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SISTEMÁTICA

Sistemática molecular de adultos y metacercarias de *Uvulifer* spp. (Digenea:

Diplostomidae) en diferentes localidades de México y Centroamérica

TESIS

QUE PARA OPTAR POR EL GRADO DE:

MAESTRA EN CIENCIAS BIOLÓGICAS

PRESENTA:

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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 junio de 2017, se aprobó el siguiente jurado para el examen de grado de **MAESTRA EN CIENCIAS BIOLÓGICAS** de la alumna **LÓPEZ JIMÉNEZ CECILIA ALEJANDRA** con número de cuenta **308181829** con la tesis titulada **"Sistemática molecular de adultos y metacercarias de *Uvulifer* spp. (Digenea: Diplostomidae) en diferentes localidades de México y Centroamérica"**, realizada bajo la dirección del **DR. JOSÉ MARTÍN GARCÍA VARELA**:

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

A T E N T A M E N T E
"POR MI RAZA HABLARA EL ESPIRITU"
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RESUMEN

Los miembros del género *Uvulifer* son diplostómidos distribuidos en todo el mundo que infectan a caracoles acuáticos y peces dulceacuícolas que fungen como primeros y segundos huéspedes intermedios, y aves piscivoras (alcedines) como huéspedes definitivos. La infección en los peces se conoce comúnmente como la enfermedad de la mancha negra o "black spot". En el presente estudio, metacercarias de *Uvulifer* fueron recolectadas de las aletas y la piel de varias especies de peces dulceacuícolas incluyendo localidades de México y Centroamérica; Guatemala, Honduras, Nicaragua y Costa Rica. Los adultos fueron colectados del intestino de dos especies de alcedines; *Chloroceryle americana* y *Megarecyle alcyon* en cuatro localidades de México. La divergencia genética entre 76 muestras (64 metacercaria y 12 adultos) se estimó a través de las regiones 28S e (ITS1+5.8S+ITS2) del DNA nuclear y de la región del citocromo oxidasa subunidad 1 (COI) del DNA mitocondrial. Los análisis filogenéticos mostraron una alta diversidad genética dentro del género *Uvulifer*, revelando la existencia de cuatro linajes que exhiben un cierto patrón de especificidad hospedatoria en el segundo huésped intermediario. Las metacercarias del linaje 1, están asociadas a peces de la familias Characidae y Cyprinidae, mientras que el adulto fue encontrado en el "martín pescador gigante norteamericano" (*Megarecyle alcyon*), que se distribuye en el centro y norte de México, sin embargo a este linaje no se le asignó un nombre debido a que los pocos adultos recolectados eran inmaduros. Las metacercarias de los linajes 2 y 3 están asociadas a peces de la familia Cichlidae y se distribuyen simpatricamente en cuatro países de Centroamérica. La falta de adultos en estos linajes impidio una descripción formal de estas especies. Las metacercarias del linaje 4 están asociadas a peces de la familia Poeciliidae y el adulto fue encontrado en el "martín pescador verde" (*Chloroceryle americana*), encontrado en vertientes del Golfo de

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México y del Océano Pacífico en México, Guatemala, Honduras y Nicaragua. Las metacercarias del linaje 4 fueron correlacionados con ejemplares adultos grávidos. Por lo tanto, se describió una nueva especie del género *Uvulifer*, que se distingue principalmente de las otras cinco especies del género conocidas para el continente americano (*U. ambloplitis, U. semicircumcisus, U. prosocotyle, U. weberi* y *U. elongatus*) por la presencia de espinas en el segmento posterior del cuerpo y por presentar un bolsa eyaculadora más grande, así como vesícula seminal y huevos más pequeños.

ABSTRACT

Members of the genus *Uvulifer* are diplostomid trematodes distributed worldwide that infect aquatic snails and freshwater fishes as first and second intermediate hosts, and fish-eating birds (kingfishers) as definitive hosts. Parasitic infection in fish is commonly referred to as the black spot disease. In the current study, metacercariae of *Uvulifer* were collected from the fins and skin of several species of freshwater fishes across of Mexico and Middle America; Guatemala, Honduras, Nicaragua, and Costa Rica. The adults were recovered from the intestine of two species from kingfishers; *Chloroceryle americana* y *Megarecyle alcyon* in four localities of Mexico. The genetic divergence among 76 samples (64 metacercaria and 12 adults) was estimated through the large subunit (28S) and (ITS1+5.8S+ITS2) of nuclear DNA and the region cytocromo oxidasa subunidad 1 (COI). Phylogenetic analyses showed an unexpected genetic diversity of the genus *Uvulifer* in Middle America, revealing the existence of four genetic lineages that exhibit some level of host specificity to their second intermediate hosts. The metacercariae of one of the lineages (Lineage 1) is associated with characids and cyprinids, while the adult was found in the Belted Kingfisher, and was distributed in central and northern Mexico. The collection of very few adult specimens prevented a formal species description. Metacercariae of lineages 2 and 3 were characteristically associated with cichlids distributed widely and even occurred in sympatry in some localities. No adults of these lineages were recovered from kingfishers and then it was not possible to describe them. The metacercariae of lineage 4 were found in poeciliids, and the adult in the Green Kingfisher, widely distributed in the Gulf of Mexico and Pacific Ocean slopes in Mexico, Guatemala, Honduras and Nicaragua. The number of specimens sampled for Lineage 4 for both, gravid adults and metacercariae, allowed us describe a new species of *Uvulifer*, which is mainly distinguished from the other

five described congeners from the Americas (*U. ambloplitis*, *U. semicircumcisus*, *U. prosocotyle, U. weberi* and *U. elongatus*) by having a hindbody covered with spines extending from the anterior end of hindbody to the level of the anterior testis, and by having smaller eggs and a bigger ejaculatory pouch

I. INTRODUCCIÓN

I.I Delimitación de especies en helmintos

La delimitación de especies de helmintos se basa principalmente en rasgos morfológicos de sus estados adultos . El reconocimiento a través de cualquier característica morfológica se conoce como concepto linneano o morfológico de especie (Mayden & Wood, 1995). La variación morfológica en especies parásitas puede deberse a tres factores; 1) distribución geográfica, 2) especie de huésped y 3) condiciones ecológicas donde se encuentra (Hanzelová *et al*., 2005). Sin embargo, determinar correctamente a las especies es un reto para los taxónomos debido a la incertidumbre sobre la validez de los caracteres morfológicos diagnósticos, la plasticidad fenotípica, la limitación de las características morfológicas que permitan asociar los estados larvarios con el estado adulto y al reconocimiento de especies crípticas (morfológicamente idénticas pero genéticamente diferentes) (León-Règagnon *et al*., 1999; Nolan & Cribb, 2005; Pérez-Ponce de León & Nadler, 2010). Actualmente se han empleado secuencias de DNA mitocondrial y nuclear, así como caracteres morfológicos y ecológicos para determinar con mayor certidumbre a las especies de helmintos (Pérez-Ponce de León *et al*., 2008; Razo-Mendivil *et al*., 2004, 2008).

I.II Marcadores moleculares en helmintos

Los marcadores más utilizados para delimitar especies o poblaciones de helmintos son los genes nucleares del DNA ribosomal (rDNA) y los genes del DNA mitocondrial. Particularmente, el rDNA se presenta en repeticiones tándem y está formado por tres subunidades altamente conservadas (18S, 5.8S y 28S), separadas por dos espaciadores transcritos internos con elevadas tasas de sustitución (ITS1 e ITS2) (Eickbush y Eickbush,

2007) (Fig. 1). Estas repeticiones en tándem se encuentran conservadas a lo largo de todo el genoma y evolucionan concertadamente, por lo que se han convertido en los marcadores moleculares predilectos para separar poblaciones, especies y géneros (Andrade-Gómez *et al*., 2016; Blasco Costa *et al*., 2017; García Varela *et al*., 2016; Hernández-Mena *et al*., 2014; Pinacho-Pinacho *et al*., 2017; Pérez-Ponce de León *et al*., 2015, 2016a; Stoyanov *et al*., 2017).
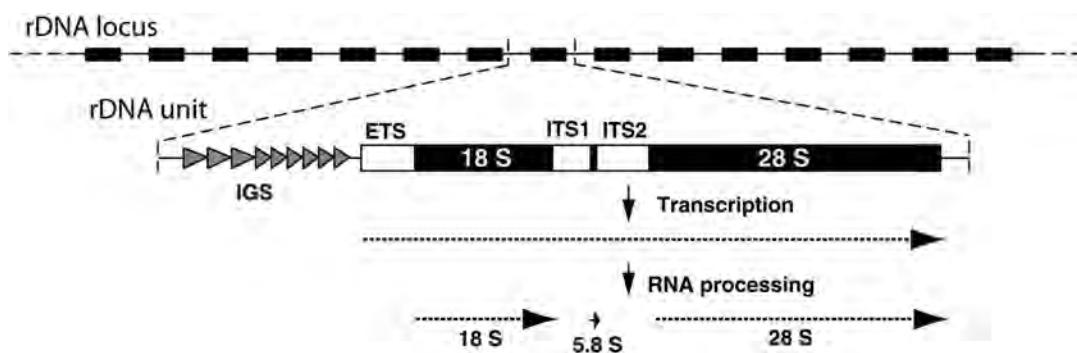
Fig. 1 Organización de genes del DNA ribosomal en eucariontes. (Tomado de Eickbush y Eickbush, 2007).

El DNA mitocondrial es una molécula circular con aproximadamente 16, 569 pares de bases con un total de 37 genes que varían dependiendo del organismo (13 RNA mensajeros, 2 RNA ribosomales y 22 RNA de transferencia). Entre las características más interesantes en términos filogenéticos y filogeográficos están su alta tasa de sustitución, su casi nula recombinación y su herencia materna, lo que permiten describir la historia matrilineal de organismos coespecíficos y con ello aplicar estimaciones de reloj molecular e inferir hipótesis de coalescencia (Vázquez-Domínguez 2007, 2009) (Fig. 2). En los grupos

de helmintos se han utilizado algunos genes mitocondriales para inferir la evolución de los grupos y separar especies (Macnish *et al*., 2002; Locke *et al*., 2015; Pérez-Ponce de León *et al*., 2016b; Soldánova *et al*., 2017).

El auge de información molecular ha caído en el problema de utilizar un solo marcador como única evidencia para diferenciar nuevos linajes o especies. Sin embargo, existe el riesgo de sobreestimar la diversidad de especies debido a las diferencias en las tasas de evolución de los marcadores moleculares utilizados (Villas *et al*., 2005). Por lo tanto, es fundamental para la descripción y diferenciación de especies desde un punto de vista taxonómico que se recurra a más fuentes de información para respaldar el reconocimiento de especies.
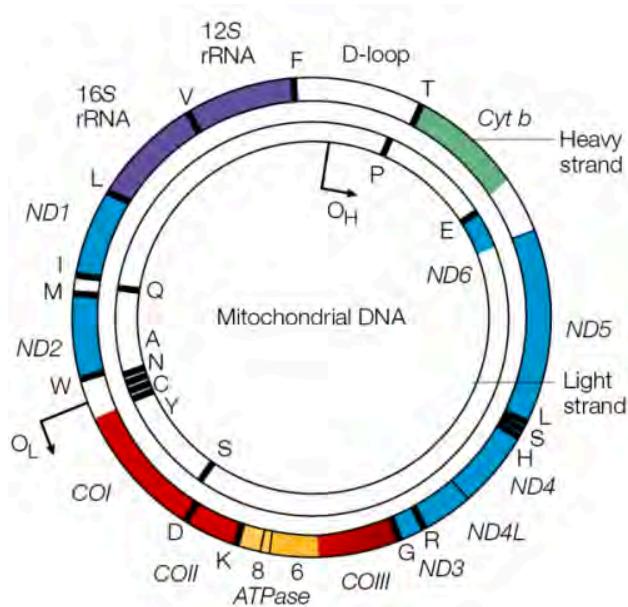
Fig. 2 Molécula de DNA mitocondrial de animales.

I.III Características del género *Uvulifer* Yamaguti, 1934

Los miembros de la Familia Diplostomidae se caracterizan por presentar un cuerpo dividido en dos segmentos: el segmento anterior contiene por lo general un par de pseudoventosas, ventosa oral, acetábulo, faringe, esófago corto, ciegos y el órgano tribocítico. El segmento posterior es de forma cilíndrica o coniforme y contiene los órganos reproductores; sin embargo, no presentan saco del cirro (Fig. 3). Esta familia está dividida en cuatro subfamilias con un total de 41 géneros con distribución cosmopolita (Niewiadomska, 2002).

Particularmente el género *Uvulifer* Yamaguti, 1934 se encuentra dentro de la subfamilia Crassiphialinae y se diferencia morfológicamente de otros géneros por presentar una bolsa eyaculadora muscular y un cono genital embebido en un pliegue con forma de prepucio (Niewiadomska, 2002). La metacercaria de este género se caracteriza por formar un quiste con pigmentación negra en la piel y aletas del segundo huésped intermediario, enfermedad comunmente conocida como "black spot". Actualmente, se han descrito 18 especies distribuidas en todo el mundo, abarcando desde regiones neotropicales hasta regiones holárticas asociadas al intestino de aves ictiófagas de la familia Cerilydae. De las 18 especies del género descritas hasta la fecha, cinco de ellas se han registrado en el continente americano: (1) *Uvulifer ambloplitis* (=*Crassiphiala ambloplitis*) descrito por Hughes (1927) del pez *Ambloplites rupestris* Rafinesque de la familia Centrarchidae en el Lago Douglas, Michigan. Posteriormente, Hunter (1933) describió los adultos de *U. ambloplitis* en alcedines *Megaceryle alcyon* Linnaeus en New York; (2) *U. prosocotyle* descrito por Dubois (1937) del martin pescador *M. torquata* L. en Brasil; (3) *U. semicircumcisus* descrito por Dubois y Rausch (1950) de *M. alcyon* colectados en

Michigan; (4) *U. weberi* descrito por Dubois (1985) del *Chloroceryle amazona* Latham en

Paraguay y posteriormente redescrito en 1988 por el mismo autor en una especie de

hospedero diferente (*C. americana* Gmelin). Finalmente, *U. elongatus* fue descrito por

Dubois (1988) de *M. torquata* en Paraguay.
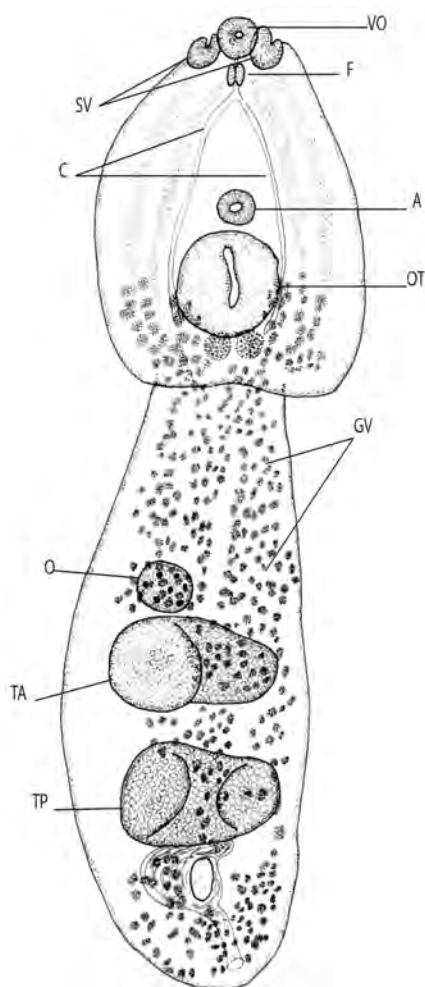
Fig. 3 Esquema general de un diplostomido. Abreviaturas: acetábulo (A), ciegos (C), faringe (F), glándulas vitelógenas (GV), órgano tribocítico (OT), ovario (O), seudoventosas (SV), testículo anterior (TA), testículo posterior (TP), ventosa oral (VO) (Modificado de Pérez del Olmo *et al.*, 2014).

I.IV Ciclo de vida de *Uvulifer*

Hunter & Hunter (1934) completaron el ciclo de vida experimental de *U. ambloplitis*. Los huevos operculados se liberan al medio acuático a través de las heces de aves infectadas con el adulto. Posteriormente, de los huevos eclosiona un miracidio ciliado que penetra a dos especies de gasterópodos acuáticos del género *Helisoma* Swainson (*H. trivolvis* y *H. companulatum*) los cuales fungen como los primeros huéspedes intermediarios. En el gasterópodo se desarrolla un esporocisto madre que da lugar a varias generaciones de esporocistos hijos, los cuales maduran hasta formar cercarias. Las cercarias emergen del gasterópodo y nadan para penetrar y enquistarse en la piel y aletas de su segundo huésped intermediario, y desarrollan la enfermedad comúnmente conocida como "black spot". Las metacercarias alcanzan el estado adulto cuando los peces son ingeridos por aves de la familia Cerylidae, comúnmente conocidos como "martines pescadores" donde maduran en el intestino (Fig. 4)
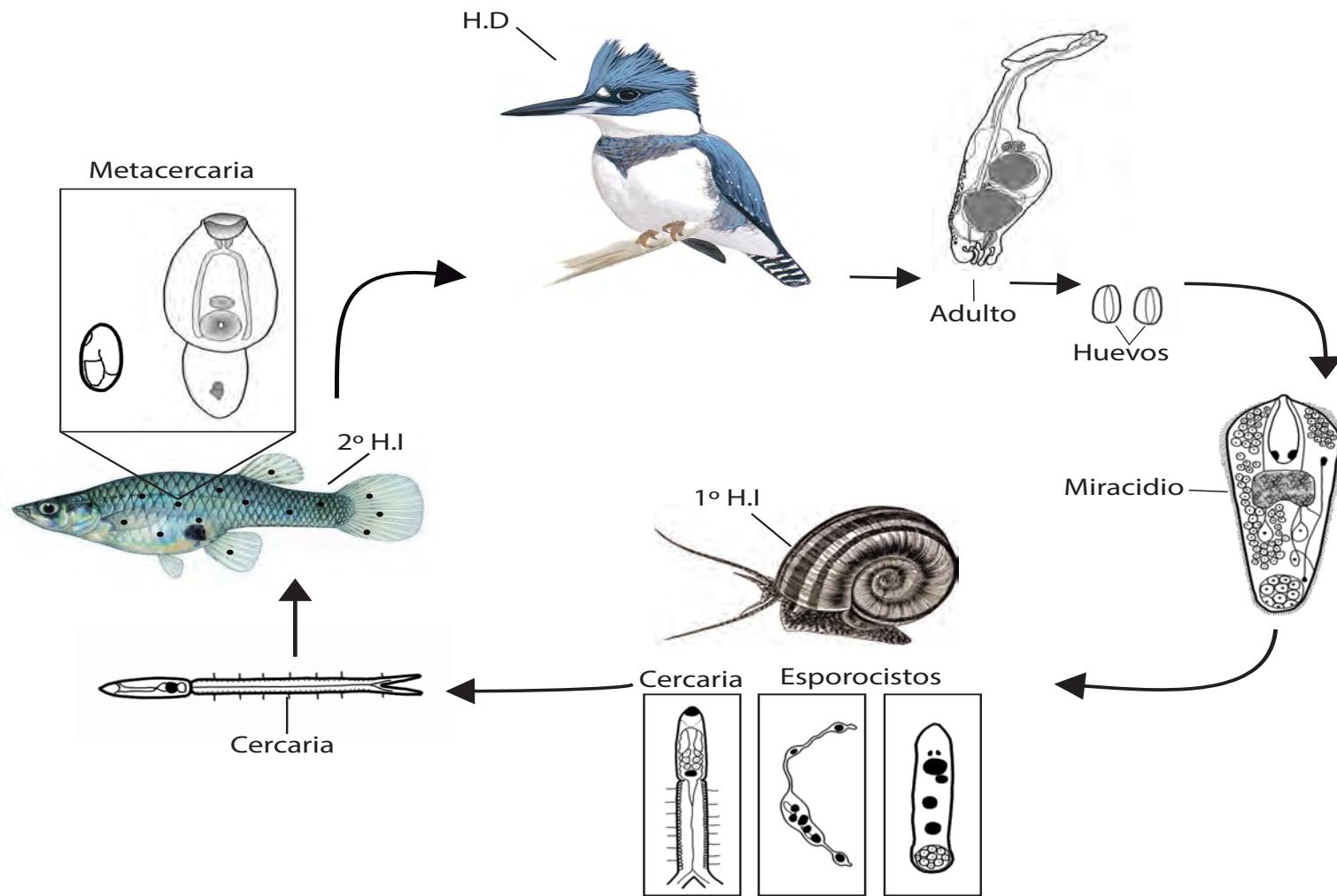
Fig. 4 Ciclo de vida de *Uvulifer ambloplitis* (Modificado de Hunter & Hunter, 1934). H.I=Hospedero intermediario; H.D=Hospedero definitivo.

I.V Registros del género *Uvulifer* en México

En México, la metacercaria de *Uvulife*r sp., fue registrada por primera vez en el pez *Micropterus salmoides* Lacepede, 1802 en la presa Vicente Guerrero, Tamaulipas (Pérez-Ponce de León *et al*., 1996). Posteriormente las metacercarias de *Uvulifer* sp., han sido registradas en 45 especies de peces en 18 estados de México, pertenecientes a 10 familias (Atherinopsidae, Cichlidae, Characidae, Cyprinidae, Eleotridae, Gobiidae, Heptapteridae, Mugilidae, Goodeidae y Poeciliidae), aunque parecen infectar preferentemente a peces de las familias Cichlidae y Poeciliidae (Pérez-Ponce de León *et al*., 2007, 2010; García Magaña & López-Jiménez, 2008; Bautista-Hernández *et al*., 2014). Por su parte, las metacercarias de *U. ambloplitis* han sido registradas en 17 especies de peces pertenecientes a 7 familias (Cichlidae, Characidae, Cyprinidae, Eleotridae, Heptapteridae, Mugilidae y Poeciliidae) (Salgado-Maldonado *et al*., 2004, 2014). Sin embargo, estos últimos estudios carecen de la identificación morfológica del estadio adulto del género *Uvulifer* y por lo tanto la identificación a nivel de especie es incierta.

II. OBJETIVOS

II.I Objetivo general

Caracterizar morfológica y molecularmente las metacercarias y adultos de *Uvulifer* spp. colectados en México y Centroamérica.

II.II Objetivos particulares

1. Realizar la descripción morfológica de las metacercarias y adultos de este género colectados de diferentes hospederos intermediarios y definitivos.

2. Analizar la subunidad mayor del DNA ribosomal nuclear y de los espaciadores transcritos internos, así como el citocromo oxidasa subunidad 1 (COI) del DNA mitocondrial.

3. Estimar las divergencias genéticas entre metacercarias y adultos de *Uvulifer* spp. y proponer una hipótesis filogenética.

III. RESULTADOS

Los resultados derivados del proyecto de Maestría se presentan en forma de manuscrito el cual fue aceptado para su publicación en la revista *Journal of Helminthology*.

Journal of Helminthology

Molecular data reveal high diversity of Uvulifer (Trematoda: Diplostomidae) in Middle America, with the description of a new species.
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Abstract:	Members of the genus Uvulifer are distributed worldwide and infect aquatic snails and freshwater fishes as first and second intermediate hosts respectively and fish-eating birds (kingfishers) as definitive hosts. Metacercariae of Uvulifer spp., were collected from the fins and skin of 20 species of freshwater fishes in Mexico, Guatemala, Honduras, Nicaragua and Costa Rica and the adults were recovered from the intestine of kingfishers in four localities of Mexico. The genetic divergence among 76 samples (64 metacercariae and 12 adults) was estimated by sequencing the 28S and 5.8 nuclear genes, as well as the internal transcribed spacers ITS1 and ITS2, and one mitochondrial gene (cox 1). Maximum likelihood and Bayesian inference analyses inferred with each data set showed a high genetic diversity within the genus Uvulifer across Middle America, revealing the existence of four genetic lineages that exhibit some level of host specificity to their second intermediate hosts. The metacercariae of lineage 1 were associated with characids and cyprinids in central and northern Mexico. Metacercariae of lineages 2 and 3 were associated with cichlids distributed widely across Middle America. The lack of adults of these lineages in kingfishers in lineages 2 and 3, or the fact that just a few adult specimens were recovered as in lineage 1 prevented a formal description of these species. The metacercariae of lineage 4 were found in poeciliids, across a distribution range comprising Mexico, Guatemala, Honduras and Nicaragua, and the adult was found in the green kingfisher in Mexico. The number of specimens sampled for lineage 4 for both, gravid adults and metacercariae, allowed us to describe a new species, Uvulifer spinatus n. sp. We describe the new species herein and we briefly discuss the genetic diversity in Uvulifer spp. and the importance of using DNA sequences to properly characterise parasite diversity

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1   Running title: High genetic diversity in *Uvulifer* across Middle America.

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4   **Molecular data reveal high diversity of *Uvulifer* (Trematoda: Diplostomidae) in**

5   **Middle America, with the description of a new species.**

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Abstract

Members of the genus *Uvulifer* are distributed worldwide and infect aquatic snails and freshwater fishes as first and second intermediate hosts respectively and fish-eating birds (kingfishers) as definitive hosts. Metacercariae of *Uvulifer* spp., were collected from the fins and skin of 20 species of freshwater fishes in Mexico, Guatemala, Honduras, Nicaragua and Costa Rica and the adults were recovered from the intestine of kingfishers in four localities of Mexico. The genetic divergence among 76 samples (64 metacercariae and 12 adults) was estimated by sequencing the 28S and 5.8 nuclear genes, as well as the internal transcribed spacers ITS1 and ITS2, and one mitochondrial gene (*cox 1*). Maximum likelihood and Bayesian inference analyses inferred with each data set showed a high genetic diversity within the genus *Uvulifer* across Middle America, revealing the existence of four genetic lineages that exhibit some level of host specificity to their second intermediate hosts. The metacercariae of lineage 1 were associated with characids and cyprinids in central and northern Mexico. Metacercariae of lineages 2 and 3 were associated with cichlids distributed widely across Middle America. The lack of adults of these lineages in kingfishers in lineages 2 and 3, or the fact that just a few adult specimens were recovered as in lineage 1 prevented a formal description of these species. The metacercariae of lineage 4 were found in poeciliids, across a distribution range comprising Mexico, Guatemala, Honduras and Nicaragua, and the adult was found in the green kingfisher in Mexico. The number of specimens sampled for lineage 4 for both, gravid adults and metacercariae, allowed us to describe a new species, *Uvulifer spinatus* n. sp. We describe the new species herein and we briefly discuss the genetic diversity in *Uvulifer* spp. and the importance of using DNA sequences to properly characterise parasite diversity.

Introduction

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48   The identification at species level of diplostomid parasites with complex life cycles

49   is challenging, particularly when identification is solely based on the metacercarial stage.

50   The morphology of these trematodes sometimes varies with the host species and the habitat

51   they occur (Graczyk, 1991; Niewiadomska & Szymanski, 1991; Pérez-Ponce de León,

52   1995; Locke *et al.*, 2010a). The novel use of DNA sequences in taxonomic studies of

53   diplostomids in combination with morphological data, is very useful for establishing a link

54   between cercariae, metacercariae and adults. Recent studies on diplostomids illustrate the

55   usefulness of such approaches, where both internal transcribed spacers ITS1, ITS2 and the

56   5.8 and the large subunit (LSU) of the ribosomal DNA, as well as the mitochondrial gene

57   cytochrome *c* oxidase subunit I (*cox 1*) have been commonly used as the most popular

58   molecular markers for the identification and delimitation of species (e.g., Georgieva *et al.*,

59   2013; Chibwana *et al.*, 2013, 2015; Blasco-Costa *et al.*, 2014; 2017; Selbach *et al.*, 2015;

60   García-Varela *et al.*, 2016a, 2016b; Stoyanov *et al.*, 2017).

61   As in other diplostomids, the adults of species of the genus *Uvulifer* Yamaguti, 1934

62   are parasites in the intestine of fish-eating birds, particularly alcedines, i.e., kingfishers,

63   across the globe. Metacercariae are found on the skin and fins of freshwater fishes; the

64   black spot disease is caused by the metacercariae of *Uvulifer*, although the metacercarial

65   stages of other diplostomids as *Crassiphiala* Van Haitsma, 1925; *Posthodiplostomum*

66   Dubois, 1936, and heterophyds of the genera *Cryptocotyle* Lühe, 1899 and *Apophallus*

67   Lühe, 1909 may produce the black spot disease (Kristoffersen, 1991; Kurochkin &

68   Biserova, 1996; Krause *et al.*, 1999; Quist *et al.*, 2007; Rodnick *et al.*, 2008). The cercaria

69   is released from a snail belonging to the genus *Helisoma* Swainson that serves as the first

70   intermediate host and it penetrates the skin and fins of multiple species of fishes, where

71   they encyst and develop into metacercariae and the fish surrounds the cyst with black

72   pigment (Niewiadomska, 2002 and references therein). Currently, the genus *Uvulifer*

73   contains 18 described species, eight of which are in Asia (*U. gracilis* Yamaguti, 1934, the

74   type-species; *U. stunkardi* (Pande, 1938) Bhalerao, 1942 [syn. *Cardiocephalus halcyonis*

75   Gupta & Dhillon, 1954 and *U. mehrai* Chatterji, 1956]; *U. ceryliformis* (Vidyarthi, 1938)

76   Bhalerao, 1942 [syn. *Crassiphiala amulai* Chatterji, 1955]; *U. bisphincter* Oshmarin, 1971;

77   *U. giriensis* Mishra & Gupta, 1980; *U. chandigarhensis* Mishra & Gupta, 1980; *U.*

78   *nanningensis* (Lung Tsu-pei, 1966) and *U. iruvettiensis* Subair & Janardanan, 2013), one in

79   Europe (*U. denticulatus* Rudolphi, 1819), four in Africa (*U. cerylou* Dollfus, 1950; *U.*

80   *murinum* Baer, 1971; *U. pseudoprosocotyle* Dubois & Beverley-Burton, 1971 and *U. cheni*

81   (Yang Fu-shi, 1965) Dubois, 1977 [syn. *Prochoanochenia cheni* Yang, 1965) and five in

82   the Americas (*U. ambloplitis* Hughes, 1927 [syn. *U. erraticus* Chandler & Rausch, 1948;

83   *U. claviformis* Dubois & Rausch, 1948 and *U. magnibursiger* Dubois & Rausch, 1950]; *U.*

84   *prosocotyle* Lutz, 1928; *U. semicircumcisus* Dubois & Rausch, 1950; *U. weberi* Dubois,

85   1985 and *U. elongatus* Dubois, 1988) (see Yamaguti, 1971; Dubois, 1970, 1977, 1985,

86   1988; Subair *et al*., 2013). In North America, only two species of *Uvulifer* have been

87   described, both from the belted kingfisher *Megaceryle alcyon* Linnaeus, *U. ambloplitis* and

88   *U. semicircumcisus* (Hunter, 1933; Dubois & Rausch, 1950). Metacercariae of both species

89   have been found in at least nine families of freshwater fishes (see Hoffman, 1999).

90   Adults of species of the genus *Uvulifer* have not been recorded in Middle America

91   thus far, and records in Mexico are based solely on metacercariae, where these parasites

92   have been indistinctly determined as *Uvulifer* sp. or as *Uvulifer ambloplitis* (see Pérez-

93 Ponce de León *et al*., 2007, 2010). Instead, the metacercaria of *Uvulifer* sp. has been

94 recorded in 18 states across Mexico, in the fins and skin of 45 fish species included in ten

95 families of freshwater fishes (Atherinopsidae, Cichlidae, Characidae, Cyprinidae,

96 Eleotridae, Gobiidae, Heptapteridae, Mugilidae, Godeidae and Poeciliidae); however, they

97 seem to infect cichlid and poeciliid fishes preferentially (Pérez-Ponce de León *et al*., 2007,

98 2010; García Magaña & López-Jiménez, 2008; Salgado-Maldonado *et al*., 2014; Bautista-

99 Hernández *et al*., 2014). Additionally, the metacercaria has been recorded as *U. ambloplitis*

100 in 17 fish species belonging to seven families (Cichlidae, Characidae, Cyprinidae,

101 Eleotridae, Heptapteridae, Mugilidae and Poeciliidae) (Salgado-Maldonado *et al*., 2004,

102 2005, 2014). However, those studies lacked of a detailed morphological study of the

103 metacercariae, and adults of *Uvulifer* were not recovered from their definitive hosts;

104 therefore the identification at species level of those specimens is doubtful and requires

105 further verification.

106   In the current research, we collected specimen adults and metacercariae identified as

107 *Uvulifer* sp. from 23 fish species and two bird species distributed across Middle America,

108 including localities of Mexico, Guatemala, Honduras, Nicaragua, and Costa Rica. The aims

109 of this study were: 1) to characterise molecularly the adults and metacercariae of *Uvulifer*

110 sp. across a wide geographic range in Middle America; 2) to link the adult and

111 metacercariae when both developmental stages are sampled, using sequences of both

112 internal transcribed spacers plus 5.8S and LSU of the nuclear ribosomal DNA, and

113 cytochrome *c* oxidase subunit 1 from mitochondrial DNA; 3) to examine the ultrastructure

114 of the body surface of adults using scanning electron microscopy to search for new

115 morphological traits that could be reliable for discriminating among species, and 4) to

116 provide a morphological description of genetically identified metacercariae and adults,

117 where possible.

Materials and methods

119 *Specimen collection*

120   Adults of *Uvulifer* sp. were collected from 11 individuals of the green kingfisher

121 *Chloroceryle americana* (Gmelin) and two of the belted kingfisher *Megaceryle alcyon*,

122 with a shotgun and were dissected within the following 2 h. Their viscera were placed in

123 separate Petri dishes with 0.75% saline solution and examined under a dissecting

124 microscope in four localities in Mexico (table 1). Avian definitive hosts were identified

125 using the field guides of Howell & Webb (1995) and the American Ornithologists' Union

126 (1998). Metacercariae were collected from the fins and skin of 20 species of fish belonging

127 to the families Poeciliidae, Profundulidae, Characidae, Cyprinidae and Cichlidae in 30

128 localities across five countries: Mexico, Guatemala, Nicaragua, Honduras and Costa Rica,

129 from December 2013 through February 2016 (fig.1; table 1). Fish were captured with seine

130 nets and electrofishing, maintained alive and transported to the laboratory, pith sacrificed,

131 and immediately examined. Collected digeneans were fixed by sudden immersion in hot

132 (steaming) 4% formalin for morphological comparisons; others were preserved in 100%

133 ethanol for DNA extraction and sequencing. Fish were identified following Miller *et al*.,

134 (2005).

135

136 *Morphological analyses*

137   The specimens preserved in hot 4% formalin were stained with Mayer`s

138 paracarmine, dehydrated in graded ethanol series, cleared in methyl salicylate, and mounted

139 as permanent slides using Canada balsam. All the specimens were examined using a bright-

140 field Leica DM 1000 LED microscope (Leica, Wetzlar, Germany). Measurements were

141 taken using the Leica Application Suite microscope software; the descriptions are presented

142 in micrometers with the range followed by the mean in parentheses. Drawings were made

143 with the aid of a drawing tube. Some of the adult individuals preserved in 4% formalin

144 were dehydrated through a graded series of ethyl alcohol, and then critical-point dried with

145 carbon dioxide. These specimens were mounted on metal stubs with silver paste, coated

146 with gold, and examined in a Hitachi Stereoscan model SU1510 (Hitachi High-

147 Technologies Mexico S.A.de C.V, Mexico) at 15 kV. Specimens were deposited in the

148 Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional

149 Autónoma de México, México City.

150

151 *DNA extraction, PCR amplification, sequencing and phylogenetic analyses*

152 Seventy-six individuals of *Uvulifer* sp. (64 metacercariae and 12 adults) were placed

153 individually in tubes and digested overnight at 56 °C in a solution containing 10 mM Tris-

154 HCl (pH 7.6), 20 mM NaCl, 100 mM Na2-EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml

155 proteinase K. Following digestion, DNA was extracted using DNAzol reagent (Molecular

156 Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. Two

157 regions of nuclear ribosomal DNA (rDNA) were amplified using the polymerase chain

158 reaction (PCR). The ITS1, 5.8S and ITS2 region was amplified using the forward primer

159 BD1, 5′-GTCGTAACAAGGTTTCCGTA-3′and the reverse primer BD2, 5′-

160 ATCTAGACCGGACTAGGCTGTG-3′ (Bowles & McManus, 1993). The D1-D3,

161 domains of the large subunit (LSU) from ribosomal DNA were amplified using the forward

162 primer BD3, 5′-GAACATCGACATCTTGAACG-3′ (Hernández-Mena *et al*., 2014), and

163 the reverse primer 536, 5′-CAGCTATCCTGAGGGAAAC-3′ (García-Varela & Nadler,

164 2005). The cytochrome *c* oxidase subunit 1 (*cox 1*) of the mitochondrial DNA was

165 amplified using the forward primer JB3, 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ and

166 the reverse primer JB4, 5′-TAAAGAACATAATGAAATTG-3′ (Bowles *et al.*, 1993). PCR

167 reactions (25 µl) consisted of 10 µM of each primer, 2.5 µl of 10 X buffer, 1.5 µl of 2 mM

168 $MgCl_2$, 0.5 µl of dNTP′s (10 mM), 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen

169 Corporation, São Paulo, Brazil) plus 2 µl of the genomic DNA plus 16.7 µl of distillated

170 water. PCR cycling parameters for rDNA amplifications included denaturation at 94 °C for

171 5 min; followed by 35 cycles of 94 °C for 1 min, annealing at 50°C for 1 min for the three

172 molecular markers, and extension at 72 °C for 1 min, followed by a post-amplification

173 incubation at 72 °C for 10 min. Sequencing reactions were performed using ABI Big Dye

174 (Applied Biosystems, Boston, Massachusetts) terminator sequencing chemistry and

175 reaction products were separated and detected using an ABI 3730 capillary DNA

176 sequencer. Contigs were assembled and base-calling differences resolved using Codoncode

177 Aligner version 5.1.2 (Codoncode Corporation, Dedham, Massachusetts). Sequences

178 obtained in the current research for ITS, LSU and *cox 1* were aligned with sequences of

179 other genera of diplostomids downloaded from Genbank, i.e., *Posthodiplostomum* Dubois

180 1936, *Ornithodiplostomum* Dubois 1936, *Diplostomum* von Nordmann 1832, *Tylodelphys*

181 Diesing, 1950, *Austrodiplostomum* Szidat & Nani 1951, *Neodiplostomum* Railliet 1919,

182 and *Alaria* Goeze 1782 and two species of the genus *Bolbophorus* Dubois 1935. In

183 addition, sequences of the strigeids *Australapatemon* Sudarikov 1959, *Parastrigea* Szidat

184 1928 and *Apharyngostrigea* Ciurea 1927 were used as outgroups, since this family is

185 considered to be closely related to Diplostomidae (see Olson *et al.,* 2003). Sequences of

186 each molecular marker were aligned separately using the software Clustal W (Thompson *et*

8
25

187   *al*., 1997). In particular, all sites were unambiguously aligned in the ITS and 28S datasets.

188   Nucleotide substitution model was selected for each molecular marker using jModelTest

189   v0.1.1 (Posada, 2008) and applying the Akaike criterion; for the ITS dataset, selected

190   model was TVM +I+G for Bayesian analysis, and GTRGAMMAI model was used for all

191   Maximum likelihood (ML) analyses. For the LSU dataset, selected model was GTR+I+G

192   and for *cox1* dataset selected model was TPM1uf +I+G. Phylogenetic trees were

193   constructed through Maximum likelihood (ML) with the program RAxML v7.0.4

194   (Stamatakis, 2006). A GTRGAMMAI substitution model was used, and 10,000 bootstrap

195   replicates were run to assess nodal support. We also estimated gene trees using MrBayes

196   3.2.2 (Ronquist *et al*., 2012), with two runs of the Markov chain (MCMC) for 10 million

197   generations, sampled every 1000 generations, a heating parameter value of 0.2 and burn-in

198   (25%). Trees were drawn using FigTree version 1.4.0 (Rambaut, 2012). The genetic

199   divergence among taxa was estimated using uncorrected "*p*" distances with the program

200   MEGA version 6 (Tamura *et al*., 2013).

201   **Results**

202   *Molecular characterization and phylogenetic analyses*

203   In this study, sequences of the ITS1, 5.8S, ITS2, and LSU from rDNA plus *cox 1* of

204   mDNA of *Uvulifer* sp. (64 metacercariae and 12 adults) from five countries: Mexico,

205   Guatemala, Honduras, Nicaragua and Costa Rica (table 1; fig. 1) were aligned with

206   sequences of the other genera of diplostomids and strigeids. The ITS data set included

207   1,037 characters with 92 sequences and yielded a single tree with similar topology to the

208   Bayesian inference (BI) consensus tree (fig. 2). Both trees showed that the genus *Uvulifer* is

209   monophyletic and was conformed by four independent lineages, all very well supported by

210 bootstrap and posterior probability values. Lineage 1 contains metacercariae from two

211 species of fish, i.e. *Astyanax mexicanus* De Filppi (Characidae), and *Gila* sp. (Cyprinidae),

212 whereas immature adults were recovered from the intestine of the belted Kingfisher

213 distributed in Mexico. Lineages 2 and 3 are represented only by metacercariae, and are

214 associated with cichlid fishes in localities across Middle America. Finally, Lineage 4 was

215 conformed by sequences of 34 metacercariae obtained from poeciliids and a single species

216 of a profundulid, whereas sequences of ten mature adults were collected from the intestine

217 of the green Kingfisher in four localities of Mexico (see table 1) and this lineage is

218 described as a new species. The LSU data set included 1, 232 characters with 28 sequences.

219 The phylogenetic analyses inferred with both methods (ML and BI) recovered the same

220 four lineages as the trees inferred with ITS (fig 3). Finally, the *cox1* data set, included 396

221 characters with 29 sequences. The ML and BI trees also supported the presence of four

222 independent lineages within *Uvulifer* sp., with strong bootstrap and posterior probability

223 values (fig. 4).

224

225 *Morphological description*

226 *Uvulifer spinatus* n. sp. (figs. 5a-6; table 2)

227 Description based on 13 adult specimens. Body distinctly bipartite. Forebody oval 20.9-30

228 (25) % of total body length, ventrally concave, covered with papillae on the ventral surface

229 of tegument. Hindbody claviform, longer than forebody HL/FL ratio= 1:2.44–3,63 (3.04),

230 FW/HW ratio 1:0.98-1.28 (1.13). Total length 1,161–1,782 (1,499). Oral sucker oval,

231 muscular, subterminal, 57–71 (61) long by 53–74 (62) wide; longer than ventral sucker;

232 sucker width ratio 1: 1.67-2.33 (1:1.99). Pseudosuckers absent. Ventral sucker subspherical

233 muscular, 21–28 (24) long by 28–35 (31) wide, located close to holdfast organ. Prepharynx

234 absent. Pharynx small, oval, muscular, 34–46 (37) long by 29–35 (32) wide. Oesophagus

235 short 25–32 long (28). Caeca long, terminating at level of posterior margin of ejaculatory

236 pouch. Holdfast organ oval 88–121 (97) long by 97–125 (108) wide, situated near to

237 posterior margin of forebody. Proteolytic gland typically with bipartite appearance, located

238 dorsally at posterior margin of holdfast organ. Testes in tandem, oval, in posterior region of

239 hindbody; anterior testis 80–144 (113) long by 91–125 (108) wide; posterior testis 78–139

240 (104) long by 89–124 (107) wide. Ovary spherical, pretesticular, 49–72 (59) long by 56–64

241 (60) wide, slightly separated from anterior testis in some specimens [6 specimens].

242 Vitellarium in hindbody, extend laterally at some distance from the anterior end of

243 hindbody up to posterior margin of ejaculatory pouch, occupying approximately ¾ of total

244 hindbody length; vitelline reservoir and Mehlis' gland intertesticular. Hindbody covered

245 with conspicouos spines extending from anterior margin to anterior testis level (fig. 5a; fig.

246 6d). Seminal vesicle small, 66–85 (75) long by 36–45 (40) wide, followed by muscular

247 ejaculatory pouch situated dorsally, 110–217 (172) long by 64–109 (80) wide. Copulatory

248 bursa with protrusible genital cone half 71–117 (89) long enclosed by ventrolateral

249 preputial fold; genital pore terminal situated dorsally (fig. 5b). Hermaphroditic duct opens

250 at apex of cone. Eggs 65–81 (73) long by 42–48 (44) wide.

251 *Taxonomic summary: adults*

252 *Type host.* *Chloroceryle americana* Gmelin (green Kingfisher) Cerylidae.

253 *Site of infection*. Intestine.

254   *Type locality*. Rio Atlapexco, Hidalgo, Mexico (21º00´55.6´´ N, 98º20´20.9´´W)

255   *Type material*. Holotype CNHE: 10322; Paratypes CNHE: 10323; Voucher CNHE: 10324

256   *Etymology*. The specific epithet refers to the presence of spines on the tegument extending

257   from the anterior end of hindbody to the level of anterior testis

258   *Morphological description: metacercariae* (fig. 5c)

259   Description is based on six metacercariae found encysted in the fins and skin of their

260   second intermediate host, the Gila topminnow *Poeciliopsis occidentalis* Baird & Girard

261   from Puente Gavilán, Sonora. 'Neascus' type metacercariae. Body distinctly bipartite, 592–

262   677 (636) long by 412–434 (424) wide. Body distinctly bipartite, with calcareous

263   corpuscles. Forebody more or less spatulate, largen thanhindbody. Hindbody bulb to oval-

264   shaped. Oral sucker elongate-oval, muscular terminal 65–75 (70) long by 51–59 (55) wide.

265   Pseudosuckers absent. Ventral sucker smaller than oral sucker, subspherical, fairly

266   muscular, located at margin anterior of holdfast organ 39–49 (44) long by 45–50 (47) wide.

267   Prepharynx absent. Pharynx small, enlogate-oval 31–34 (34) long by 21–23 (22) wide.

268   Oesophagus short. Caeca long, extending to hindbody to anterior level of primordial

269   copulatory bursa. Holdfast organ oval 92–110 (102) long by 86–128 (97) wide. Proteolytic

270   gland located dorsally at posterior margin of holdfast organ. Primordial testes 2, tandem,

271   anterior testis slightly smaller than posterior. Primordial ovary, oval located between testes.

272   Primordial copulatory bursa ovoid; genital pore terminal.

273   *Taxonomic summary: metacercariae*

274   *Type host: Poeciliopsis occidentalis* Baird and Girard

275   *Site of infection.* Fins and skin.

276   *Type locality.* Puente Gavilán, Sonora (29°19.5′00′′N, 110°32.1′00′′W).

277   *Voucher material.* CNHE: 10325

278   *Remarks*

279   The new species belongs to the genus *Uvulifer* because it possesses a bipartite body,

280   forebody oval, hindbody claviform, longer than forebody, ventral sucker smaller than oral

281   sucker, vitellarium in hindbody. Genital cone half-enclosed in prepuce-like folds (see

282   Niewiadomska, 2002). Yamaguti (1934) erected the genus *Uvulifer* with *U. gracilis* as the

283   type species, from specimens collected from the crested Kingfisher (*Ceryle lugubris*

284   Temminck) from Japan. Currently, 18 species of the genus *Uvulifer* have been described

285   worldwide. In the Americas, only five species of *Uvulifer* have been reported, all of them as

286   parasites of alcedines, i.e., *U. ambloplitis* and *U. semicircumcisus* from *Ceryle alcyon* in

287   U.S.A., *U. prosocotyle* in *Ceryle torquata* Linnaeus and *Chloroceryle amazona* Latham in

288   Venezuela and Brasil, *U. weberi* from *Chloroceryle amazona* and *C. americana* in

289   Paraguay, and *U. elongatus* in *Megaceryle torquata* also from Paraguay (Yamaguti, 1971;

290   Dubois, 1985, 1988) (see table 2). The new species described herein, *Uvulifer spinatus* n.

291   sp., can be differentiated from the five species of the Americas by having a tegument

292   covered with spines extending from the anterior part of hindbody to the level of anterior

293   testis (see fig. 5a-6e,f). Additionally, the new species differs from the three species

294   described from South America (*U. prosocotyle, U. weberi,* and *U. elongatus*) by having

295   testes and vesicle seminal smaller (see table 2). Finally, the new species can be further

296   distinguished from the other two congeneric species from North America, i.e., *U.*

ambloplitis and *U. semicircumcisus*, by having smaller eggs and a longer ejaculatory pouch (see table 2).

Discussion

The phylogenetic tree inferred with ITS, LSU and *cox 1* data sets showed that *Uvulifer* is an independent clade with strong bootstrap and posterior probability support values (100/1.0). The genetic divergence between *Uvulifer* and other genera of Diplostomidae ranged from 12 to 19% for ITS, from 5 to 8 % for LSU and from 13 to 18 % for *cox 1*. Our analysis showed a high genetic diversity within the genus *Uvulifer* across Middle America. We detected four major lineages, all well supported (see figs. 2-4). The lowest genetic divergence was found between the Lineage 2 and 3, and ranged from 2 to 3.4% for ITS, from 1.3 to 1.4% for LSU and from 9.3 to 9.6 % for *cox 1*; the highest genetic divergence was found between Lineage 1 and Lineage 4 (described herein as a new species, *Uvulifer spinatus* n. sp.), ranging from 5.7 to 7.8 % for ITS, from 1.4 to 1.6% for LSU and from 9.6 to 12.5 % for *cox 1*. Those values of genetic divergence of ITS and *cox 1* among lineages are similar to those previously found among species of *Diplostomum* (*D. mergi* Dubois, 1932, *D. huronense* (La Rue, 1927)*, and *D. indistinctum* (Guberlet, 1923), with 2 to 4.5% for ITS, and among species of *Tylodelphys* (*T. clavata* von Nordmann, 1832), *T. mashonense* (Sudarikov, 1971)*, Tylodelphys* sp.*, and *T. aztecae* García-Varela, Sereno-Uribe, Pinacho-Pinacho, Hernández-Cruz & Pérez-Ponce de León, 2016) with divergence values between 3 and 9% (see García-Varela *et al*., 2016b and references therein), and

318   among species of *Tylodelphys* the genetic divergence for *cox 1* ranged from 8 to 16.5 %

319   (see Blasco-Costa *et al*., 2017).

320   The intraspecific genetic divergence within lineages 1, 2 and 3, and within *Uvulifer*

321   *spinatus* n. sp ranged from 0 to 1.4 % for ITS, from 0 to 1.8 % for *cox 1*, and for LSU the

322   sequences of all isolates were identical. These ranges of intraspecific genetic divergence are

323   also similar to those previously described for congeneric diplostomids; *Tylodelphy*s sp., *T.*

324   *aztecae* and *T. mashonense* showed a divergence from 0 to 1.4% for ITS (see Chibwana *et*

325   *al*., 2013, 2015; García-Varela *et al*., 2016b), and among isolates of *D. baeri* Dubois, 1937

326   varied from 0 to 0.4% (see Blasco-Costa *et al*., 2014). Finally among isolates of

327   *Tylodelphys* spp., the genetic divergence ranged from 0.2 to 1.2 % (see Blasco-Costa *et al*.,

328   2017).

329   The identification of the metacercariae found encysted in the fins and skin in freshwater

330   fishes of Mexico has been problematic. The metacercariae of *Uvulifer* causing the black

331   spot disease have been indistinctly determined taxonomically either as *Uvulifer* sp., or as *U.*

332   *ambloplitis*. This diplostomid has been recorded from at least 45 species of fish belonging

333   to 10 unrelated families (Atherinopsidae, Cichlidae, Characidae, Cyprinidae, Eleotridae,

334   Gobiidae, Heptapteridae, Mugilidae, Godeidae and Poeciliidae) (see Pérez-Ponce de León

335   *et al*., 2007). However, most of the records of the metacercariae of *Uvulifer* in Mexico from

336   hosts of two fish families, including cichlids in 18 of the 45 host species (40 %), and eight

337   species of poeciliids (19 %) (see Pérez-Ponce de León *et al*., 2007 and references therein;

338   García Magaña & López-Jiménez, 2008; Pérez-Ponce de León *et al*., 2010; Salgado-

339   Maldonado *et al*., 2014; Bautista-Hernández *et al*., 2014).

340   In this study, the adult and metacercarial stages of species of *Uvulifer* were

341   characterised for the first time using an integrative taxonomic approach, combining

342   morphology and DNA sequences, and we were able to link the metacercariae with the

343   adults, at least in two of the four recognized lineages widely distributed across Middle

344   America. In addition, the adults of *Uvulifer* seem to be very specific to alcedines across the

345   world, and our study revealed an apparently well-defined host specificity pattern of the

346   metacercariae in their second intermediate hosts, a pattern that has been found in other

347   metacercariae in freshwater fishes (see Locke *et al.*, 2010a; Pérez-Ponce de León *et al.*,

348   2016). The metacercariae of *Uvulifer spinatus* n. sp. is only found parasitising poeciliids;

349   Lineage 1 is associated with Characidae (*Astyanax mexicanus*) and Cyprinidae (*Gila* sp.),

350   two unrelated families of freshwater fish, with Neotropical and Nearctic affinity,

351   respectively. Unfortunately, adults recovered from the intestine of the belted Kingfisher

352   were immature and prevented the formal taxonomic description of this species. Lineages 2

353   and 3 were found parasitizing exclusively cichlids across a wide geographic range

354   comprising Mexico, Honduras, Nicaragua and Costa Rica, and even in two localities, one in

355   Mexico and one in Honduras, both lineages occurred in sympatry (see table 1; fig.1). The

356   association of a metacercaria and a cichlid fish across the same geographical region was

357   also found in a genetic lineage of *Clinostomum* (see Pérez-Ponce de León *et al.*, 2016).

358   Interestingly, the metacercariae of the most widely distributed species of *Uvulifer* in

359   North America, and the agent causative of the black spot disease in many freshwater fish

360   species in the U.S.A. and Canada (*U. ambloplitis*) has been found mainly in centrarchiids

361   (Hoffman & Putz, 1965; Berra & Ray-Jean, 1978; Lemly & Esch, 1983, 1984a, 1984b,

362   1985; Camp, 1988; Wilson & Camp, 2003). In Mexico, four species of the family

363   Centrarchidae, i.e., *Lepomis machochirus* Rafinesque, *L. megalotis* Rafinesque,

364   *Micropterus salmoides* Lacépède and *Pomoxis annularis* Rafinesque, have been examined

365   to a certain extent for helminth parasites. During the course of this investigation we also

366   analysed 10 specimens of *M. salmoides* from Purificacion River in northern Mexico (see

367   Locality 13; table 1; fig 1.). However, the metacercariae of *Uvulifer* has not been reported

368   from any species of that host family (see Pérez-Ponce de León *et al*., 2007, 2010; Pérez-

369   Ponce de León & Choudhury, 2010) which represents a typically Nearctic fish group.

370   Instead, we found the metacercariae infecting neotropical freshwater fish species such as

371   characids, cichlids and poeciliids, and even some endemic species such as atherinopsids.

372   This study reinforces the view that metacercariae of some trematodes show a preference to

373   infect certain fish species, and it seems that such host specificity is more strongly related to

374   the physiological compatibility of host and parasite species, than to ecological factors

375   (Hoffman & Putz, 1965; Locke *et al*., 2010b; Perez-Ponce de León *et al*., 2016). In

376   addition, to better understand the life cycle of species of *Uvulifer*, as well as to elucidate

377   some aspects of their evolutionary history and basic biology, it is also necessary to

378   characterize, morphologically and molecularly, the cercarial stage released by their snail

379   intermediate host (see Blasco-Costa & Poulin, 2017). An assessment of all the stages of the

380   life cycle will increase our chances to describe the cryptic diversity patterns among

381   *Uvulifer* spp.

382   Without molecular evidence, and without adult forms obtained from fish-eating birds,

383   the genetic lineages of *Uvulifer*, and the new species uncovered in our study, would have

384   been probably considered to represent a single species, and would have been designated as

385   *Uvulifer* sp., or even *U. ambloplitis* as previously recorded in other studies (see Pérez-

17

386 Ponce de León *et al*., 2007; Salgado-Maldonado *et al*., 2014; Bautista-Hernández *et al*.,

387 2014). The data generated in this study, and the use of an integrative taxonomy approach,

388 represent the first step of a more detailed study on the taxonomy, evolution and

389 biogeography of the genus *Uvulifer*. Sequencing work of *Uvulifer* metacercariae from

390 centrarchiids across USA and Canada are required to test the host specificity hypothesis for

391 the metacercariae. Finally, the formal description of the other three lineages detected in our

392 study requires further sampling of the alcedine definitive hosts to obtain gravid specimens

393 to conduct the morphological study.

394

408 **Conflict of interest**

409 None.

410 **Ethical standards**

411 Specimens in Mexico were collected under the Cartilla Nacional de Colector Científico

412 (FAUT 0202 and 0057) issued by the Secretaría del Medio Ambiente y Recursos Naturales

413 (SEMARNAT), to MGV and GPPL, respectively.

414

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599 **Table captions**

600 Table 1. Specimens information including collections sites, geographical coordinates, host

601 species, Life-Cycle stage, Adult (A), Metacercariae (M); Genbank accession numbers

602 of ITS, 28S and *cox*1. The number of the localities corresponds with the numbers in

603 Figure 1.

604 Table 2. Comparative morphometrics (in microns) of adult worms of *Uvulifer spinatus* n.

605 sp. with congeneric species from the Americas.

606 **Figure captions.**

607 Fig.1. Sampling sites of specimens of *Uvulifer* in Middle America. Localities with a circle

608 represent Lineage 1 (●), Lineage 2 is represented with the symbol (■), Lineage 3 (◆), and

609 the new species described *Uvulifer spinatus* n. sp. (★). Localities with a shading represent

610 two lineages occurring in sympatry. Collection sites are numbered according to Table 1.

611 Fig.2. Maximum likelihood tree inferred with ITS1, 5.8S and ITS2 data set. Numbers near

612 internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI).

613 Fig.3. Maximum likelihood tree inferred with LSU data set. Numbers near internal nodes

614 show ML bootstrap clade frequencies and posterior probabilities (BI).

615 Fig.4. Maximum likelihood tree inferred with *cox 1* data set. Numbers near internal nodes

616 show ML bootstrap clade frequencies and posterior probabilities (BI).

617 Fig.5. *Uvulifer spinatus* n. sp. Holotype, (a) Adult, obtained from the intestine of

618 *Chloroceryle americana* in Mexico, scale bar= 200 μm; (b) Enlarged lateral view of

619 terminal genitalia of paratype CNHE 10323, scale bar=200 μm; (c) Metacercaria from

620 *Poeciliopsis occidentalis* in Mexico, scale bar=100 μm. Abbreviations: OS, oral sucker; F,

621 faringe; OE, oesophagus; VS, ventral sucker; HO, holdfast organ; PG, proteolityc gland; C,

622 caeca; S, spines; V, vitellarium; E, eggs; OV, ovary; AT, anterior testis; PT, posterior testis;

623 VR, vitelline reservoir; MG, Mehlis' gland; SV, seminal vesicle; EP, ejaculatory pouch; U,

624 uterus; CG, genital cone; GP, genital pore; CB, copulatory bursa.

625

626 Fig.6. Scanning electron micrographs of adult *Uvulifer spinatus* n. sp. (a) Entire specimen

627 from *Chloroceryle americana* from Mexico, scale bar=400 μm; (b) Forebody, scale

29

628 bar=100 µm; (c) Tegument of the ventral surface of forebody showing papillae, scale bar=5

629 µm; (d) Holdfast organ, scale bar=20 µm; (e) Hindbody, scale bar=25 µm; (f) Spines, scale

630 bar=10 µm; (g) Copulatory bursa, scale bar=25 µm.

Table 1

Table 1. Specimens information including collections sites, geographical coordinates, host species, Life-Cycle stage, Adult (A), Metacercaria (M); Genbank accession numbers of ITS, 28S and *cox*1. The number of the localities corresponds with the numbers in the Figure 1.

CS	Locality	Coordinates	Host	Family	Life-Cycle stage	GenBank accession number			Taxa
						ITS	28S	*cox*1	
	Mexico Hidalgo State								
1	Río Malila	20°43′60′′N 48°43′00′′W	*Xiphophorus malinche*	Poeciliidae	M	MF568606			*U. spinatus* n. sp.
2	Río Atlapexco	21°00′55.6′′N 98°20′20.9′′W	*Megaceryle alcyon*	Cerylidae	A A	MF568630 MF568631	MF568567 MF568569	MF568659 MF568660	Lineage 1
			Poecilia mexicana	Poeciliidae	M M	MF568604 MF568605			*U. spinatus* n. sp.
			Chloroceryle americana	Cerylidae	A	MF568632		MF568676	*U. spinatus* n. sp.
3	San Felipe Orizatlán	21°09′57.7′′N 98°36′20.1′′W	*Xiphophorus cortezi*	Poeciliidae	M	MF568633			*U. spinatus* n. sp.
			Herichthys labridens	Cichlidae	M	MF568634	MF568570	MF568663	Lineage 2
4	Laguna de Metztitlan	20° 41′ 45.1′′N 98° 50′ 11.5′′W	*Herichthys labridens*	Cichlidae	M M	MF568635 MF568636	MF568574 MF568571	MF568661 MF568664	Lineage 2
	Jalisco State								
5	Río Grande	19° 14′ 44.9′′N 102° 46′ 15.7′′W	*Cichlasoma istlanum*	Cichlidae	M	MF568612			Lineage 3
	Morelos, State								
6	Huizatlan	18° 28′ 50.4′′N 99° 09′ 56.1′′W	*Amatitlania nigrofasciata*	Cichlidae	M M	MF568637 MF568638	MF568576 MF568575	MF615998 MF568670	Lineage 3

No	Location	Coordinates	Species	Family		GenBank			Notes
					M	MF568639			
7	Río Amacuzac Oaxaca State	18° 35′ 51.6′′ N 99° 22′ 36.9′′W	*Amatitlania nigrofasciata*	Cichlidae	M M	MF568640 MF568641	MF568579 MF568578	MF568669 MF568671	Lineage 3
8	Río Cuilapam	16°59′54.2′′N 96°47′38.4′′W	*Poecilia sphenops*	Poeciliidae	M	MF568609			*U. spinatus* n.sp.
			Profundulus oaxacae	Profundulidae	M	MF568611			*U. spinatus* n. sp.
9	Arroyo La Manzanita	17°06′18.3′′N 96°47′23.2′′W	*Profundulus oaxacae*	Profundulidae	M	MF568610			*U. spinatus* n. sp.
10	Presa Los Ocotes	16º36′57′′N 96º43′13′′W	*Chloroceryle americana*	Cerylidae	A A A A A	MF568583 MF568584 MF568585 MF568586 MF568587			*U. spinatus* n. sp.
			Poeciliopsis gracilis	Poeciliidae	M	MF568588			*U. spinatus* n. sp.
11	Matias Romero San Luis Potosi State	16°47′30.8′′N 95°00′59′′W	*Xiphophorus helleri*	Poeciliidae	M M	MF568600 MF568601			*U. spinatus* n. sp.
12	Axtla de Terrazas Tamaulipas State	21°28′2.7′′N 98°57′11.3′′W	*Astyanax mexicanus*	Characidae	M M	MF568628 MF568629	MF568568		Lineage 1
13	Río Purificacion	24°05′21.4′′N 99°09′54′′W	*Chloroceryle americana*	Cerylidae	A A	MF568616 MF568617	MF568581 MF568580	MF568680 MF568677	*U. spinatus* n. sp.
			Herichthys cyanoguttatus	Cichlidae	M	MF568621	MF568572		Lineage 2
			Poecilia formosa	Poeciliidae	M	MF568618			*U. spinatus* n. sp.

No.	Locality	Coordinates	Species	Family					
14	Río Conchos	24°45′56.1′′N 97°59′55.5′′W	*Chloroceryle americana*	Cerylidae	A	MF568622		MF568682	*U. spinatus* n. sp.
					A	MF568623		MF568678	
					A	MF568624			
15	Soto La Marina	23°42′55′′N 98°49′07′′W	*Poecilia mexicana*	Poeciliidae	M	MF568614	MF568582	MF568681	*U. spinatus* n. sp.
					M	MF568615			
16	Puente Guemez	23°54′43.2′′N 99°06′48.6′′W	*Poecilia formosa*	Poeciliidae	M	MF568619		MF568679	*U. spinatus* n. sp.
					M	MF568620			
17	Río Frio	22°58′11.3′′N 98°59′36.1′′W	*Herichthys cyanoguttatus*	Cichlidae	M	MF568627	MF568573	MF568662	Lineage 2
			Herichthys labridens	Cichlidae	M	MF568625			Lineage 3
					M	MF568626			
18	Río Aldama	22°52′42.1 N 98°11′51.9′′W	*Herichthys cyanoguttatus*	Cichlidae	M	MF568613			Lineage 3
	Sonora State								
19	Puente Gavilan	29°19.5′00′′N 110°32.1′00′′W	*Poeciliopsis occidentali*	Poeciliidae	M	MF568654			*U. spinatus* n. sp.
					M	MF568655			
			Gila sp.	Cyprinidae	M	MF568658			Lineage 1
20	Puente La Ventana	28°22.6′00′′N 108°53.8′00′′W	*Poeciliopsis* sp.	Poeciliidae	M	MF568656			*U. spinatus* n. sp.
					M	MF568657			
	Costa Rica								
21	Río Orosi	11°02′50′′N 85°22′48′′W	*Hypsophrys nematopus*	Cichlidae	M	MF568607		MF568666	Lineage 2
					M	MF568608		MF568667	
	Guatemala								
22	Puente San Sare	14°44′52′′N 90°06′33′′W	*Heterandria* sp.	Poeciliidae	M	MF568589			*U. spinatus* n. sp.
					M	MF568590			
23	Río Encarnación	14°56′8′′N 90°03′90′′W	*Poecilia* sp.	Poeciliidae	M	MF568594			*U. spinatus* n. sp.
					M	MF568595		MF568674	
						MF568596			
24	Hacienda La Vega	14°54′59.6′′N 90°06′1′′W	*Heterandria* sp.	Poeciliidae	M	MF568591			*U. spinatus* n. sp.
					M	MF568592			
					M	MF568593			
	Honduras								

No.	Locality	Coordinates	Species	Family	M	Accession 1	Accession 2	Accession 3	Lineage
25	Los Potrerillos	14°32′31″N 87°52′54.9″W	*Alfaro huberi*	Poeciliidae	M	MF568597			*U. spinatus* n. sp.
					M	MF568598		MF568675	
					M	MF568599			
26	Río Chiquila	15°13′50″N 88°35′19″W	*Thorichthys* sp.	Cichlidae	M	MF568602			Lineage 2
					M	MF568603			
									Lineage 3
	Nicaragua								
27	Blue Fields	12°00′10″N 83°96′39″W	*Alfaro cultratus*	Poeciliidae	M	MF568642			*U. spinatus* n. sp.
			Poecilia mexicana	Poeciliidae	M	MF568643			*U. spinatus* n. sp.
					M	MF568644			
28	Río Perez	11°45′0.8″N 84°14′11.4″W	*Amatitlania siquia*	Cichlidae	M	MF568645			Lineage 3
					M	MF568646	MF568577	MF568673	
					M	MF568647			
29	San Carlos	11°08′54.7″N 84°42′50.1″W	*Amphilophus longimanus*	Cichlidae	M	MF568648		MF568672	Lineage 3
					M	MF568653			
30	Palo de Arquito	11°07′12.3″N 84°36′5.3″W	*Hypsophrys* sp.	Cichlidae	M	MF568649			Lineage 2
					M	MF568650		MF568665	
			Archocentrus centrarchus	Cichlidae	M	MF568651		MF568668	Lineage 2
					M	MF568652			

Table 2

Table 2. Comparative morphometrics (in microns) of adult worms of *Uvulifer spinatus* n. sp. with congeneric species from the Americas. L (length); W (widht).

| Species	*U. ambloplitis* Hunter, 1933	*U. ambloplitis* [*syn. U. erraticus* Chandler & Rausch, 1948]	*U. ambloplitis* [*syn. U. claviformis* Dubois & Rausch, 1948]	*U. ambloplitis* [*syn. U. magnibursiger* Dubois & Rausch, 1950]	*U. semicircumcisus* Dubois and Raush, 1950	*U. prosocotyle* (Lutz, 1928) Dubois, 1937	*U. weberi* Dubois, 1985	*U. weberi* Dubois, 1988	*U.elongatus* Dubois, 1988	*U. spinatus* n. sp.
Locality	New York, USA	Ohio, USA	Michigan, USA	Michigan, USA	Michigan, USA	Brazil	Paraguay	Paraguay	Paraguay	Rio Atlapexco, Hidalgo, Mexico
Host	*Megaceryle alcyon*	*Toxostoma rufum*	*Megaceryle alcyon*	*Megaceryle alcyon*	*Megaceryle alcyon*	*Megaceryle torquata*	*Chloroceryle amazona*	*Chloroceryle americana*	*Megaceryle torquata*	*Chloroceryle americana*
Body (L)		1,080–1,550	2,200–2,530	2,190–2,750	1,600–1,860	950	700–1,400	400–1,750	2,200–3,300	1,161–1,782
Forebody (L)	480–620	415–462	430	450–480	390–520	210–480	270–330	240–390	330–500	276–439
Forebody (W)	260–290	164–193	210	210–240	270–320	140–220	200–230	210–270	360–440	204–227
Hindbody (L)	1,360–1,700	638–1,100	1,680–2,100	1,710–2,250	1,260–1,620	580–720	420–1,060	500–1,070	1,800–2,500	800–1,327
Hindbody (W)	280–490	193–264	355–370	340–420	270–400	160–220	160–210	140–250	250–360	110–195
Oral sucker (L)	84	24–35	39	53–64	48–65	65–77	45–57	40–57	52–68	52–71
Oral sucker (W)	120	–	77	85–91	60–80	77–100	48–57	47–60	68–92	53–74
Pharynx (L)	52–63	45	36	43–45	–	43–70	28–40	30–32	45–55	34–46
Pharynx (W)	40–45	–	38	41–50	–	24–31	16–26	16–28	30–37	29–35
Ventral sucker (L)	44–52	–	41	35	40–49	22–33	21–42	22–28	85–100	21–28
Ventral sucker (W)	45–56	–	44	38	–	31–43	31–52	30–36	100–120	28–35
Holdfast organ (L)	83–120	88–90	80–100	–	120–170	36–100	63–85	60–95	130–185	88–121

Holdfast organ (W)	87–130	–	108	–	–	36–110	63–95	60–80	130–140	97–125
Vesicle seminal (L)	180–280	–	–	160	–	–	–	–	105–440	66–85
Vesicle seminal (W)	170	–	–	100	–	–	–	–	60–95	36–25
Anterior testis (L)	400	150–160	115	225–260	260–350	84–120	85–140	105	200–360	80–144
Anterior testis (W)	290	–	165	270–295	180–300	96–132	140–190	115	105–170	91–125
Posterior testis (L)	330	150–160	115	225–270	290–400	77–135	100–110	120	170–300	78–139
Posterior testis (W)	290	–	180	270–285	200–295	96–130	160–190	140	110–160	89–124
Ovary (L)	58–91	70–77	100	105–135	108–120	36–60	65–85	60–80	85–120	49–72
Ovary (W)	100–120	–	–	125–153	–	43–67	80–115	60–85	85–90	56–64
Ejaculatory pouch (L)	88–120	–	160	–	90–120	30–38	63–220	80–210	115–200	110–217
Ejaculatory pouch (W)	65–70	–	130	–	63–85	24–30	42–95	45–120	45–70	64–109
Eggs (L)	90–99	96–103	98–105	95	80–102	–	78–90	78–89	80–90	65–81
Eggs (W)	56–66	58–61	62–75	66	53–67	–	45–50	42–52	42–50	42–48

Figure 1

54
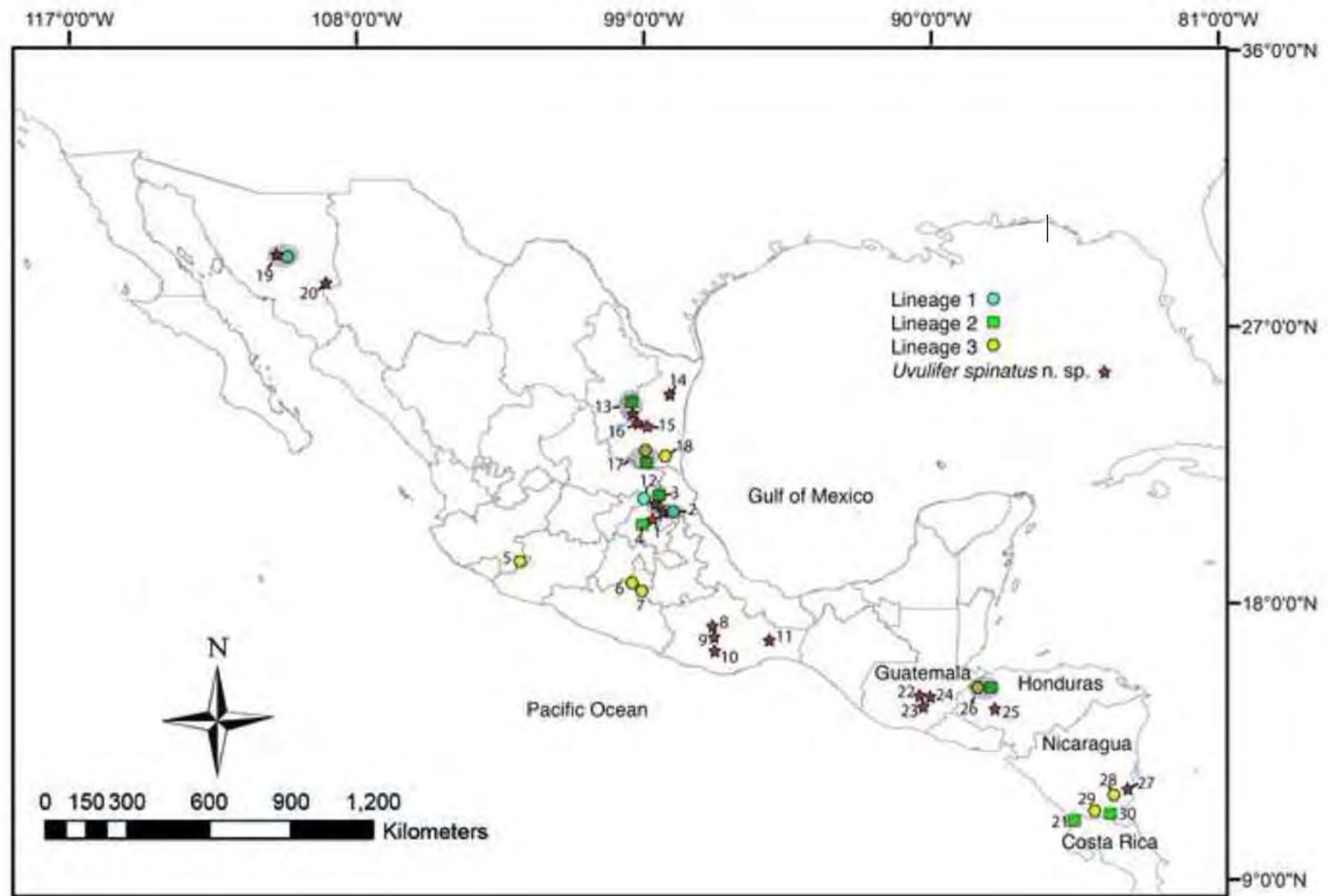
Figure 2   Click here to download Figure Fig.2.tiff ⬇

Figure 3

Figure 4

Figure 4

Figure 5

Figure 6
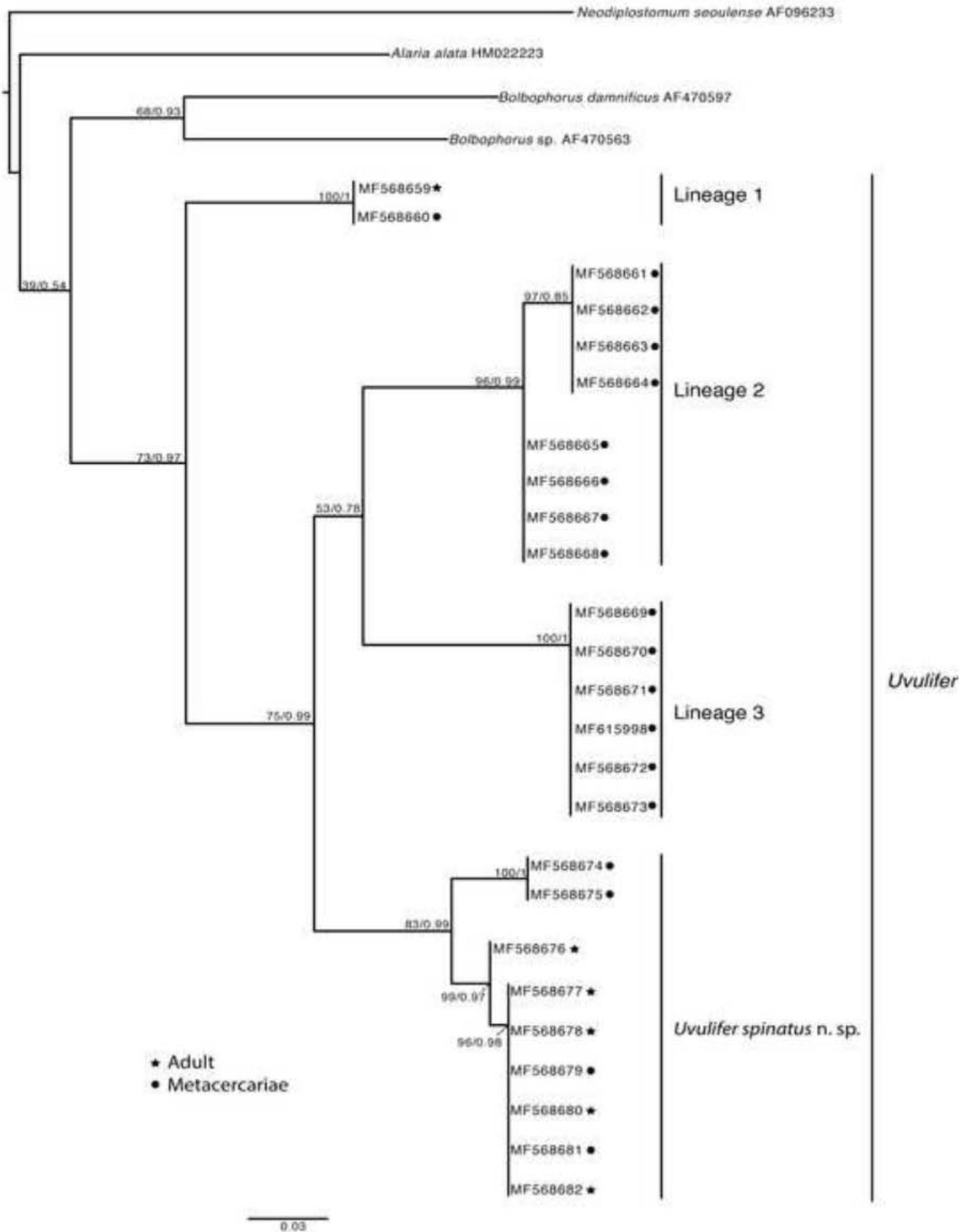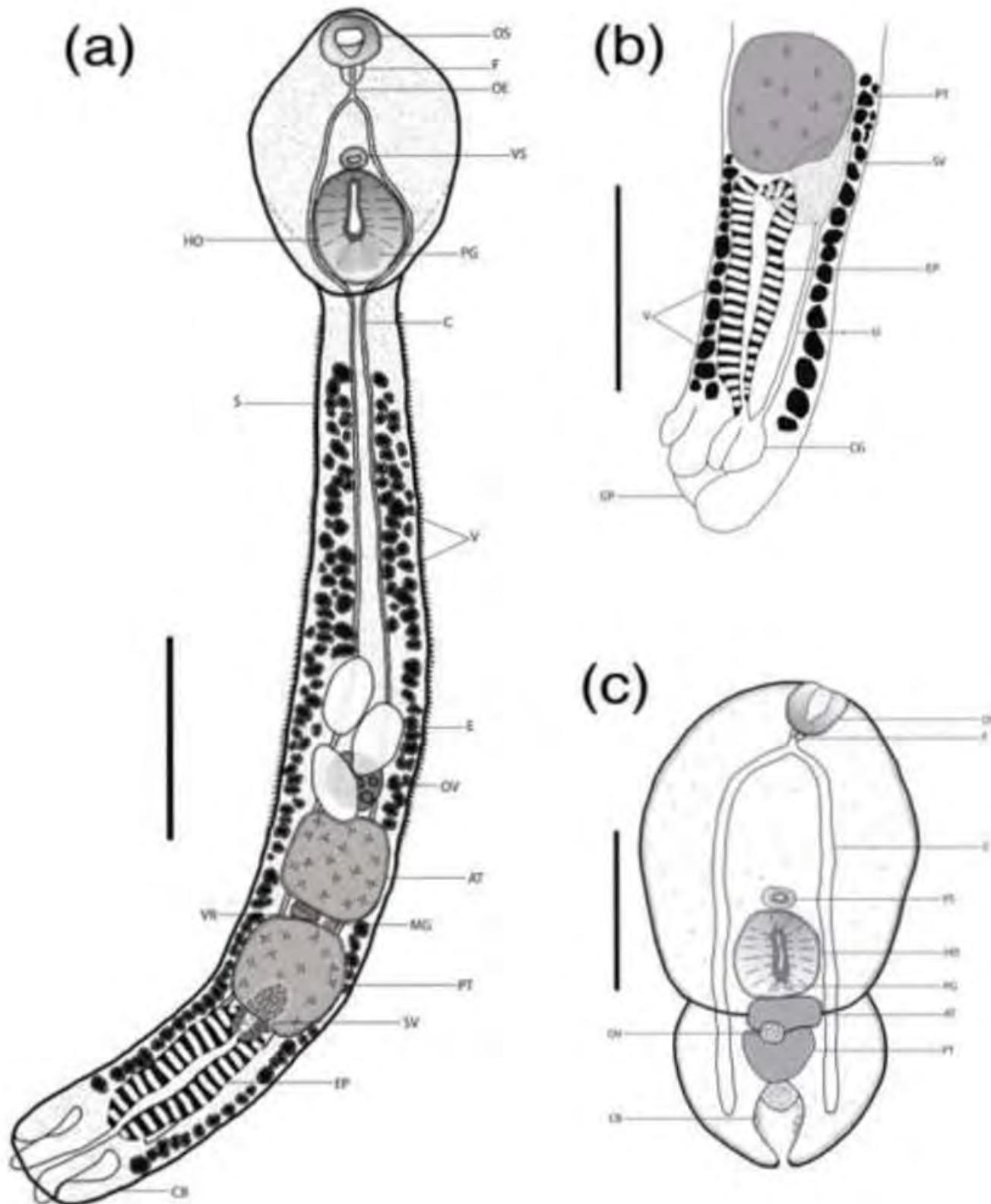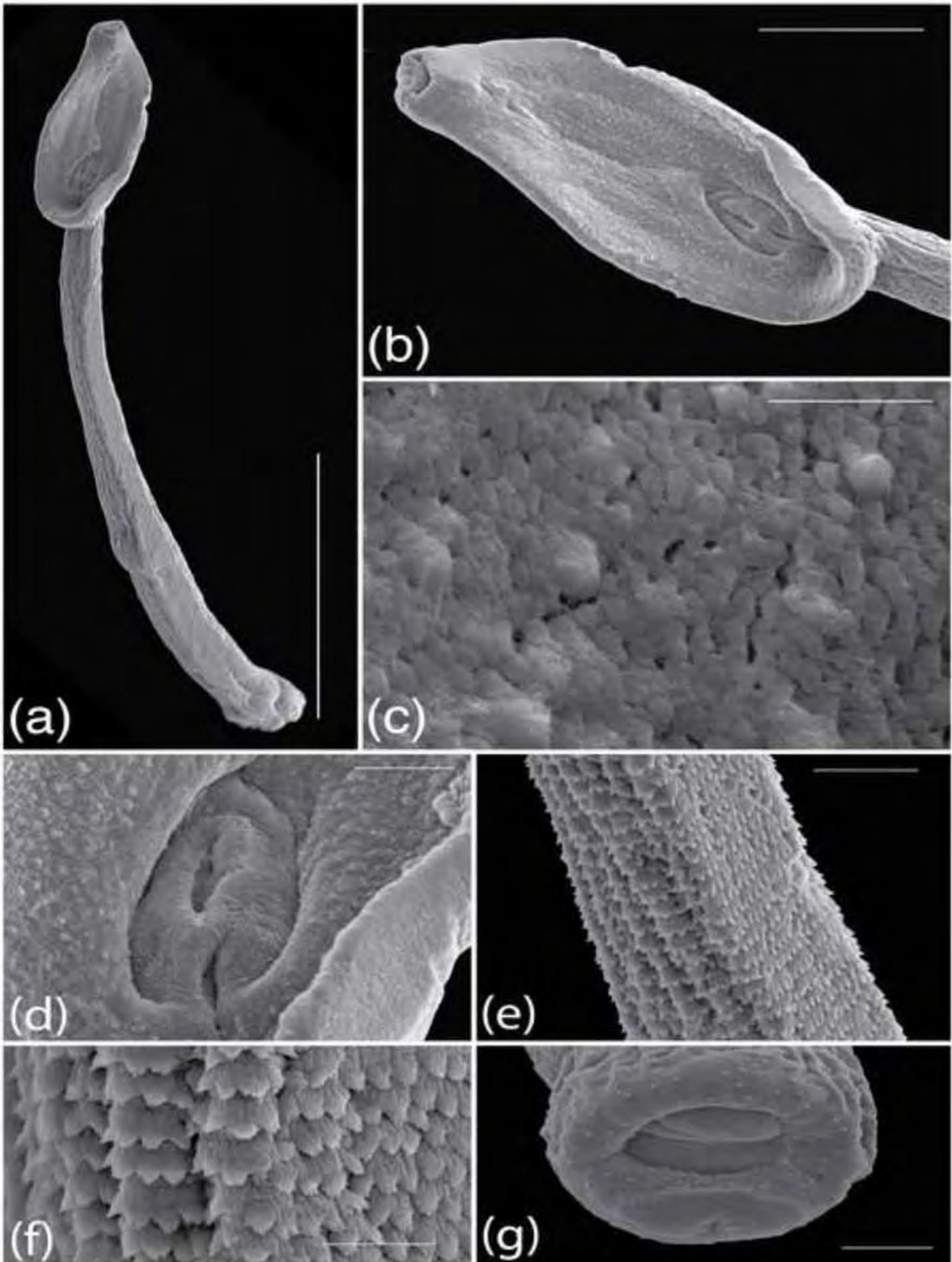
IV. DISCUSIÓN GENERAL

Actualmente, el uso de herramientas moleculares ha permitido la exploración de información genética de los organismos de manera general y particularmente de los helmintos, así como el desarrollo de estrategias metodológicas con el propósito de delimitar a las especies (Carstens *et al*., 2013).

El genéro *Uvulifer* ha sido un grupo controversial en México, ya que se ha identificado a partir de estadios larvales y se ha clasificado indistintamente como *U. ambloplitis* o *Uvulifer* sp. El género se ha registrado en 45 especies de peces pertenecientes a 10 familias, en donde el 40% se ha encontrado principalmente en cílidos y un 19% en poeciilidos (García Magaña & López-Jiménez, 2008; Pérez-Ponce de León *et al*., 2007, 2010; Salgado-Maldonado *et al*., 2014; Bautista-Hernández *et al*., 2014). Sin embargo, la carencia de formas adultas de este trematodo en el país pone en duda la identificación a nivel de especie de los registros citados anteriormente.

En este trabajo se realizó una prospección molecular del género *Uvulifer* a través de la colecta de los segundos hospederos intermediarios y definitivos, con el propósito de explorar la variación genética entre los individuos distribuidos en México y en cuatro países de Centroamérica (Guatemala, Honduras, Niacaragua y Costa Rica) (Fig. 5). En este sentido, el resultado de los análisis filogenéticos del género *Uvulifer* obtenidos mediante el estudio de tres marcadores moleculares (ITS1, 5.8S, ITS2), 28S y COI muestran consistentemente cuatro linajes independientes con valores altos de apoyo tanto de boostrap como de probabilidades posteriores (Fig. 6-8).

Con base en caracteres morfológicos del estadio adulto colectados del hospedero *Chloroceryle americana* en cuatro localidaes de Mexico y de las metacercarias colectadas en poeciliidos y profundúlidos se describió una nueva especie, los adultos de esta especie presentan un carácter nunca antes registado en otras espcies congenéricas como lo es la presencia de espinas en el segmento posterior del cuerpo (Fig. 9-10).

Los análisis filogenéticos revelaron un cierto patrón de especificidad hospedatoria hacia el segundo huésped intermediario. El linaje 1 esta asociado a dos especies de peces no relacionados filogenéticaente (*Astyanax mexicanus* De Filippi y *Gila* sp.). Genéticamente las metacercarias son idénticas a los adultos colectados del hospedero *Megaceryle torquata*, sin embargo por la inmadurez de los ejemplares no se describe la especie en el presente trabajo. Los linajes 2 y 3 están asociados a peces de la familia Cichlidae distribuidos en tres países de Centroamérica (Honduras, Nicaragua y Costa Rica) y en 13 localidades de México. La falta de adultos en estos linajes impidió una descripción formal de estas especies. El linaje 4 fue descrito como una nueva especie, la cual está asociada primordialmente a peces de las familias Poeciilidae y Profundulidae distribuidas en tres países de Centroamérica (Guatemala, Honduras y Nicaragua) y varias localidades de México. El patrón hospedatorio entre las metacercarias y sus segundos huéspedes intermediarios aparentemente tiene un componente filogenético que debe ser puesto a prueba con más ejemplares asociados a diferentes huéspedes. Cabe señalar que un patrón de especificidad hospedatoria similar ha sido detectado en otros grupos de tremátodos de los géneros *Diplostomum* y *Clinostomum* (Locke *et al*., 2010; Pérez-Ponce de León *et al*., 2016b).

Hoffman y Putz (1965) propusieron un patrón de especificidad entre *U. ambloplitis* y peces de la familia Centrarchidae; interesantemente, en nuestro estudio también reconocimos un patrón de especificidad en los cuatro linajes propuestos. En el presente trabajo, se revisaron 10 individuos de peces centrárquidos (*Micropterus salmoides* Lacepede) en una localidad del estado de Tamaulipas (Río Purificación) en busca de metacercarias, sin embargo, no se encontró ningún pez parasitado. Además, han sido examinadas otras especies de centrárquidos en la región Neártica de México y hasta ahora ninguna metacercaria de *Uvulifer* ha sido registrada (Pérez-Ponce de León *et al.,* 2007, 2010; Pérez-Ponce de León & Choudhury, 2010). Esta nueva información apoya la hipótesis de que *U. ambloplitis* esta asociado a peces de centrarquidos de Norteamérica. La información generada en el presente trabajo permitió reconocer diferentes linajes del género *Uvulifer* para México y Centroamérica que no habian sido detectados por las fuentes de información tradicionales (taxonomía alfa).

V. CONCLUSIONES

1. Los análisis filogenéticos derivados de los genes nucleares y mitocondriales, reconocieron 4 linajes independientes con altos valores de boostrap y probabilidades posteriores.

2. Se reconoció una nueva especie del género *Uvulifer* a partir de metacercarias y adultos maduros. La nueva especie se distingue morfológicamente de las otras 5 especies de *Uvulifer* descritas previamente en el Continente Americano por presentar espinas en la parte posterior del cuerpo y por presentar un bolsa eyaculadora más grande, así como vesícula seminal y huevos más pequeños.

3. Se reconoció un patrón de especificidad hospedatoria. Las metacercarias del linaje 1 están asociadas con peces de las familias Characidae y Cyprinidae. Metacercarias de los linajes 2 y 3 están asociadas con peces de la familia Cichlidae, finalmente el linaje 4 está asociado a peces de las familias Poeciilidae y Profundulidae.

4. Los 4 linajes detectados en este trabajo tienen un amplio rango de distribución, probablemente debido a la migración del hospedero definitivo. Por ejemplo el linaje 1 se distribuye en los estados de Sonora, San Luis Potosí e Hidalgo en México. Los linajes 2 y 3 se distribuyen en 4 paises: México, Honduras, Nicaragua y Costa Rica. Finalmente, el linaje 4 se distribuye en 4 paises: México, Guatemala, Honduras y Nicaragua.

5. Con la descripción de la nueva especie de *Uvulifer* se incrementa a 19 especies conocidas del género asociadas con aves de la familia Cerylidae.

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