponce de León *et al.*, 2016; Caffara *et al.*, 2017), *Clinostomatopsis* Dollfus, 1932 (two species), and the monotypic *Clinostomoides* Dollfus, 1959; Euclinostominae Yamaguti, 1958, with *Euclinostomun* Travassos, 1928 containing eight species parasitizing birds (Caffara *et al.*, 2016); Nephrocephalinae Travassos, 1928, with two genera parasitic in crocodilians, i.e. *Odhneriotrema* Travassos, 1928 and *Nephrocephalus* Odhner, 1902, with two and one species, respectively (Woodyard *et al.*, 2017); and Ithyoclinostominae Yamaguti, 1958, with the monotypic *Ithyoclinostomum dimorphum* (Diesing, 1850) Witenberg, 1925, as a parasite of birds. Even though great progress has been made in recent years to increase the generic library for species of *Clinostomum*, the most species-rich genus in the family, in studies aimed at establishing robust species delimitation criteria, few attempts have been made to generate sequence data to perform a phylogenetic analysis at the family level (see Woodyard *et al.*, 2017).

During survey work on the helminth fauna of Middle American freshwater fishes, unusually large clinostomid metacercariae were found unencysted in the body cavity of some cichlid species in several locations of Mexico and Costa Rica. Based on the size of the metacercariae and the position of the genital complex in the posterior fourth of the body, we first identified the species as *Clinostomum heluans* Braun, 1899. An additional piece of information was that *C. heluans* had been previously recorded as a parasite of the great