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

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ABSTRACT

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

1. Introduction

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

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

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

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

(*continued on next page*)

Table 1 (*continued*)

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

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

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

[a] Measured from the published figure.

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

In the current research, we analyzed, for the first time, the *cox* 1 gene to delineate a few species within the genus *Saccocoelioides* distributed in Middle America associated primarily with freshwater fishes. Our analyses inferred with *cox* 1 clearly distinguished species previously recognized within *Saccocoelioides* with two nuclear molecular markers. The combination of mitochondrial and nuclear markers plus morphological and ecological characteristics allowed us to recognize a new species of *Saccocoelioides* associated with freshwater and brackish fishes from the Gulf of Mexico. In addition, we provide new morphological data and extend the distribution range of *S. tkachi*, a parasite of freshwater fishes from Middle America.

2. Materials and methods

2.1. Specimen collection

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

2.2. Morphological analyses

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

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

2.3. Amplification and sequencing of DNA

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

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

2.4. Alignments and phylogenetic analyses

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

3. Results

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

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

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

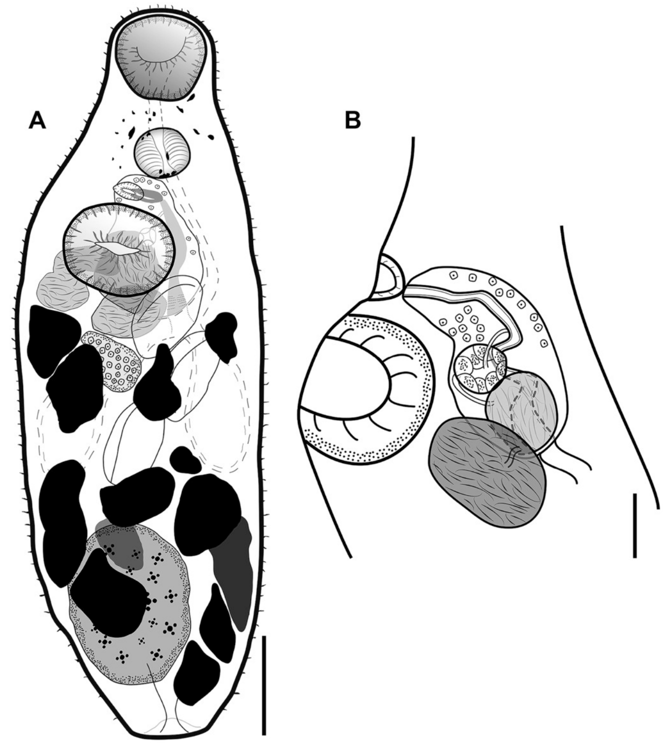

Fig. 1. *Saccocoelioides macrospinosus* n. sp., from *Poecilia catemaconis* (A) whole worm, holotype, ventral view; (B) Hermaphroditic sac, paratype, ventral view; Scale bars = 100 μm (A); 50 μm (B).

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

Type host: *Poecilia catemaconis* Miller, 1975

Additional host: *Mugil curema* Valenciennes, 1836

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

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

Site of infection: Intestine

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

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

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

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

3.2. Remarks

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

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

3.3. Morphological description

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

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

Type host: *Astyanax aeneus* Günther, 1860.

Additional host: *Astyanax fasciatus* Cuvier, 1819.

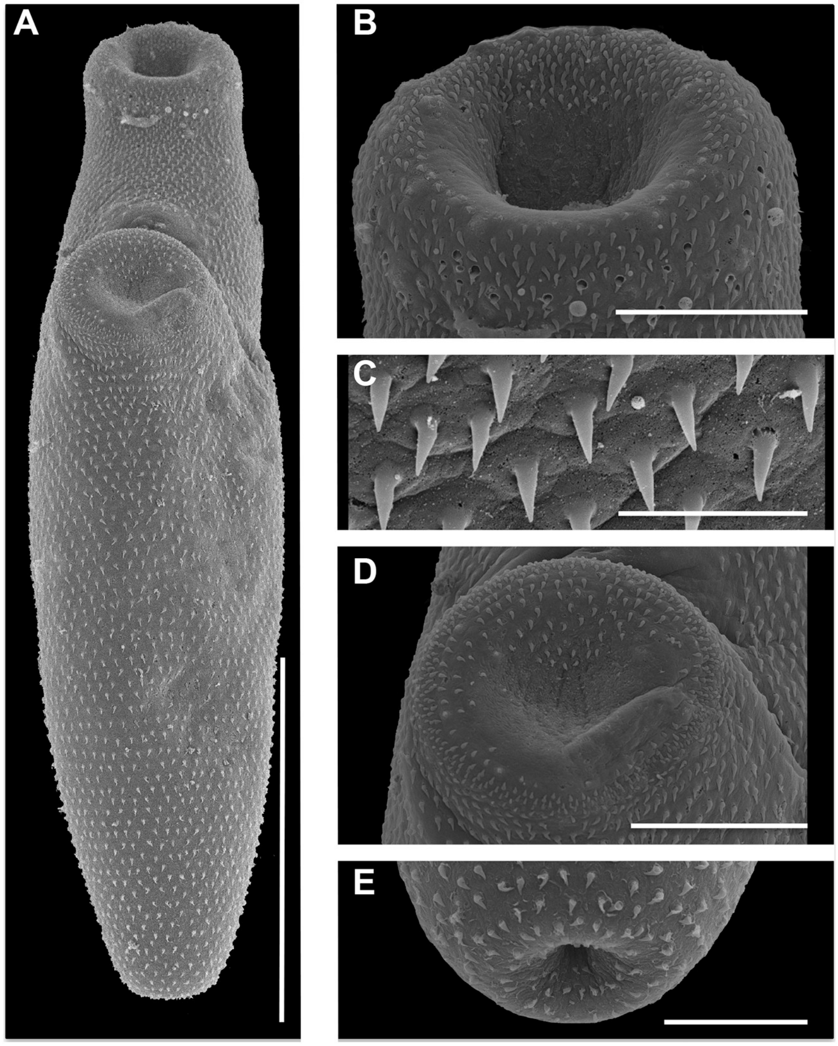

Fig. 2. Scanning electron micrographs of paratype of *Saccocoelioides macrospinosus* n. sp., from *Poecilia catemaconis* (A), Whole worm; (B) Oral sucker; (C) Tegumental spines; (D) Ventral sucker; (E) Pore terminal. Scale bars = 200 μm (A); 40 μm (B); 10 μm (C); 50 μm (D); 30 μm (E).

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

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

Site of infection: Intestine

Voucher material: CNHE 11130.

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

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

3.4. Remarks

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

3.5. Phylogenetic analyses

3.5.1. Nuclear genes

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

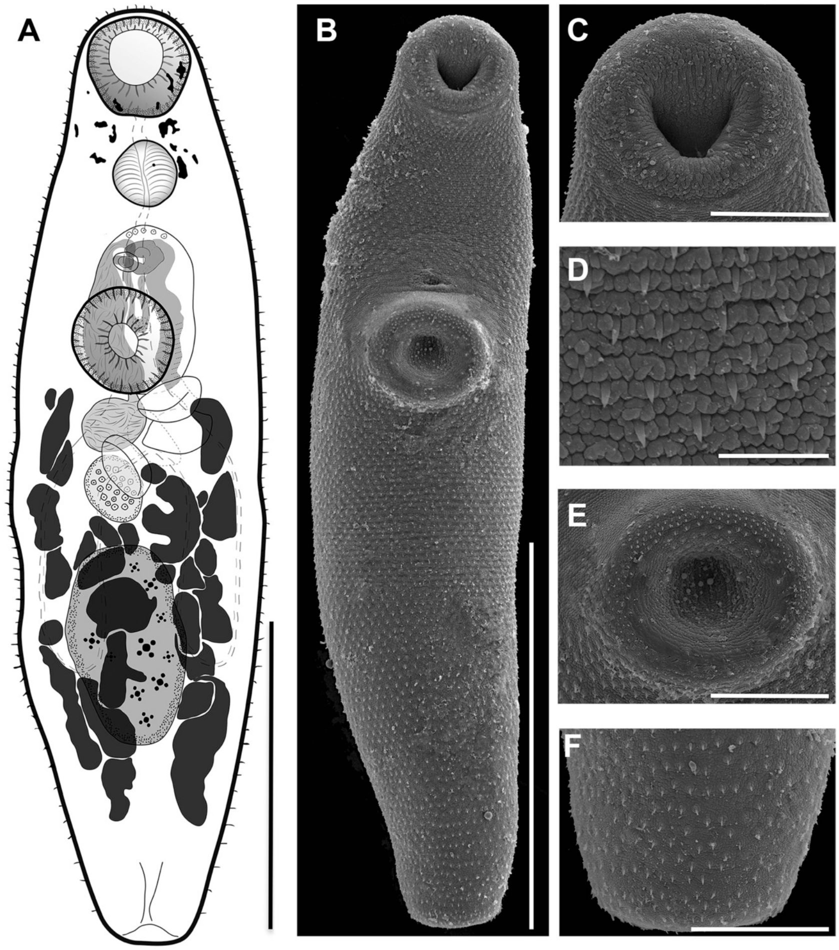

Fig. 3. *Saccocoelioides tkachi* from *Astyanax aeneus* (A) whole worm voucher, ventral view; Scanning electron micrographs of voucher (B), Whole worm; (C) Oral sucker; (D) Tegumental spines; (E) Ventral sucker; (F) Posterior region. Scale bars = 300 μm (A–B); 50 μm (C); 10 μm (D); 50 μm (E–F).

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

3.5.2. Mitochondrial gene

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

3.5.3. Nuclear and mitochondrial genes

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

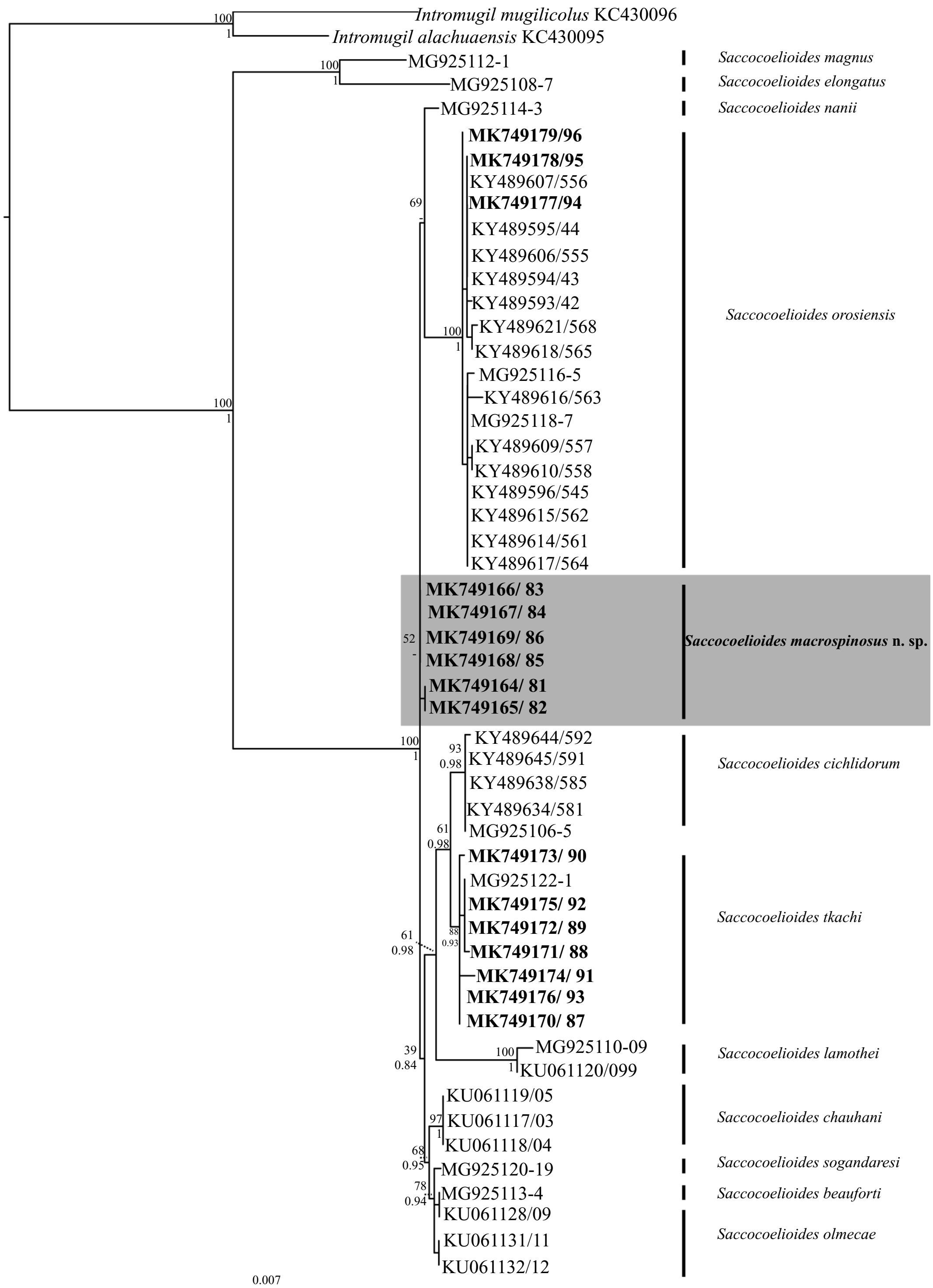

Fig. 4. Maximum likelihood tree and consensus Bayesian Inference trees inferred with the combined (ITS2 + LSU) data set; numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI).

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

4. Discussion

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

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

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

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

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

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

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

5. Conclusions

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

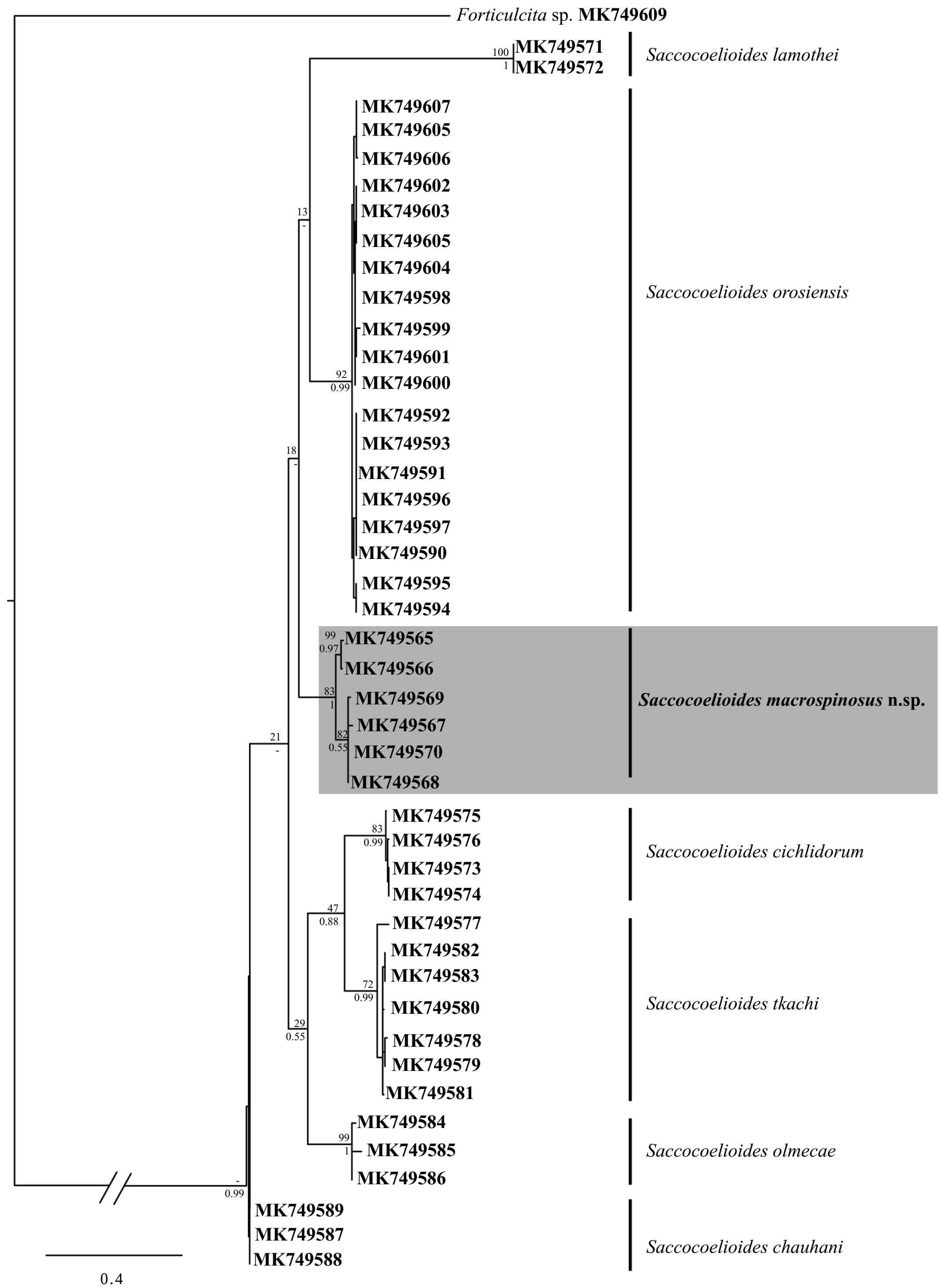

Fig. 5. Maximum likelihood tree and consensus Bayesian Inference trees inferred with *cox 1* data set; numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI).

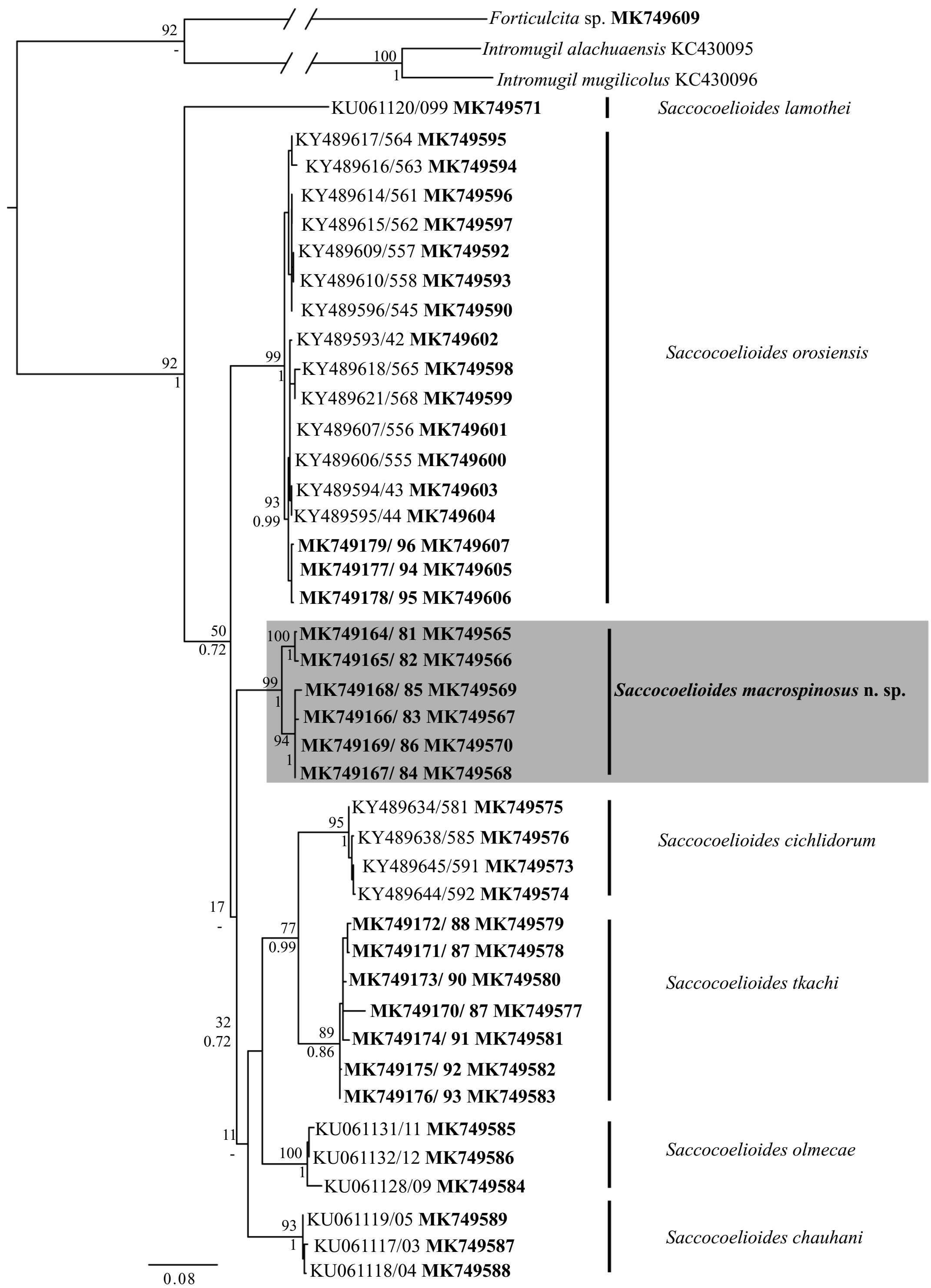

Fig. 6. Maximum likelihood tree and consensus Bayesian Inference trees inferred with the concatenated (*cox 1* + ITS2 + LSU) data set; numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI).

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

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Conflict of interest

None.

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