Parasitology Research (2020) 119:1713–1728
https://doi.org/10.1007/s00436-020-06680-w

FISH PARASITOLOGY - ORIGINAL PAPER

Two new species of *Gyrodactylus* von Nordmann, 1832 parasitizing *Cnesterodon decemmaculatus* (Poeciliidae) from the southern limit of the family in the Neotropical region

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Received: 15 February 2019 / Accepted: 26 March 2020 / Published online: 13 May 2020

Abstract

In this study, we followed an integrative taxonomy approach to describe two new species of *Gyrodactylus* von Nordmann, 1832, and to identify specimens of *G. breviradix* Vega, Razzolini, Arbetman, and Viozzi, 2019, all three collected from ten spotted live-bearer *Cnesterodon decemmaculatus* (Jenyns, 1842), an endemic and widespread poeciliid from the Pampean region, which is the southernmost occurring species of the Poeciliidae in the Americas. Gyrodactylids were first characterized morphologically and mophometrically, and when possible, sequences of the Internal Transcribed Spacers (ITS1-5.8S-ITS2) and the cytochrome oxidase II (COII) were used to delimit species. *Gyrodactylus breviradix*, *Gyrodactylus marplatensis* n. sp., and *Gyrodactylus pampeanus* n. sp. were found on the fins and body surface of *C. decemmaculatus* in La Tapera Creek, Mar del Plata, Buenos Aires province, Argentina. A phylogenetic analysis combining newly generated sequences of one of the new species, *G. marplatensis* n. sp., and of *G. breviradix*, along with those available in GenBank for a further 36 species of *Gyrodactylus*, revealed that *G. marplatensis* n. sp. is a sister taxon of *Gyrodactylus decemmaculati* Vega, Razzolini, Arbetman, and Viozzi, 2019. Genetic distances for the ITS and COII gene were estimated among *Gyrodactylus* spp. and further supported the validity of the new species. Overall, morphometric and molecular data coincided in delimiting the new taxa, thus demonstrating the value of integrative taxonomy for the erection of new species of *Gyrodactylus* and species identification.

Keywords *Gyrodactylus* · Poeciliidae · morphometry · ITS · COII · Non-Metric Multidimensional Scaling (nMDS)

Introduction

Members of Poeciliinae sensu Parenti (1981) (Cyprinodontiformes: Poeciliidae) comprise approximately 275 species in 27 genera, which are widely distributed in the Americas, from the southern USA to Argentina, as well as on many islands throughout the Caribbean (Lucinda 2003; Reznick et al. 2017). The highest diversity occurs in Central America (Hrbek et al. 2007), especially in Mexico, where 81 species are known (Miller 2005), whereas only 5 species have been reported in Argentina (Mirande and Koerber 2015). Due to their diversity and broad distribution, poeciliids have played a prominent role in studies of biogeography (Hrbek et al. 2007), which have recently postulated that the family originated in South America, although its major diversification dates to a later colonization of Central America (Reznick et al. 2017). In addition to their remarkable biological diversity, variation in reproductive traits displayed by this group of fishes, ranging from egg laying to live-bearing and including morphological

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Section Editor: Shokoofeh Shamsi

urn:lsid:zoobank.org:pub:67B0676C-51EC-4E0D-89B8-FB8428F3C64E

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and behavioral sexual dimorphism, makes them excellent subjects for research on their evolutionary history (Endler 2011).

Recently, 13 species of monogeneans of the genus *Gyrodactylus* von Nordmann, 1832 parasitizing poeciliid fishes from Mexico (Rubio-Godoy et al. 2010; García-Vásquez et al. 2015, 2019), and two from Argentina (Vega et al. 2019) have been characterized. Previous phylogenetic studies on species of *Gyrodactylus* infecting poecillids evidenced their polyphyletic origin (García-Vásquez et al. 2015, 2019), revealing that the diversity of *Gyrodactylus* in poeciliids is largely unknown and highlighting the potential of host-parasite systems for studies on coevolution and cophylogeny. Moreover, gyrodactylids infecting goodeid and profundulid fishes in Mexico, two other families of native cyprinodontiform hosts that are morphologically and ecologically similar to poeciliids, have been recently characterized (Rubio-Godoy et al. 2016; García-Vásquez et al. 2018a, b, 2019). Data suggest that these three fish families are infected by different lineages of morphologically similar and phylogenetically close parasite species, some of which are shared by these hosts. Species of *Gyrodactylus* possess several biological traits such as alternation of asexual, parthenogenetic, and sexual reproduction, explosive population growth, direct life cycle, high host specificity, and proved colonizing capability through host switching, which makes them interesting models for research on fish-parasite coevolutionary relationships (Huyse and Volckaert 2005).

The ten spotted live-bearer, *Cnesterodon decemmaculatus* (Jenyns 1842), is an endemic and widespread poeciliid from the Pampean region (Lucinda 2005),that represents the southernmost occurring species of the family in the Americas. This poeciliid has been recently used as a model to examine historical processes shaping the genetic structure of freshwater fishes in the region (Bruno et al. 2016; Ramos-Fregonezi et al. 2017) and has been reported to harbor three undescribed species of *Gyrodactylus* in Patagonian rivers (Argentina) where it has been recently introduced (Rauque et al. 2018); two of which were recently described (Vega et al. 2019). Consequently, on the basis of the current knowledge on the host biogeography, characterization of its gyrodactylid fauna is a first step towards the understanding of factors driving the diversity, phylogeny, and phylogeography of gyrodactylid-poeciliid systems, not only at local scales but across the entire Neotropical region when new information becomes available. Therefore, the aim of this paper is to describe two new species of *Gyrodactylus* collected from *C. decemmaculatus* in the Pampean region, Argentina.

Materials and methods

Sample collection and preparation

Specimens of *Cnesterodon decemmaculatus* ($n = 120$) were collected with hand nets (net mesh 0.28 mm) at La Tapera creek, Mar del Plata, Buenos Aires province, Argentina (37° 56′ 40″ S – 57° 32′ 22″ W) on August 10, 2016, austral winter. Fish were sacrificed by spinal cord severing with the aid of dissecting needles under a stereo microscope. Gyrodactylids were removed using surgical needles, and specimens were then fixed in either 95% ethanol or 5% formaldehyde and processed individually. Haptors of specimens fixed in ethanol were excised using a scalpel and subjected to partial proteolytic digestion to remove tissue enclosing the haptoral armature following Rubio-Godoy et al. (2012). Digestion was arrested by the addition of a 50:50 formaldehyde/glycerine solution. Bodies were fixed individually in 95% ethanol and stored at − 20 °C and labeled for subsequent molecular analyses (Harris and Cable 2000). Type material was mounted in Canada Balsam and also in Hoyer's medium. Morphological analyses were performed on both ethanol-(digestion method as described in García-Vásquez et al. 2015) and formaldehyde-preserved specimens (which were rinsed in tap water for several hours), which were wet-mounted and measured (Fannes et al. 2015; Shinn et al. 2004).

Morphological analysis

Images of the haptoral attachment hooks were captured using Leica BFC 295 and Leica ICC50 HD digital cameras interfacing with Leica DM 2500 and DM750 microscopes (magnification of 10 × 100 with oil immersion, 100 objective lens for hamuli, and the marginal hooks with phase contrast). Twenty-eight point-to-point measurements of haptoral structures based on Shinn et al. (2004) and García-Vásquez et al. (2015) were taken on images using the ImageJ (Java 1.6.0_12) software, including for the hamulus (H): total length (HTL), aperture distance (HAD), proximal shaft width (HPSW), point length (HPL), distal shaft width (HDSW), shaft length (HSL), inner curve length (HICL), aperture angle (HAA), point curve angle (HPCA), inner aperture angle (HIA), and root length (HRL); for the ventral bar (VB): total length (VBTL), total width (VBTW), process-to-mid length (VBPML), median length (VBML), process length (VBPL), and membrane length (VBMBL); for the dorsal bar (DB): length (DBL), width (DBW), and attachment point length (DBAPTL); and for the marginal hook (MH): total length (MHTL), shaft length (MHSHL), sickle length (MHSL), sickle proximal width (MHSPW), sickle toe length (MHSTL), sickle distal width (MHSDW), aperture (MHAD), and instep/arch height (MHIH). All measurements are given in micrometers (Table 1). Specimens found in the present study were morphometrically compared with those species recorded in poeciliids: *G. actzu* García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015; *G. apazapanensis* García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015; *G. breviradix* Vega, Razzolini, Arbetman, and Viozzi, 2019; *G. bullatarudis* Turnbull, 1953; *G. chiapaneco* García-

Table 1 Measurements of *Gyrodactylus* species collected from *Cnesterodon decemmaculatus* from La Tapera creek, Mar del Plata, Argentina. Measurements are given in micrometers (mean ± standard deviation followed by the range in parentheses); *n*, number of structures measured

Measurement	*G. breviradix*	*G. marplatensis n.* sp.	*G. pampeanus* n. sp.
Hamulus	*n* = 13	*n* = 8	*n* = 9
HTL	42.5 ± 1.8 (37.1–44.8)	67.5 ± 2.6 (63.4–70.7)	53.7 ± 1.4 (52.0–55.7)
HA	15.3 ± 0.9 (13.6–17.4)	21.8 ± 1.4 (20.6–25.1)	25.5 ± 3.1 (18.0–28.9)
HPSW	7.1 ± 0.7 (6.1–8.5)	9.2 ± 0.8 (7.6–10.4)	7.5 ± 0.6 (6.5–8.4)
HPL	21.3 ± 1.0 (19.6–23.0)	27.1 ± 1.1 (24.6–28.3)	21.8 ± 1.1 (20.5–23.6)
HDSW	3.7 ± 0.2 (3.4–4.3)	4.9 ± 0.4 (4.0–5.3)	3.9 ± 0.6 (3.4–5.2)
HSL	27.9 ± 1.0 (25.4–28.9)	38.0 ± 2.1 (33.9–41.1)	36.2 ± 1.2 (34.0–37.7)
HICL	2.2 ± 1.1 (0.5–4.0)	4.8 ± 1.5 (3.2–6.9)	1.5 ± 1.1 (0.5–3.7)
HAA	35.8 ± 2.5 (33.0–42.3)	38.8 ± 1.6 (36.7–41.7)	49.1 ± 5.0 (37.2–53.5)
HPCA	9.5 ± 4.5 (3.4–16.1)	15.4 ± 4.8 (9.7–22.3)	5.4 ± 3.3 (1.6–12.1)
HIA	41.0 ± 3.4 (36.8–50.2)	44.7 ± 2.0 (41.9–47.4)	54.4 ± 5.0 (42.6–58.2)
HRL	12.7 ± 1.4 (9.7–14.9)	27.4 ± 2.0 (24.6–31.1)	20.5 ± 1.8 (17.9–22.6)
Ventral bar	*n* = 11	*n* = 6	*n* = 7
VBTL	25.9 ± 2.1 (21.4–28.0)	38.3 ± 3.2 (35.1–42.7)	22.4 ± 2.5 (18.6–26.3)
VBTW	23.4 ± 2.5 (19.7–27.0)	44.5 ± 3.1 (38.7–49.9)	20.1 ± 1.3 (18.0–22.3,)
VBPML	10.0 ± 0.6 (9.2–11.1)	12.7 ± 1.1 (11.2–14.5)	3.5 ± 1.0 (2.4–5.3)
VBML	4.9 ± 0.9 (3.0–6.6)	8.2 ± 1.7 (6.3–12.1)	5.2 ± 0.9 (4.2–6.7)
VBPL	7.7 ± 0.7 (6.5–8.8)	9.2 ± 1.3 (7.2–10.7)	2.0 ± 0.8 (0.8–3.3)
VBMBL	10.6 ± 1.6 (7.6–13.6)	23.8 ± 1.5 (20.9–25.8)	14.5 ± 2.5 (11.2–18.8)
Dorsal bar	*n* = 4	*n* = 2	*n* = 2
DBL	20.3 ± 0.8 (19.4–21.3)	26.6 ± 3.3 (24.2–28.9)	1.42 ± 0.2 (1.3–1.6)
DBW	1.4 ± 0.1 (1.3–1.5)	2.2 ± 0.5 (1.8–2.5)	20.9 ± 1.1 (20.1–21.7)
DBAPTL	5.4 ± 0.7 (4.5–6.3)	7.3 ± 0.0 (7.3–7.3)	8.0 ± 0.2 (7.8–8.1)
Marginal hook	*n* = 15	*n* = 6	*n* = 8
MHTL	27.9 ± 1.6 (24.8–30.5)	29.8 ± 0.7 (29.0–30.9)	25.7 ± 2.0 (21.4–27.8)
MHSHL	23.7 ± 1.6 (20.5–25.8)	24.1 ± 0.6 (23.2–24.9)	20.3 ± 1.9 (16.1–21.8)
MHSL	4.6 ± 0.2 (4.3–5.0)	6.3 ± 0.2 (6.0–6.5)	6.1 ± 0.2 (5.7–6.5)
MHSPW	3.9 ± 0.2 (3.5–4.2)	4.7 ± 0.1 (4.7–4.8)	3.9 ± 0.4 (3.4–4.4)
MHSTL	1.4 ± 0.1 (1.2–1.7)	2.1 ± 0.1 (2.0–2.3)	1.9 ± 0.2 (1.6–2.1)
MHSDW	2.6 ± 0.3 (2.3–3.2)	3.2 ± 0.3 (2.7–3.5)	2.1 ± 0.4 (1.6–2.9)
MHAD	3.5 ± 0.2 (3.1–3.9)	5.7 ± 0.4 (5.3–6.5)	5.3 ± 0.3 (4.8–5.6)
MHIH	0.6 ± 0.1 (0.4–0.8)	0.6 ± 0.1 (0.4–0.7)	0.5 ± 0.3 (0.2–1.1)

Mophometric variables according to García-Vásquez et al. [8] and Shinn et al. [17] (see text for full names)

Vásquez, Pinacho-Pinacho, Guzmán-Valdivieso, Salgado-Maldonado, and Rubio-Godoy, 2019; *G. decemmaculati* Vega, Razzolini, Arbetman, and Viozzi, 2019; *G. costaricensis* Kritsky and Fritts, 1970; *G. cytophagus* Paperna, 1968; *G. guatopotei* García-Vásquez, Pinacho-Pinacho, Guzmán-Valdivieso, Salgado-Maldonado, and Rubi o-Godoy, 2019; *G. jarocho* Rubio-Godoy, Paladini, García-Vásquez, and Shinn, 2010; *G. lhkahuili* García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015; *G. microdactylus* García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015; *G. milleri* Harris and Cable, 2000; *G. pictae* Cable, von Oosterhout, Barson, and Harris, 2005; *G. poeciliae* Harris and Cable, 2000; *G. pseudobullatarudis* García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015; *G. takoke* García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015; *G. tlaloci* García-Vásquez, Pinacho-Pinacho, Guzmán-Valdivieso, Salgado-Maldonado, and Rubio-Godoy 2019; *G. turnbulli* Harris, 1986; *G. unami* García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015; *G. xalapensis* Rubio-Godoy, Paladini, García-Vásquez, and Shinn, 2010; and *G. xtachuna* García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015. Morphometric measurements of all previously mentioned species of *Gyrodactylus* were obtained from Rubio-Godoy et al. (2010) and García-Vásquez et al. (2015, 2019), except for measurements of *G. breviradix* and *G.decemmaculati* that were obtained from type material (MACN-Pa 680/1 and

MACN-Pa 677/1–2, respectively), and images kindly provided by the authors (Vega et al. 2019).

Moreover, the following species parasitizing neotropical goodeids and profundulids were also compared: *G. iunuri* García-Vásquez, Guzmán-Valdivieso, Razo-Mendivil, and Rubio-Godoy, 2018; *G. katamba* García-Vásquez, Guzmán-Valdivieso, Razo-Mendivil, and Rubio-Godoy, 2018; *G. lamothei* Mendoza-Palmero, Sereno-Uribe, and Salgado-Maldonado, 2009; *G. montealbani* García-Vásquez, Rubio-Godoy, Guzmán-Valdivieso, and Razo-Mendivil, 2018; *G. tepari* García-Vásquez, Guzmán-Valdivieso, Razo-Mendivil, and Rubio-Godoy, 2018; and *G. tomahuac* Rubio-Godoy, Razo-Mendivil, García-Vásquez, Freeman, Shinn, and Paladini, 2016. Measurements used in these analyses were taken from García-Vásquez et al. (2018a, b) and Rubio-Godoy et al. (2016).

Statistical analysis

Morphometric similarities between specimens found in the present study and their 30 known congeners parasitizing poeciliid, goodeid, and profundulid fishes from the Neotropical region were simultaneously compared by Non-Metric Multidimensional Scaling (nMDS) analyses. Analyses were based on Euclidean distances of the 25 point-to-point measurements of haptoral structures (H, MH, and VB), when data were available. Only those specimens of this study for which all the measurements were obtained were included in the analyses. In the case of described species, average values were used, depending on the availability of data in the literature (Rubio-Godoy et al. 2010, 2016; García-Vásquez et al. 2015, 2018a, b, 2019). In the case of *G.* breviradix, the VB could not be measured from type material neither images provided by the authors. Vector overlays were used to identify those point-to-point measurements determining the similarity between species. Hierarchical agglomerative cluster analysis, with group-average linking (Clarke and Warwick 2001), was applied to the same matrices, and those resemblance levels that include all specimens of each new species were overlaid on the nMDS plot.

Phylogenetic analysis

Bodies of excised specimens, whose haptors were morphometrically characterized, were placed individually in 1.5 ml Eppendorf tubes for genomic DNA extraction using the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, California) following the manufacturer's instructions. Two regions, the ribosomal region spanning the 3′end of the 18S rRNA gene, ITS1, 5.8S rRNA gene, and ITS2, and the 5′end of the 28S rRNA were amplified by PCR adding the forward primer BD1 (5′-GTCGTAACAAGGTTTCCGTA-3′) and the reverse primer BD2 (5′-ATCTAGACCGGACTAGGCTGTG-3′) (Bowles et al. 1995), and the cytochrome oxidase II (COII) gene was amplified using the primer pairs COX2 F1 (5′-TACATAYCGCCCGTCAATYT-3′) and COX2 R1 (5′-TCARTAYCACTGDCGDCCYA-3′) (Xavier et al. 2015). All PCR reactions were performed following the protocols of García-Vásquez et al. (2015) and Xavier et al. (2015). Amplicons were visualized on GelRed (Biotium, San Francisco, California) stained 1% agarose gel and then unincorporated nucleotides, and primers of each PCR amplicon were removed using ExoSap-IT (USB Corporation, Ohio). Sequencing reactions were carried out with the use of BigDye terminator chemistry, incorporating the same primers used in PCR reactions, and cleaned by filtration with Sephadex G50. The sequenced products were read on an ABI PRISM 3100–automated DNA sequencer (Applied Biosystems, Foster City, California).

Electropherograms were visually inspected with FinchTV (Geospiza Inc., Seattle, Washington), and overlapping fragments of forward and reverse sequences were assembled with BioEdit v. 7.0.9 (Hall 1999). Sequences were deposited in GenBank and their accession numbers are cited in the description of each species. Partial sequences obtained from the ITS and COII were aligned separately with sequences of other species of *Gyrodactylus* (see Table 2) available in GenBank using ClustalW with default parameters implemented in MEGA 7.0 (Kumar et al. 2016). The best-fitting nucleotide substitution model (GTR + I + G for both ITS and COII) was estimated with the Akaike Information Criterion (AIC) implemented in MEGA 7.0 (Kumar et al. 2016). Phylogenetic trees were reconstructed with unique sequences, inferred by Maximum Likelihood (ML) and Bayesian Inference (BI) analyses. For ML analyses, the program RAxML v7.0.4 (Stamatakis 2006) was used. A GTRGAMMAI substitution model was used for ML analyses, and 1000 bootstrap replicates were run to assess nodal support. BI trees were generated using MrBayes v3.2 (Ronquist et al. 2012), running two independent MC3 runs of four chains for 10 million generations and sampling tree topologies every 1000 generations. "Burn-in" periods were set to 2.5 million generations according to the standard deviation of split frequencies values (p 0.01). Posterior probabilities of clades were obtained from 50% majority rule consensus of sample trees after excluding the initial 25% as "burn-in". The genetic divergence among species of *Gyrodactylus* was estimated using uncorrected "p" distances with MEGA 7.0 (Kumar et al. 2016). Finally, trees were drawn using FigTree version 1.3.1 (Rambaut 2006).

Results

Three species of *Gyrodactylus*, two new taxa (*Gyrodactylus marplatensis* n. sp. and *Gyrodactylus pampeanus* n. sp.) and *G. breviradix* were found parasitizing the tegument of 46 of

Table 2 List of species of *Gyrodactylus* included in the phylogenetic analyses

Species	Host	Locality	GenBank		Reference
			ITS	COII	
Gyrodactylus actzu García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015	*Poecilia mexicana*	Veracruz, Mexico	KM514475	–	García-Vásquez et al. (2015)
Gyrodactylus anisopharynx Popazoglo and Boeger, 2000	*Corydoras paleatus*	Brazil: Rio Iguacu, Agua Azul, Lapa, Parana	–	MH043346, MH043345	Razzolini et al. (2019)
Gyrodactylus apazapanensis García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015	*Poecilia mexicana*	Veracruz, Mexico	KM514463	–	García-Vásquez et al. (2015)
Gyrodactylus breviradix Vega, Razzolini, Arbetman, and Viozzi, 2019	*Cnesterodon decemmaculatus*	Neuquen River, Vista Alegre, Neuquen, Argentina	MK312260, MK312259	MN066553, MN066552	Vega et al. (2019)
Gyrodactylus breviradix	*Cnesterodon decemmaculatus*	Mar del Plata, Argentina	MK965395–397	MN927192	Present study
Gyrodactylus bueni Bueno-Silva and Boeger 2014	*Scleromystax macropterus*	Fortuna River, Pontal do Paraná, Paraná, Brazil	–	GU131214, GU131215	Bueno-Silva and Boeger (2014)
Gyrodactylus bullatarudis Turnbull, 1956	*Poecilia reticulata*	Trinidad	AY692024, AJ011410	KP168350, KP168373, KP168379, KP168380	Cable et al. (2005); Xavier et al. (2015)
Gyrodactylus carolinae Boeger, Ferreira, Vianna, and Patella, 2014	*Characidium lanei*	Brazil: Rio Marumbi, Morretes, Parana	–	MH043351, MH043350	Razzolini et al. (2019)
Gyrodactylus chiapaneco García-Vásquez, Pinacho-Pinacho, Guzmán-Valdivieso, Salgado-Maldonado, and Rubio-Godoy, 2019	*Tlaloc labialis*, *Pseudoxiphophorus bimaculatus*	Chiapas, Mexico; Guanajuato, Mexico	MK912264, MK912265-MK912266	–	García-Vásquez et al. (2019)
Gyrodactylus corydori Bueno-Silva, and Boeger, 2009	*Corydoras ehrhardti*	Brazil	–	GU131197, GU131198	Unpublished
Gyrodactylus decemmaculati Vega, Razzolini, Arbetman, and Viozzi, 2019	*Cnesterodon decemmaculatus*	Arroyón stream, Cinco Saltos, Argentina	MK299423, MK299424, MK299425, MK312457, MK312458	MN066555, MN066554, MN066556	Vega et al. (2019)
Gyrodactylus guatopotei García-Vásquez, Pinacho-Pinacho, Guzmán-Valdivieso, Salgado-Maldonado, and Rubio-Godoy, 2019	*Poeciliopsis hnilickai*	Chiapas, Mexico	MK912263	MN927193	García-Vásquez et al. (2019); Present study
Gyrodactylus iunuri García-Vásquez, Guzmán-Valdivieso, Razo-Mendivil, and Rubio-Godoy, 2018	*Goodea atripinnis*	Querétaro, Mexico	KR815853	–	García-Vásquez et al. (2018b)
Gyrodactylus jarocho Rubio-Godoy, Paladini, García-Vásquez, and Shinn, 2010	*Xiphophorus hellerii*	Veracruz, Mexico	KJ621984	–	Rubio-Godoy et al. (2010); García-Vásquez et al. (2015)
Gyrodactylus katamba García-Vásquez, Guzmán-Valdivieso, Razo-Mendivil, and Rubio-Godoy, 2018	*Goodea atripinnis*	Querétaro, Mexico	KR815854	–	García-Vásquez et al. (2018b)
Gyrodactylus lamothei Mendoza-Palmero, Sereno-Uribe, and Salgado-Maldonado, 2009	*Girardinichthys multiradiatus*	Estado de Mexico, Mexico	KX555666, KX555668	–	Rubio-Godoy et al. (2016)

Table 2 (continued)

Species	Host	Locality	GenBank		Reference
			ITS	COII	
Gyrodactylus lhkahuili García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015	*Poecilia mexicana*	Veracruz, Mexico	KM514477	–	García-Vásquez et al. (2015)
Gyrodactylus lilianae Razzolini, Murari, Baldisserotto, and Boeger, 2019	*Rhamdia quelen*	State of Rio Grande do Sul, Brazil	–	MH043348, MH043349	Razzolini et al. (2019)
Gyrodactylus major Bueno-Silva and Boeger 2014	*Scleromystax macropterus*	Fortuna River, Pontal do Paraná, Paraná, Brazil	–	GU131210,GU131209	Bueno-Silva and Boeger (2014)
Gyrodactylus marplatensis n. sp.	*Cnesterodon decemmaculatus*	Mar del Plata, Argentina	MK965393–394	MN927194, MN927195	Present study
Gyrodactylus microdactylus García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015	*Poecilia mexicana*	Veracruz, Mexico	KM514474	–	García-Vásquez et al. (2015)
Gyrodactylus mojarrae Mendoza-Palmero, Blasco-Costa, and Pérez-Ponce de León, 2019	*Thorichthys maculipinnis*	Veracruz, Mexico	MK573785	–	Mendoza-Palmero et al. (2019)
Gyrodactylus montealbani García-Vásquez, Rubio-Godoy, Guzmán-Valdivieso, and Razo-Mendivil, 2018	*Profundulus oaxacae*	Oaxaca, Mexico	MG883700, MG883696, MG883701, MG883703	–	García-Vásquez et al. (2018a)
Gyrodactylus pictae Cable, von Oosterhout, Barson, and Harris, 2005	*Poecilia reticulata*	Trinidad	AY692023	–	Cable et al. (2005)
Gyrodactylus poeciliae Harris and Cable 2000	*Poecilia caucana*	Venezuela	AJ001844	KP168345, KP168344, KP168335, KP168328, KP168329, KP168327, KP168285	Harris and Cable (2000); Xavier et al. (2015)
Gyrodactylus pseudobullatarudis García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015	*Xiphophorus hellerii*	Veracruz, Mexico	KM514436, KM514439	–	García-Vásquez et al. (2015)
Gyrodactylus samirae Popazoglo and Boeger, 2000	*Corydoras ehrhardti*	Brazil	–	GU131200	Unpublished
Gyrodactylus scleromystaci Bueno-Silva and Boeger 2014	*Scleromystax barbatus*	Marumbi River, Morretes, Paraná, Brazil	–	GU131217, GU131216	Bueno-Silva and Boeger (2014)
Gyrodactylus superbus Popazoglo and Boeger, 2000	*Corydoras ehrhardti*	Brazil	–	GU131203, GU131202	Unpublished
Gyrodactylus tlaloci García-Vásquez, Pinacho-Pinacho, Guzmán-Valdivieso, Salgado-Maldonado, and Rubio-Godoy, 2019	*Tlaloc labialis*, *Poeciliopsis hnilickai*	Chiapas, Mexico	MK912267-MK912268	–	García-Vásquez et al. (2019)
Gyrodactylus takoke García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015	*Pseudoxiphophors bimaculatus*	Veracruz, Mexico	KM514447, KM514455-KM514457, KM514462	GHbAB2, GHbF2, GHbF3	García-Vásquez et al. (2015); Present study
Gyrodactylus tepari García-Vásquez, Guzmán-Valdivieso, Razo-Mendivil, and Rubio-Godoy, 2018	*Goodea atripinnis*	Querétaro, Mexico	KR815856-KR815859	–	García-Vásquez et al. (2018b)
Gyrodactylus tomahuac Rubio-Godoy, Razo-Mendivil, García-Vásquez, Freeman, Shinn, and Paladini, 2016	*Goodea atripinnis*	Querétaro, Mexico	KR815849, KJ621983, KR815847, KR815852	–	Rubio-Godoy et al. (2016)
Gyrodactylus turnbulli Harris 1986	*Poecilia reticulata*	Unknown	EF445942, AJ001846		

Table 2 (continued)

Species	Host	Locality	GenBank		Reference
			ITS	COII	
				KP168394, KP168391, KP168403, KP168410, KP168404, KP168406	Harris (1986); Xavier et al. (2015)
Gyrodactylus unami García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015	*Poeciliopsis gracilis*	Veracruz, Mexico	KM514469, KM514470	–	García-Vásquez et al. (2015)
Gyrodactylus xalapensis Rubio-Godoy, Paladini, García-Vásquez, and Shinn, 2010	*Pseudoxiphophorus bimaculatus*	Veracruz, Mexico	KJ621985	GPN16_3	Rubio-Godoy et al. (2010); Present study
Gyrodactylus xtachuna García-Vásquez, Razo-Mendivil, and Rubio-Godoy, 2015	*Poecilia mexicana*	Veracruz, Mexico	KM514442- KM514443, KM514445-KM514446	–	García-Vásquez et al. (2015)
Gyrodactylus zapoteco García-Vásquez, Pinacho-Pinacho, Martínez-Ramírez, and Rubio-Godoy, 2018	*Profundulus oaxacae*	Oaxaca, Mexico	MG883704	–	García-Vásquez et al. (2018a)
Gyrodactylus sp. B Mendoza-Palmero, Blasco-Costa, and Pérez-Ponce de León, 2019	*Gobiomorus dormitor*	Palo de Aquita, Nicaragua	MK573789	–	Mendoza-Palmero et al. (2019)

the 120 (38%) specimens of *C. decemmaculatus* examined. *Gyrodactylus breviradix* was the most prevalent (14%) species infecting *C. decemmaculatus* from La Tapera Creek. Measurements of haptoral structures of the two new species and *G. breviradix* are given in Table 1. Molecular and multivariate analyses (nMDS) of morphometric data corroborated the presence of *G. breviradix* and of *G. marplatensis* n. sp. on *C. decemmaculatus* from the Pampean region. *Gyrodactylus pampeanus* n. sp. was characterized by its morphometry (nMDS) and morphology only, as no molecular data were obtained. Finally, the phylogenetic position of both *G. breviradix* and *G. marplatensis* n. sp. is presented.

Nomenclatural acts

This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank Life Science Identifiers (LSIDs) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http:/zoobank.org/". The LSID for this publication is: urn:lsid:zoobank:pub: 67B0676C-51EC-4E0D-89B8-FB8428F3C64E. In addition, species profiles including taxonomic traits, host details, and other metadata are provided on www.gyrodb.net (Harris et al. 2008, Shinn et al. 2011).

Descriptions

***Gyrodactylus marplatensis* n. sp. (Fig. 1; Table 1).**
urn:lsid:zoobank.org:act:2513F514-76FC-4E3E-9DAC-39BDB01E6420.

Type host *Cnesterodon decemmaculatus* (Jenyns, 1842) (Cyprinodontiformes: Poeciliidae).

Site of infection Fins and body surface.

Type locality La Tapera Creek, Mar del Plata, Buenos Aires province, Argentina (37° 56′ 40″ S – 57° 32′ 22″ W).

Type material Holotype (accession no. MLP-HE XXX) and seven paratypes (accession no. MLP-HE XXX) were deposited in the Helminthological Collection of the Museo de La Plata (HCMLP), La Plata, Argentina. Two additional paratypes (acc. no. CNHE 11065) are deposited in Colección Nacional de Helmintos (CNHE), Mexico City, Mexico.

DNA reference sequences Sequences obtained from 2 individuals are deposited in GenBank (accession nos.: MK965393-965394 for ITS, and accession nos.: MN927194-MN927195 for COII).

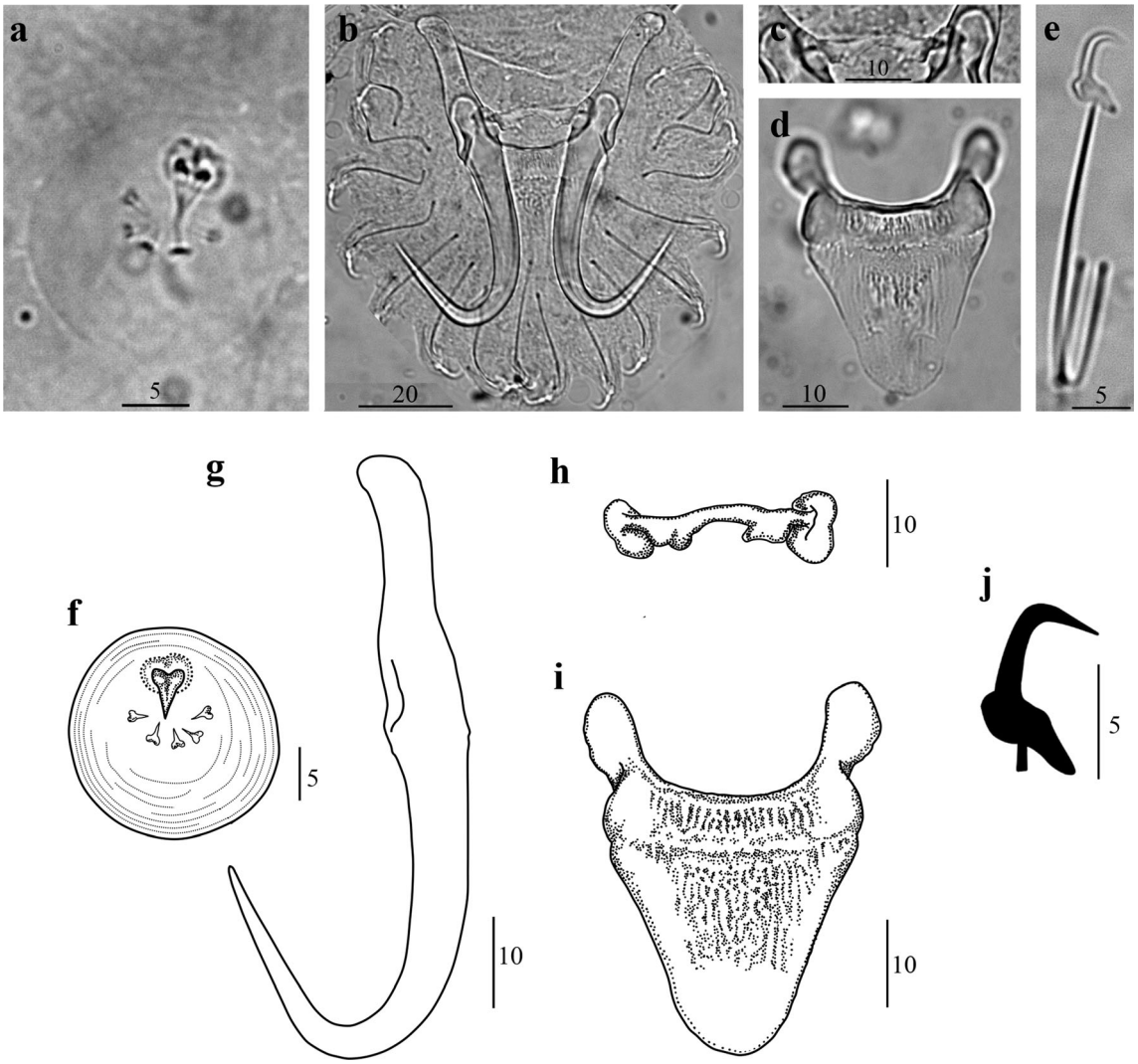

Fig. 1 Light micrographs and drawings of *Gyrodactylus marplatensis* n. sp. **a** Male copulatory organ (MCO). **b** Haptoral complex. **c** Dorsal bar. **d** Ventral bar. **e** Marginal hook at a glance. **f** MCO. **g** Hamulus. **h** Dorsal bar. **i** Ventral bar. **j** Marginal hook sickle

Etymology The specific name refers to the type locality of the new species: Mar del Plata, Argentina.

Prevalence 8.4%

Number of specimens collected 9

Description (based on 8 specimens)

Body (based on 2 specimens with fully extended body) 341.3–427.2 long and 69.7–114.8 wide. Haptor circular, not clearly delineated from the body, 77.0–85.6 long, and 82.3–82.7 wide. Pharynx ($n = 1$) almost spherical 37.9 long, 36.8 wide, anterior, and posterior bulb not clearly delimited. Male copulatory organ (MCO, $n = 1$), spherical, 18.8 long × 18.9 wide, armed with one big principal hook (4.2 long), and a single ring of 5 small thin spines (all similar in size) (2.3, $n = 2$), positioned off centre, arranged 1 on the right, 1 central (the point of the principal hook point ends in the top of the point of the spine) and 3 on the left side, and pointing to the direction of the principal hook point, adjacent to the posterior end of pharynx (Fig. 1a, f). Hamulus ($n = 8$), total length 67.5 (63.4–70.7) long, roughly same thickness through all length and widening slightly at dorsal bar attachment point, shaft 38.0 (33.9–41.1) long; point 27.1 (24.6–28.3) long, constituting approximately half of the shaft length; proximal shaft width 9.2 (7.6–10.3), distal shaft width 4.9 (4.0–5.3); aperture distance 21.8 (20.6–25.1); aperture angle 38.8 (36.7–41.6); root 27.4 (24.6–31.1) long (Fig. 1b, g), with rounded end. Dorsal bar ($n = 2$), 1.8–2.5 long, 24.2–28.9 wide, becoming thinner at the middle and with postero-lateral protuberances, immediately before attachment points, becoming narrower when getting close to the attachment points; oval attachment points 24.2–28.9 long (Fig. 1c, h). Ventral bar ($n = 6$), 38.3 (35.1–42.7) long, 44.5 (38.7–49.9) wide; processes prominent 9.2 (7.1–10.7) long with proximal rounded ends leaning to the

hamuli; median portion 8.2 (6.3–12.1) long, trapezoidal, and with semi-triangular ends extended before the start of the ventral bar processes, central region of the median ventral bar proper angled slightly towards its centre; membrane lingulate 23.8 (20.9–25.8) long, with rounded posterior edge (Figs. 1d, i). Marginal hook ($n = 6$), 29.8 (29.0–30.8) long; slim shaft 24.1 (23.2–24.8) long and slightly curved at its end, sickle base together with the bridge are angled towards the toe, semi-curved, and short bridge; sickle 6.3 (6.0–6.5) long, with erected sickle shaft ending in a deep opened curvature, sickle point extends before the limit of the toe; triangular toe 2.1 (2.0–2.3) long, slightly curved at the end, facing downwards; small squared heel; distal width 3.1 (2.7–3.5); aperture 5.7 (5.3–6.5) 1ong; instep height 0.6 (0.4–0.7) long, curved in shaft attachment point (Figs. 1e, j).

Remarks *Gyrodactylus marplatensis* n. sp. exhibited a unique combination of morphometric features of haptoral attachment structures that allows differentiating it from its congeners parasitizing poeciliids, goodeids, and profundulids (Fig. 4). Only *G. jarocho,* a parasite of *Xiphophorus hellerii* from Mexico (Rubio-Godoy et al. 2010), showed MH morphometry similar to the new species (Fig.4b). Nonetheless, these species are readily differentiated by the shape of the marginal hook, as well as that of their ventral bars and hamuli (Fig. 4a, c). Indeed, the ventral bar of *G. jarocho* has long, narrow, and pointed processes, whereas in *G. marplatensis* n. sp., these are rounded and short. Moreover, the lateral margins of the ventral bar membrane in *G. jarocho* are not attached to the external edges of the ventral bar median portion, while in *G. marplatensis* n. sp., they are (Fig. 4c). Moreover, although measurements of the MH, H, and VB of *G. decemmaculati*, parasite of *C. decemmaculatus* from Patagonia, were never included within the region that delimits intraspecific variation of *G. marplatensis* (Fig. 4), the three structures from *G. decemmaculati* were located close to those of *G. marplatensis* in each nMDS plot (Fig. 4a, b, c).

Gyrodactylus pampeanus n. sp. (Fig. 2, Table 1).

urn:lsid:zoobank.org:act:E0DA95B6-0E10-47C3-A9F5-F7D7BDE6615D.

Type host *Cnesterodon decemmaculatus* (Jenyns, 1842) (Cyprinodontiformes: Poeciliidae).

Site of infection Fins and body surface.

Type locality La Tapera Creek, Mar del Plata, Buenos Aires province, Argentina (37° 56′ 40″ S – 57° 32′ 22″ W).

Type material Holotype (accession no. MLP-HE XXX) and five paratypes (accession no. MLP-HE XXX) were deposited in the Helminthological Collection of the Museo de La Plata (HCMLP), La Plata, Argentina. One additional paratype (acc. no. CNHE 11066) deposited in Colección Nacional de Helmintos (CNHE), Mexico City, Mexico.

DNA reference sequences No sequences were obtained from the specimens processed.

Etymology The species names refers to the region where the species was found, the Pampa, Argentina.

Prevalence 5.6%

Number of specimens collected 9

Description (based on 9 specimens) Body (based on 3 specimens with fully extended body) 395.7 (349.0–424.6) long, 99.5 (76.4–131.5) wide. Haptor circular 95.3 (93.1–97.5) long, 73.9 (72.6–75.2) wide. Pharynx ($n = 2$) spherical 52.9–61.5 long, 46.3–57.2 wide. MCO was visible in one specimen, but the arrangement and number of spines were difficult to recognize, only the principal hook was visible. Hamulus ($n = 9$), 53.7 (52.0–55.7) long, with curved shaft 36.2 (34.0–37.7) long; point 21.8 (20.5–23.6) long and slender, constituting less than half of the shaft length; proximal shaft width 7.5 (6.5–8.4) narrow; aperture distance 25.5 (18.0–28.9) long; wide aperture angle 49.1° (37.2°–53.5°); straight root 20.5 (17.9–22.6) long, proximal end rectangular, and almost the same with than the hamulus shaft (Fig. 2a, e). Dorsal bar ($n = 2$), 1.3–1.6 long, and straight, with uniform length along its width, becoming narrower in the hamulus attachment points, 20.1–21.7 width, oval attachment points 7.8–8.1 long (Fig. 2b, f). Ventral bar ($n = 7$), 22.4 (18.6–26.3) long "V"-shaped, 20.0 (18.0–22.3) wide; small and short processes 2.0 (0.8–3.3) long, pointed laterally with curved ends; median portion 5.2 (4.2–6.7) long, rectangular with rounded postero-lateral ends; membrane "V"-shaped, with truncate distal end, 14.5 (11.2–18.8) long (Fig. 2c, g). Marginal hook ($n = 8$), 25.7 (21.4–27.8) long; shaft slim 20.3 (16.1–21.8) long; sickle 6.1 (5.7–6.5) long, with erected shaft developing a small curve ending in a short point facing slightly upwards, ending just at the level of the bridge, sickle point 2.1 (1.6–2.9) long, short, and angled bridge, toe 1.9 (1.6–2.1) long, trapezoidal, and straight at the level of the sickle base, with a sort of light curvature where the shaft attaches to the sickle, heel rounded, and short; distal width 2.1 (1.6–2.8); aperture 5.3 (4.8–5.6); instep height 0.5 (0.2–1.1) (Fig. 2d, h).

Remarks *Gyrodactylus pampeanus* n. sp. is readily distinguishable from its congeners parasitizing poeciliids, goodeids, and profundulids, by showing a particular

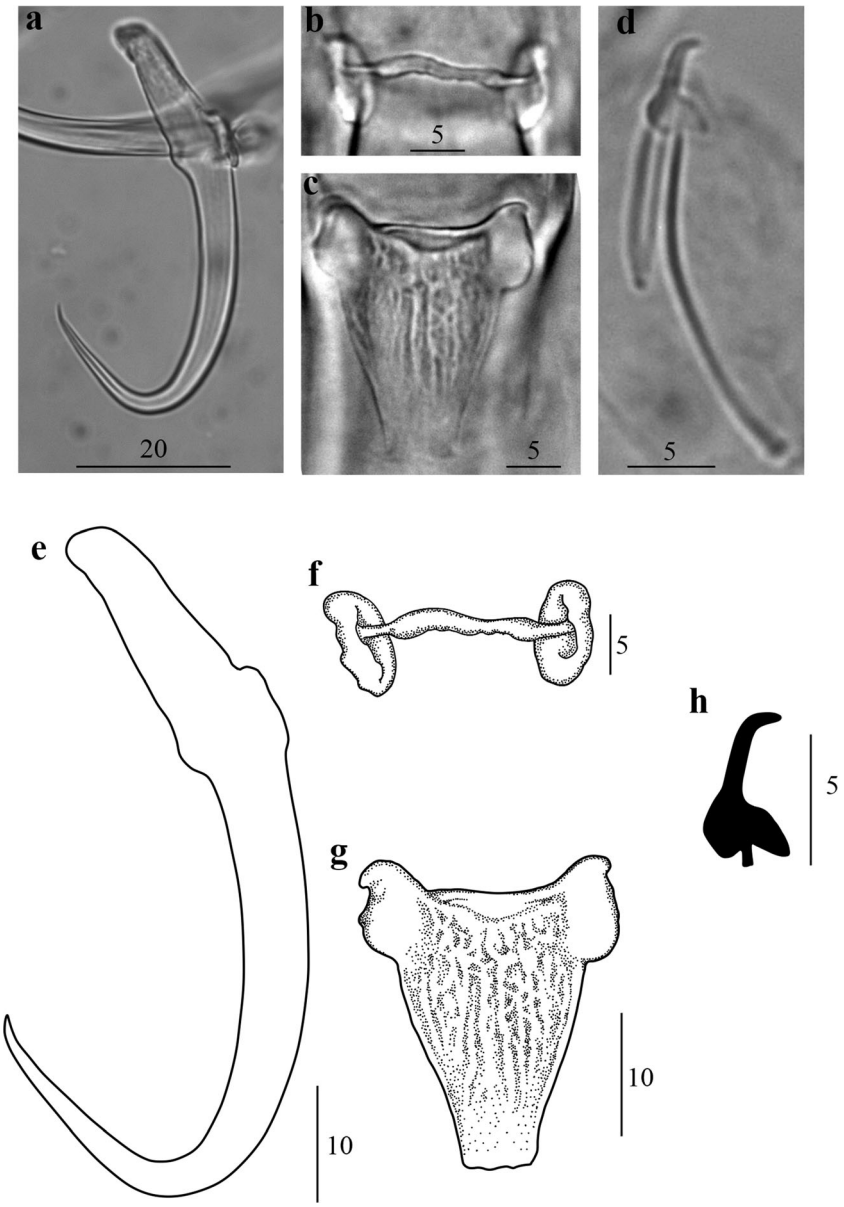

Fig. 2 Light micrographs and drawings of *Gyrodactylus pampeanus* n. sp. **a** Haptoral complex. **b** Dorsal bar. **c** Ventral bar. **d** Marginal hook at a glance. **e** Hamulus. **f** Dorsal bar. **g** Ventral bar. **h** Marginal hook sickle

combination of morphometric characteristics of haptoral attachment structures (Fig. 4). Despite this, its MH shows morphometric similarities with several congeners (Fig. 4b), among which it is morphologically most similar to *G. costaricensis* described from *Poecilia sphenops* from Costa Rica (Kritsky and Fritts 1970). However, the MH point is slim in *G. costaricensis*, whereas it is thicker in *G. pampeanus* n. sp.; also, the heel is straight in its union with the marginal hook shaft in *G. costaricensis* while in *G. pampeanus* n. sp., it forms a deep curve with the marginal hook shaft. Moreover, the new species differs from its congeners in the morphology and morphometry of both the hamuli (Fig. 4a) and ventral bar (Fig. 4c).

Gyrodactylus breviradix Vega, Razzolini, Arbetman, and Viozzi, 2019 (Fig. 3; Table 1).

Site of infection Fins and body surface.

Locality La Tapera Creek, Mar del Plata, Buenos Aires province, Argentina (37° 56′ 40″ S – 57° 32′ 22″ W).

Voucher specimens Six specimens (accession no. MLP-HE XXX) deposited in the Helminthological Collection of the Museo de La Plata (HCMLP), La Plata, Argentina; two specimens (acc. no. CNHE 11064) deposited in Colección Nacional de Helmintos (CNHE), Mexico City.

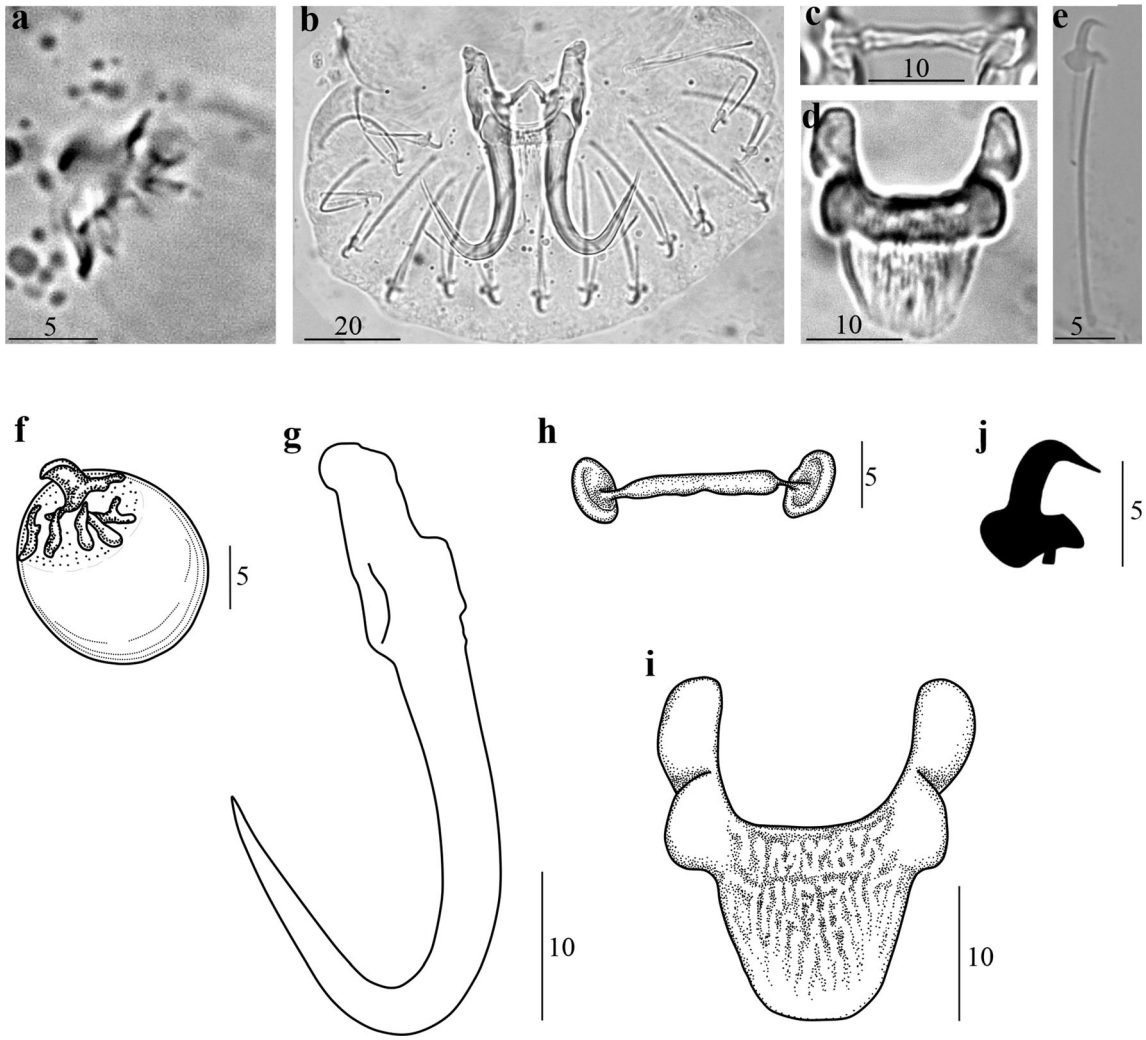

Fig. 3 Light micrographs and drawings of *Gyrodactylus breviradix*. **a** Male copulatory organ (MCO). **b** Haptoral complex. **c** Dorsal bar. **d** Ventral bar. **e** Marginal hook at a glance. **f** MCO. **g** Hamulus. **h** Dorsal bar. **i** Ventral bar. **j** Marginal hook sickle

DNA reference sequences Sequences obtained from 3 individuals are deposited in GenBank (accession nos.: MK965395-965397 for ITS and MN927192 for COII).

Prevalence 14%

Number of specimens collected 15

General measurements Body (based on 4 specimens with fully extended body) 550.7 (475.3–623.8) long, 116.3 (97.9–140.3) wide. Pharynx ($n = 3$) ovoid 45.8 (42.4–47.5) long, 34.2 (30.9–36.9) wide. Male copulatory organ (MCO, $n = 3$), 18.8 (16.1–21.1) long, 15.05 (13.9–15.9) wide, principal hook (6.18 long, $n = 1$), spines (3.08 long, $n = 4$) (Fig. 3a, f). Haptor (based on 4 specimens with fully extended haptor) 83.0 (80.0–86.3) long, 73.4 (66.6–81.0) wide. Measurements of H, MH, and VB given in Table 1 (Fig. 3j).

Remarks nMDS biplots showed that the H and MH of the specimens found in *C. decemmaculatus* from La Tapera creek were similar both morphologically and morphometrically to those of *G. breviradix* described from the same host species in Patagonia, indicating that they are conspecifics. Unfortunately, the morphometry of the VB could not be included in the analyses, because the low quality of available images from type specimens provided by Vega et al. 2019 did not allow obtaining reliable measurements. Multivariate morphometric analyses indicated that *G. breviradix* is similar to *G. takoke* for all the three structures measured, and to *G. xalapensis* for H and MH (Fig. 4a, b). However, *G. breviradix* can be differentiated from *G. takoke* by having a straight and shorter marginal hook sickle, whereas it is longer and angled downwards in *G. takoke* and *G. xalapensis*; furthermore, the marginal hook sickle base is at the level of the sickle base line in *G. takoke* and *G. xalapensis*, whereas it is angled upwards in *G. breviradix*; finally, the toe is trapezoid in

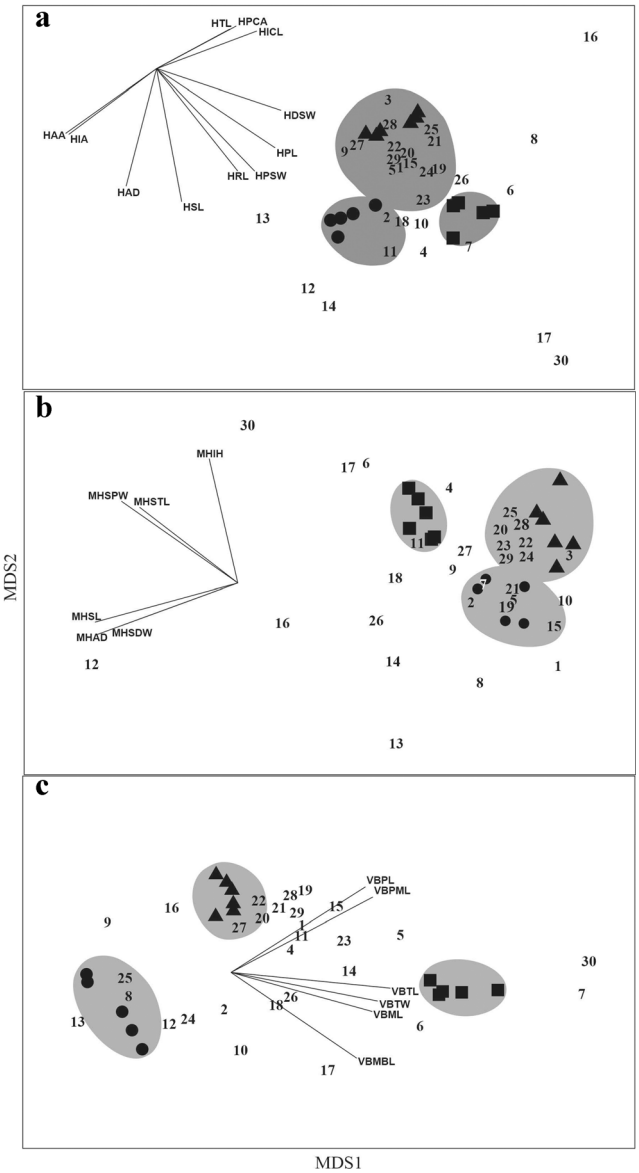

Fig. 4 Metric Multidimensional Scaling (MDS) ordination plot considering hamulus (**a**), marginal hook (**b**), and ventral bar (**c**) point-to-point measurements of known *Gyrodactylus* species infecting poeciliid, goodeid, and profundulid fishes. Symbols represent individual worms of the three species found in the present study: *Gyrodactylus breviradix* (triangle), *Gyrodactylus marplatensis* n. sp. (square), and *Gyrodactylus pampeanus* n. sp. (circle). Numbers represent averaged values of the following *Gyrodactylus* species: (1) *G. actzu*; (2) *G. apazapanensis*; (3) *G. breviradix*; (4) *G. bullatarudis*; (5) *G. chiapaneco*; (6) *G. decemmaculati*; (7) *G. costaricensis*; (8) *G. cytophagus*; (9) *G guatopotei*; (10) *G. iunuri*; (11) *G. jarocho*; (12) *G. katamba*; (13) *G. lamothei*; (14) *G. lhkahuili*; (15) *G. microdactylus*; (16) *G. milleri*; (17) *G. montealbani*; (18) *G. pictae*; (19) *G. poeciliae*; (20) *G. pseudobullatarudis*; (21) *G. rasini*; (22) *G. takoke*; (23) *G. tlaloci*; (24) *G. tepari*; (25) *G. tomahuac*; (26) *G. turnbulli*; (27) *G. unami*; (28) *G. xalapensis*; (29) *G. xtachuna*; and (30) *G. zapoteco*. Results of a hierarchical agglomerative overlaid on the nMDS biplot with distances (2.95 for hamuli, 1.5 for ventral bar, and 2.7 for marginal hook) represented by shaded areas. Vector overlays are Pearson correlations of measurements with the canonical analysis of principal coordinate axes. Vector names as in Table 1

shape in both *G. takoke* and *G. xalapensis* while it is square-shaped in *G. breviradix*. (Figs. 3e, j, 4b).

Phylogenetic analyses

Sequences of ITS were obtained from three specimens of *G. breviradix* and from two specimens of *G. marplatensis* n. sp.. Unfortunately, sequences from *G. pampeanus* n. sp. were impossible to obtain, probably due to inadequate specimen fixation. The phylogenetic tree of the ITS dataset included three unique sequences of *G. breviradix*, two unique sequence of *G. marplatensis* n. sp., and 57 sequences of 27 species of *Gyrodactylus* retrieved from GenBank (see Table 2). *Gyrodactylus mojarrae* and *Gyrodactylus* sp. B, parasites of non-cyprinodontiform Neotropical fishes were used as outgroup. Phylogenetic hypotheses produced by BI and ML analyses are shown in Fig. 5a. Strong nodal support of bootstrap and posterior probability values were obtained for specimens of *G. marplatensis* n. sp. and *G. breviradix*, respectively, in all analyses. *Gyrodactylus breviradix* formed a clade with high branch support (95/0.99) with the poeciliid fish-infecting *G. xalapensis* and *G. takoke*, as a sister species. *Gyrodactylus marplatensis* n. sp. appears in a further clade, grouped with *G. decemmaculati* and *G. guatopotei*, both parasites of poeciliids. Genetic divergence for ITS1-5.8S-ITS2 among *G. marplatensis* n. sp., *G. breviradix* and the other 27 *Gyrodactylus* species showed a great amount of nucleotide variation, ranging from 0.1 to 54.49%. Values of nucleotide inter-specific variation between *G. breviradix*, *G. xalapensis*, and *G. takoke* ranged from 11.9 to 13.3% and 10.8 to 13.5%, respectively. Nucleotide variation between *G. marplatensis* n. sp., *G. decemmaculati* and *G. guatopotei* was 1.7% and 7.9%, respectively. The intra-specific variation of *G. breviradix* was of 0–5% and in *G. marplatensis* n. sp. was null.

The COII data set included 47 sequences with 262 nucleotides. Sequences of COII were obtained from one specimen of *G. breviradix* and two specimens of *G. marplatensis* n. sp. Phylogenetic hypotheses produced by BI and ML analyses are shown in Fig. 5b. *Gyrodactylus breviradix* and *G. marplatensis* n. sp. were found to be reciprocally monophyletic in all analyses, with strong nodal support of bootstrap and posterior probability values. *Gyrodactylus breviradix* formed a clade with high branch support (98/0.99). *Gyrodactylus marplatensis* n. sp. appears in a further clade, grouped with *G. decemmaculati* and *G. guatopotei*. Genetic divergence for COII among 18 *Gyrodacylus* spp. ranged from 0.1 to 41.26%. Nucleotide variation between *G. marplatensis* n. sp., *G. decemmaculati* and *G. guatopotei* was of 11.1% and 24.8%, respectively. The intra-specific

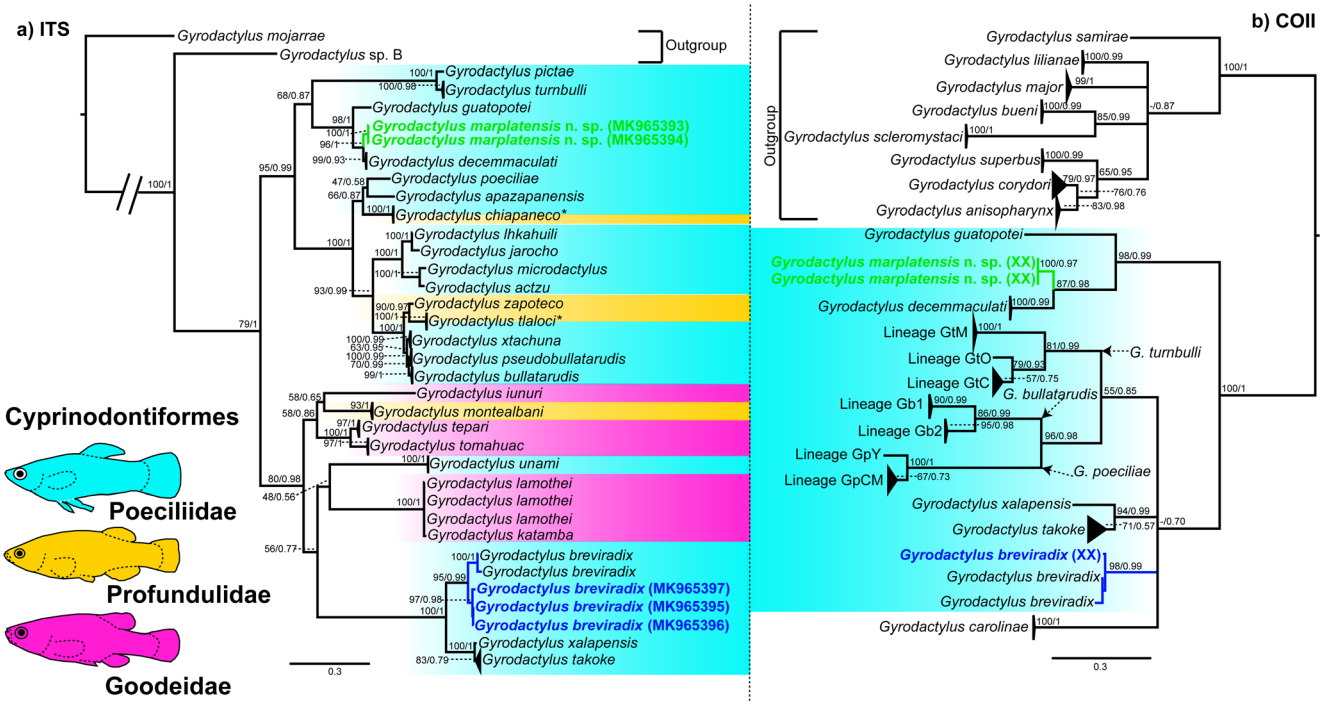

Fig. 5 Phylogenetic hypothesis for *Gyrodactylus* spp. of *Cnesterodon decemmaculatus* using ITS data (**a**), and COII data (**b**). Phylogenetic trees inferred through Maximum Likelihood (ML) and Bayesian Inference (BI). Numbers near internal nodes show bootstrap and the posterior probability of clade frequencies. Scale bars indicate the number of substitutions per site. The species characterized in the present study are shown in color: *Gyrodactylus breviradix* (blue), *Gyrodactylus marplatensis* n. sp. (green). Single asterisk indictaes both *G. chiapaneco* and *G. tlaloci* naturally infect fishes from the poeciliid and profundulid families

variation of *G. breviradix* was of 0.4 to 2.1% and in *G. marplatensis* n. sp. was null.

Discussion

In the present study, the combination of traditional morphological comparisons and different point to point morphometric measurements under multivariate statistical procedures allowed an easy discrimination and identification of species of *Gyrodactylus*. Although a broad set of morphometric characters are often provided in the descriptions of *Gyrodactylus* species (e.g., Shinn et al. 2004; Rubio-Godoy et al. 2010), many papers including recent ones restrict morphometric and morphological comparisons to marginal hook characteristics, since these structures have frequently proven to be enough for differentiating *Gyrodactylus* congeners (Malmberg 1970; Shinn et al. 1996; Kay et al. 1999; Cunningham et al. 2001; Rubio-Godoy et al. 2010; García-Vásquez et al. 2015). However, significant variability can occur, clouding the capabilities of such structures for species discrimination. Indeed, intra-specimen differences between the second and eighth marginal hooks have been recorded for some species (Huyse and Volckaert 2002; Rubio-Godoy et al. 2010). Furthermore, the shape and size of haptoral hard structures can be subjected to phenotypic plasticity (Olstad et al. 2009) associated with host and environmental parameters whose effect can vary between structures (ventral bar, hamuli or marginal hook), and species (Harris 1998; Geets et al. 1999; Huyse and Volckaert 2002; Dávidová et al. 2005). Therefore, researchers should be cautious when restricting the identification and differentiation of *Gyrodactylus* species to evidence retrieved from marginal hooks only. In the present study, the diagnostic structures that better differentiated the species from their congeners differed among the two new species, highlighting the need of simultaneously analyzing all possible morphometric data of haptoral structures, as demonstrated in several previous studies (Kay et al. 1999; McHugh et al. 2000; Shinn et al. 2000, 2004; Huyse and Volckaert 2002). This is especially applicable to gyrodactylids infecting poeciliids, which have a broad distribution that includes different biogeographic regions and are, therefore, exposed to highly contrasting environmental variables.

In general, a considerable agreement was observed among morphological, morphometric, and genetic methods in the differentiation and identification of the species reported here, demonstrating the usefulness of combining complementary methodologies for the delimitation of species of *Gyrodactylus*. However, the genetic distances, recorded for new ITS sequences of *G. breviradix*, showed a high variability (0–5%) when compared with specimens from Patagonia

(Vega et al. 2019), which is also reflected in their division in two clades in the phylogenetic tree. ITS differentiation values higher than 4% have been considered indicative of cryptic species for some morphologically indistinguishable species of *Gyrodactylus* (Razo-Mendivil et al. 2016). However, there is no consensus on the level of differentiation of ITS that reflects the presence of different taxa for this genus. Distances higher than 1% could be indicative of interspecific differentiation, when these differences are accompanied with some kind of meaningful ecological differentiation (Ziętara and Lumme 2003). Nevertheless, levels of variation up to 1.84% have been reported for specimens of a single species infecting two poeciliid hosts in three different river basins (García-Vásquez et al. 2015). On the other hand, for mtDNA, a variability smaller than 10% has been suggested to be intra-specific (Kuusela et al. 2008). The variation observed inCOII sequences of *G. breviradix* (0.4 to 2.1%) are within that range, being similar to those of some congeners infecting poeciliids, such as *G. turnbulli* and *G. poeciliae*, whose CCOII sequences vary between 0.4–3.4% and 0.4–2.3%, respectively (Xavier et al. 2015). Because of the contrasting results from mitochondrial and nuclear sequences, and considering that no morphological/morphometrical differences were observed among specimens from both regions, the new material is provisionally regarded as *G. breviradix*, until further research, based on larger samples, wider geographic areas, and additional genetic markers, allows a definitive identification of this species.

For *G. marplatensis* n. sp., different haptoral features or their combinations were responsible for morphological similarities among genetically closely related species, confirming that sclerites other than the marginal hooks are also important for species delimitation.

The extant diversity of the genus *Gyrodactylus* is the result of a combination of two kinds of evolutionary events, namely co-evolution, promoted by the direct life-cycle and high host-specificity of their representatives, and speciation by host switching, facilitated by the ability for auto-infection of their members (Brooks and McLennan 1993; Huyse and Volckaert 2002). Phylogenetic hypotheses of *Gyrodactylus* species infecting poeciliids propose that they constitute a polyphyletic group (García-Vásquez et al. 2015, 2019), a finding which is supported in the present study.

In South America, only 5 species of *Gyrodactylus* have been reported on native poeciliids: *G. turnbulli* from *Poecilia reticulata* from Peru (An et al. 1991), *G. milleri* and *G. poeciliae* from *Poecilia caucana* from Venezuela (Harris and Cable 2000), and recently, *G. decemmaculati* and *G. breviradix* from *Cnesterodon decemmaculatus*, a host recently introduced in Patagonia, Argentina (Vega et al. 2019). The present study adds two new species to the gyrodactylid fauna of poeciliids, representing the southernmost record of the genus in natural and native populations of poeciliids in the Americas. The present findings illustrate the potential for finding several more species in South America, as *C. decemmaculatus* is now known to harbor four species of *Gyrodactylus*.

Acknowledgments We thank Delfina Canel and Manuel Irigoitia for the assistance with field collections and Dr. Rocio Vega for providing the images of *G. breviradix* and *G. decemmaculati* for the measurement. We also want to thank to Gina Galllo (INECOL) for the help with the illustrations and to Dr. Delfina Cantatore for the help with phylogenetic analyses.

Funding information This work was supported by Grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) – Argentina (PIP no. 112-201501-00973), Fondo para la Investigación Ciencia y Tecnología (FonCyT)– Argentina (PICT 2015 no. 2013, PICT-2016-4175) and Universidad Nacional de Mar del Plata (UNMdP) – Argentina (EXA869/16).

Compliance with ethical standards

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

Ethical approval All applicable institutional, national, and international guidelines for the care and use of animals were followed. Permit for fishing provided by Ministerio de Asuntos Agrarios de la Provincia de Buenos Aires, Argentina (Disposición 164, August 23, 2012).

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