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Phylogenetic relationships and further unknown diversity of diplostomids (Diplostomida: Diplostomidae) parasitic in kingfishers

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Research Paper

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Abstract

Kingfishers (Alcedinidae Rafinesque) are common inhabitants of wetlands and are known to be definitive hosts to a wide range of digeneans that parasitize fish as second intermediate hosts. Among these digeneans, members of the Diplostomidae Poirier, 1886 (diplostomids) are particularly common. Recent studies of diplostomids collected from kingfishers have revealed that they are probably more diverse than currently known. This particularly concerns the genera *Crassiphiala* Van Haitsma, 1925 and *Uvulifer* Yamaguti, 1934. In the present work, we studied seven diplostomid taxa from kingfishers in Brazil, the USA and the Philippines. Partial DNA sequences of the nuclear large ribosomal subunit (28S) and mitochondrial cytochrome *c* oxidase I (*cox*1) genes were obtained, and 28S sequences were used to study the phylogenetic interrelationships of these diplostomids. We provide the first DNA sequences from *Uvulifer semicircumcisus* Dubois et Rausch, 1950 and a member of *Subuvulifer* Dubois, 1952. *Pseudocrassiphiala* n. gen. is erected for a previously recognized species-level lineage of *Crassiphiala* and a new generic diagnosis of *Crassiphiala* is provided. *Crassiphiala jeffreybelli* n. sp., *Crassiphiala wecksteini* n. sp. and *Pseudocrassiphiala tulipifera* n. sp. are described, and a description of newly collected, high-quality specimens of *Crassiphiala bulboglossa* Van Haitsma, 1925 (the type-species of the genus) is provided.

Introduction

Kingfishers (Alcedinidae Rafinesque) are known to be definitive hosts of a wide range of digeneans that use fishes as second intermediate hosts (Van Haitsma, 1925; Hoffman, 1956; Boyd & Fry, 1971; Merino *et al.*, 2003; Muzzall *et al.*, 2011). Among these digeneans, members of the Diplostomidae Poirier, 1886 are most common worldwide, including in the New World (Hoffman, 1956; Muzzall *et al.*, 2011; López-Jiménez *et al.*, 2018; Achatz *et al.*, 2019a, b, 2021a, b). Members of nine diplostomid genera are known to parasitize kingfishers (Dubois, 1968; Niewiadomska, 2002; Achatz *et al.*, 2021a, b); recent studies on diplostomids of kingfishers have revealed that they are certainly more diverse than previously known. This particularly concerns the genera *Crassiphiala* Van Haitsma, 1925 and *Uvulifer* Yamaguti, 1934 (López-Jiménez *et al.*, 2018; Achatz *et al.*, 2019a, b, 2021a, b). Members of both genera are known to encyst on the skin/fins of fishes and often cause 'black spot disease' in their fish second intermediate hosts (Van Haitsma, 1925; Hunter, 1933; Hoffman, 1956). Their association with potential health concerns of fishes has led to interest in revealing the identities of these digeneans.

Herein, we obtained DNA sequences from seven diplostomid taxa infecting kingfishers, including *Crassiphiala* and *Uvulifer* spp., collected in North and South America as well as the Philippines. The newly generated partial sequences of the nuclear large ribosomal subunit (28S) gene were used to study the interrelationships of these diplostomids. We used sequences of the more variable mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene for differentiation among closely related species. We provide descriptions of a new diplostomid genus and three new species of diplostomids. Additionally, we provide an amended diagnosis of *Crassiphiala* and a description of *Crassiphiala bulboglossa* Van Haitsma, 1925, the type-species of the genus, using newly collected high-quality specimens. We have generated the first DNA sequence data of a member of *Subuvulifer* Dubois, 1952.

Materials and methods

Adult diplostomids were collected from the intestines of a belted kingfisher *Megaceryle alcyon* (Linnaeus) in North Dakota, USA, a ringed kingfisher *Megaceryle torquata* (Linnaeus) and a green kingfisher *Chloroceryle americana* (Gmelin) in Pantanal, Mato Grosso State, Brazil, *M. torquata* in Lábrea, State of Amazonas, Brazil and a white-throated kingfisher *Halcyon smyrnensis* (Linnaeus) from the Mindoro Island, Philippines. A single *M. alcyon* from North Dakota was collected using the federal collecting permit MB072162-0. A single *M. torquata* from Lábrea was collected as a part of the biodiversity survey funded by the National Science Foundation, USA, based on the collecting permit 37740-4 from the Ministry of the Environment (Ministério do Meio Ambiente), Brazil. Birds in Pantanal were collected based on the collecting permit 10698-1 and birds in the Philippines were obtained for parasitological examination from Dr Carl Oliveros as part of the biodiversity survey funded by the National Science Foundation.

Metacercariae of *Uvulifer semicircumcisus* Dubois et Rausch, 1950 were collected from the skin and fins of the Northern redbelly dace *Chrosomus eos* Cope in Minnesota, USA. The metacercariae were removed from the host and directly fixed in 70% ethanol.

Specimens for morphological study were stained with aqueous alum carmine and permanently mounted following the protocol of Lutz *et al.* (2017). Morphology was studied using an Olympus BX51 compound microscope (Tokyo, Japan) equipped with differential interference contrast optics and a digital imaging system; drawings were prepared with the aid of a drawing tube. All measurements are provided in micrometres. Type series and morphological vouchers of adult specimens are deposited in the Museu Paraense Emílio Goeldi (MPEG), Belém, Brazil and the collection of the H. W. Manter Laboratory, University of Nebraska, Lincoln, Nebraska, USA (table 1). Previously deposited vouchers identified as *Crassiphiala* spp. and sequenced by Achatz *et al.* (2019b) were re-examined.

Table 1. Hosts, GenBank accession numbers and museum accession numbers assigned by the Museu Paraense Emílio Goeldi (MPEG) and Harold W. Manter Laboratory (HWML) for diplostomids studied in present work.

Taxa	Life stage	Host species	Geographic origin	Museum no.	GenBank accession numbers	
					28S	*cox*1
Crassiphiala bulboglossa	A	*Megaceryle alcyon*	North Dakota, USA	HWML 216888–216893	–	**OP688075–OP688082**
C. bulboglossa[a]	A	*Megaceryle alcyon*	Minnesota, USA	–	MN200254	MN193952
C. bulboglossa[a]	M	*Chrosomus eos*	Minnesota, USA	–	MN200255	MN193953
C. bulboglossa[a]	M	*Umbra limi*	Minnesota, USA	–	MN200256	MN193954, MN193955
Crassiphiala jeffreybelli n. sp.	A	*Megaceryle torquata*	Pantanal, Brazil	MPEG 000335–000339 HWML 216896	–	**OP688085**
C. jeffreybelli n. sp.	A	*Chloroceryle americana*	Pantanal, Brazil	–	**OP687981**	**OP688086**
Crassiphiala wecksteini n. sp.	A	*Megaceryle torquata*	Pantanal, Brazil	MPEG 000349–000354 HWML 216014, 216894, 216895	**OP687979, OP687980**	**OP688083, OP688084**
C. wecksteini n. sp.[b]	A	*Megaceryle torquata*	Pantanal, Brazil	HWML 216014	MN200261	MN193959, MN193960
Pseudocrassiphiala tulipifera n. sp.	A	*Megaceryle torquata*	Lábrea, Brazil	–	**OP687982**	**OP688087**
P. tulipifera n. sp.[c]	A	*Megaceryle torquata*	Pantanal, Brazil	MPEG 000340–000348 HWML 216013, 216897, 216898	MN200258–MN200260	MN193957, MN193958
Pseudocrassiphiala sp. VVT1	A	*Chloroceryle americana*	Pantanal, Brazil	–	**OP687983**	**OP688088**
Subuvulifer glandulaxiculus	A	*Halcyon smyrnensis*	Philippines	HWML 216918–216925	**OP687984–OP687986**	**OP688089–OP688092**
Uvulifer semicircumcisus	M	*Chrosomus eos*	Minnesota, USA	–	**OP687987**	**OP688093**
U. semicircumcisus	A	*Megaceryle alcyon*	North Dakota, USA	HWML 216926–216927	–	**OP688094–OP688096**

Abbreviations for life stage: A, adult; C, cercaria; M, metacercaria. GenBank accession numbers for new sequences generated in the present study are in bold.
[a]Previously published as *Crassiphiala* sp. lineage 2 of Achatz *et al.* (2019b).
[b]Previously published as *Crassiphiala* sp. lineage 5 of Achatz *et al.* (2019b).
[c]Previously published as *Crassiphiala* sp. lineage 4 of Achatz *et al.* (2019b).

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The DNA extraction as well as the amplification and sequencing of partial 28S and *cox*1 genes were performed as previously described by Achatz *et al.* (2019b, 2022a). Newly generated contiguous sequences were assembled with the assistance of Sequencher version 4.2 software (GeneCodes Corp., Ann Arbor, Michigan, USA) and deposited in GenBank (table 1).

Phylogenetic relationships of the diplostomid taxa studied in the present work were estimated based on an alignment of partial 28S sequences. The alignment included newly generated sequences from members of *Crassiphiala*, *Uvulifer*, *Subuvulifer* and the new genus (table 1) and previously published sequences of 37 diplostomids and 14 strigeids. The alignment included representatives from all currently sequenced genera of the Diplostomidae and Strigeidae Railliet, 1919; we only included sequences that were at least 1,100 base pairs (bp) long to avoid significant loss of data. Only two representatives of the Proterodiplostomidae Dubois, 1936 were included because the monophyly of the family has been previously demonstrated (Tkach *et al.*, 2020). *Suchocyathocotyle crocodili* (Yamaguti, 1954) was used as the outgroup for the analyses based on the study by Achatz *et al.* (2019c).

ClustalW implemented in MEGA7 was used to align newly generated and previously published sequences (Kumar *et al.*, 2016). The alignment was trimmed to the length of the shortest sequence; ambiguous positions were excluded from each analysis.

The best-fitting nucleotide substitution models for the alignment were determined using MEGA7. The analysis used the general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + G + I) model. Bayesian Inference (BI) as implemented in MrBayes v3.2.6 software and Maximum Likelihood (ML) as implemented in MEGA7 were used to perform the phylogenetic analyses (Ronquist & Huelsenbeck, 2003; Kumar *et al.*, 2016). The BI analysis was performed as follows: Markov chain Monte Carlo (MCMC) chains were run for 3,000,000 generations with sample frequency set at 1,000. This number of generations was considered sufficient as the standard deviation stabilized below 0.01. Log-likelihood scores were plotted and only the final 75% of trees were retained to produce the consensus trees. Nodal support of ML analysis was estimated by performing 1,000 bootstrap pseudoreplicates. Pairwise comparisons were performed using MEGA7.

Results

Molecular phylogenies

Upon trimming to the length of the shortest sequence, the alignment was 1,115 bp long; 33 nucleotide sites were excluded due to ambiguous homology. The strongly supported topologies were identical between the BI and ML analyses. The Diplostomidae and Strigeidae were clearly non-monophyletic throughout the basal polytomy (fig. 1); at the same time, the representatives of the Proterodiplostomidae formed a strongly supported clade (100% BI; 99% ML). These results are generally similar to those published and discussed in previous molecular phylogenetic studies (e.g. Blasco-Costa & Locke, 2017; Hernández-Mena *et al.*, 2017; Achatz *et al.*, 2019b, c, 2021a, b, 2022a, b; Tkach *et al.*, 2020; Locke *et al.*, 2021). Therefore, below we focus on details related to the clades that contain newly generated data.

Members of *Subuvulifer*, *Crassiphiala*, *Uvulifer*, *Posthodiplostomoides* Williams, 1969 and the new genus formed a strongly supported clade (100% BI; 97% ML) in the basal polytomy of the Diplostomoidea consisting of five clades/branches with unresolved relationships, each representing a single genus (fig. 1). *Subuvulifer* and *Posthodiplostomoides* were represented in the tree by a single species each, *Subuvulifer glandulaxiculus* Pearson et Dubois, 1985 and *Posthodiplostomoides kinsellae* Achatz, Chermak, Martens, Pulis et Tkach, 2021. The clade containing two species of *Pseudocrassiphiala* n. gen. was strongly supported (100% BI; 99% ML).

The strongly supported (100% BI; 99% ML) clade containing *Crassiphiala* spp. (weak support in BI and ML) included two clusters: *Crassiphiala jeffreybelli* n. sp. + *Crassiphiala* sp. lineage 1 of Achatz *et al.* (2019b) (99% BI; 100% ML) and *C. bulboglossa* + *Crassiphiala wecksteini* n. sp. + *Crassiphiala* sp. lineage 3 of Achatz *et al.* (2019b) (100% BI; 99% ML).

Lastly, the clade of *Uvulifer* spp. included a well-supported (99% BI; 92% ML) grouping of *Uvulifer pequenae* Achatz, Curran, Patitucci, Fecchio et Tkach, 2019 + *Uvulifer spinatus* López-Jiménez, Pérez-Ponce de León et García-Varela, 2018 and a weakly supported clade (low BI support; 57% ML) of *Uvulifer elongatus* Dubois, 1988 + a strongly supported cluster (100% BI; 99% ML) of *Uvulifer ambloplitis* (Hughes, 1927) + *U. semicircumcisus*.

Descriptions of new taxa

At the time of publication of the previous diagnosis of *Crassiphiala* (Niewiadomska, 2002), the genus was considered monotypic. We provide a new diagnosis of the genus to accommodate the features of the new species described herein as well as *Crassiphiala ceryliformis* Vidyarthi, 1938. The latter species was originally placed in *Crassiphiala* and subsequently transferred into *Uvulifer*; we return it to *Crassiphiala* based on morphological evidence (see detailed discussion below).

Family Diplostomidae Poirier, 1886
Genus *Crassiphiala* Van Haitsma, 1925

Diagnosis. Body distinctly bipartite; prosoma generally flattened or with slight concavity, much shorter than cylindrical opisthosoma. Tegument unarmed. Oral sucker present; ventral sucker and pseudosuckers absent. Holdfast organ elliptical or bulbous, with median opening, may occupy entire width of prosoma. Pharynx present; caeca reach level of seminal vesicle. Testes two, tandem. Seminal vesicle compact, winding, with pouch-like expansion at proximal end. Ejaculatory pouch absent. Ejaculatory duct typically short, may be dilated resulting in pouch-like appearance, joins distal part of metraterm to form a short hermaphroditic duct. Hermaphroditic duct opens at apex of genital cone. Genital cone with prepucial (=prepuce-like) fold, opens into genital atrium. Genital atrium with terminal opening. Ovary pretesticular; oötype intertesticular. Vitellarium distributed throughout opisthosoma; vitelline reservoir intertesticular. Excretory pore subterminal on ventral side. In kingfishers. Nearctic, Neotropics, Indomalaya.

Type species: Crassiphiala bulboglossa Van Haitsma, 1925.

Other species: Crassiphiala ceryliformis Vidyarthi, 1938, *Crassiphiala jeffreybelli* n. sp. Achatz, Von Holten, Fecchio et Tkach, *Crassiphiala wecksteini* n. sp. Achatz, Von Holten, Fecchio et Tkach.

Crassiphiala bulboglossa Van Haitsma, 1925 (figs 2 and 3)

Taxonomic summary

Type host: *Megaceryle alcyon* (Linnaeus) (Coraciiformes: Alcedinidae).

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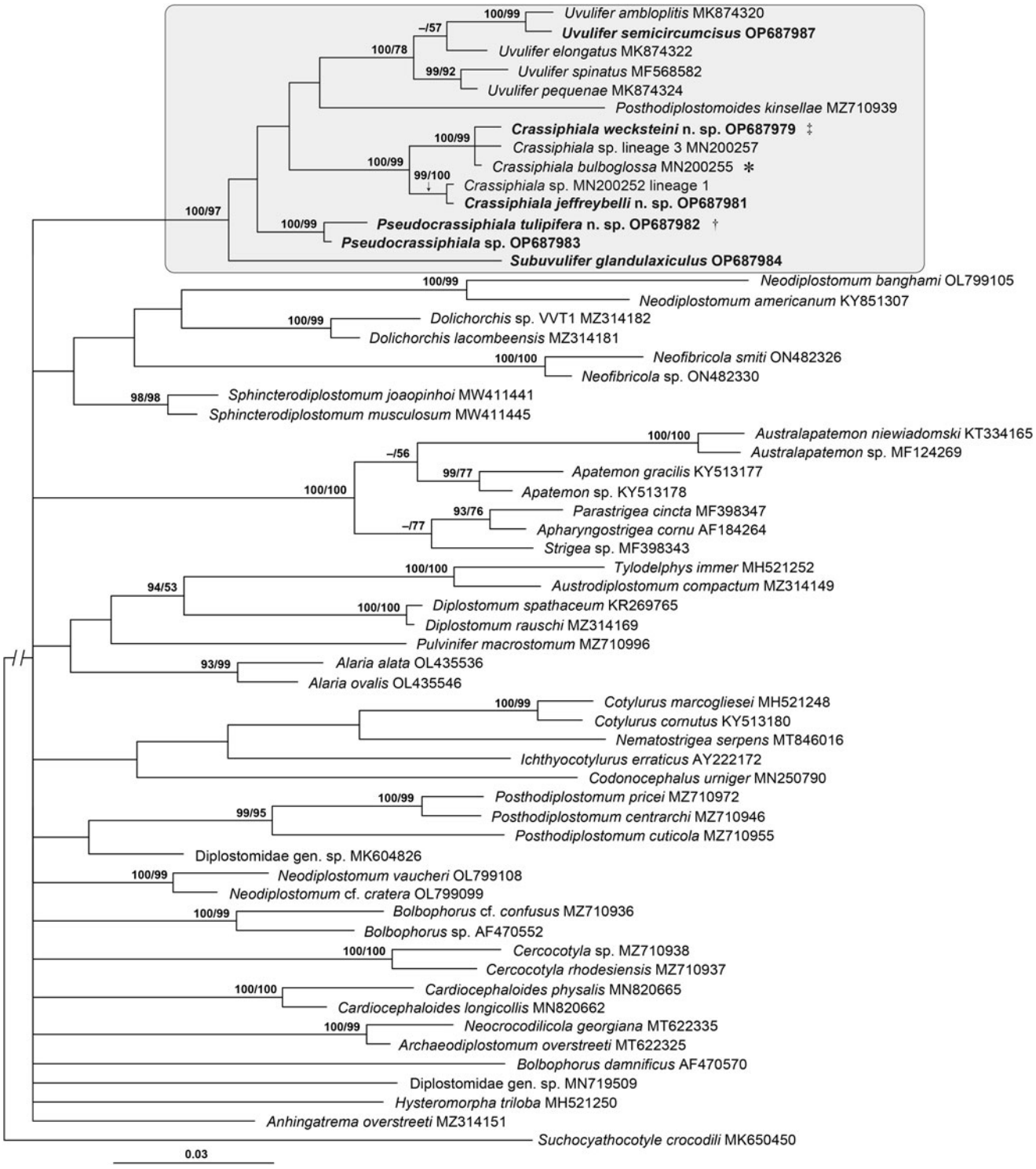

Fig. 1. Phylogenetic interrelationships among 59 diplostomoideans based on Bayesian Inference (BI) and Maximum Likelihood (ML) analyses of partial 28S rDNA gene sequences. Topology from the BI analysis is provided. Bayesian inference posterior probability/ML bootstrap values are provided above internodes. The BI posterior probability values lower than 90% and ML bootstrap values lower than 50% are not shown. The new sequences generated in this study are in bold. The scale bar indicates the number of substitutions per site. The clade containing digeneans studied in the present work is in the shaded box. GenBank accession numbers are provided after names of taxa. *Previously published as *Crassiphiala* sp. lineage 2 of Achatz *et al.* (2019b). ‡Previously published as *Crassiphiala* sp. lineage 5 of Achatz *et al.* (2019b). †Previously published as *Crassiphiala* sp. lineage 4 of Achatz *et al.* (2019b).

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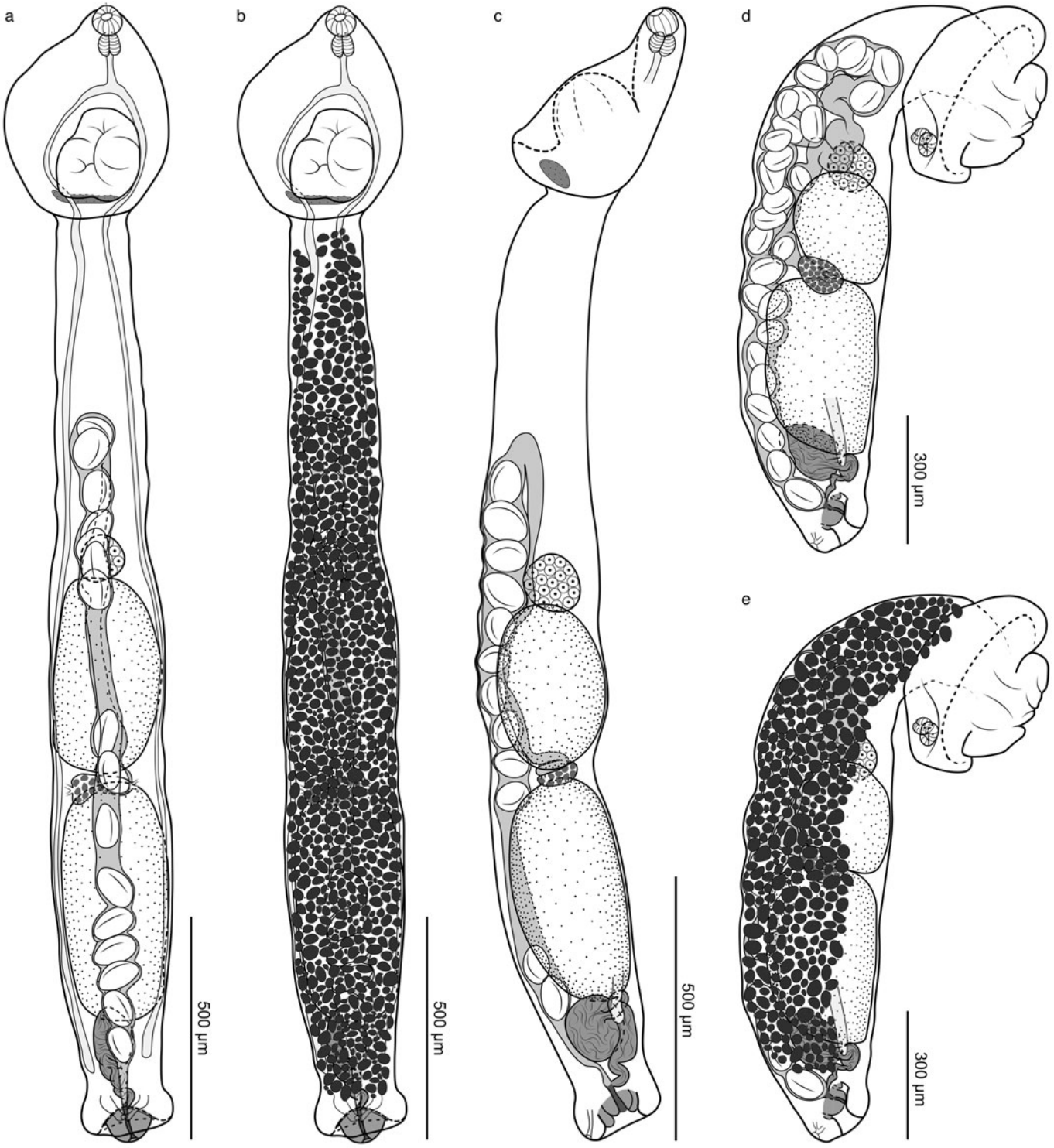

Fig. 2. *Crassiphiala bulboglossa.* (a) hologenophore 1, ventral view with vitellarium omitted; (b) hologenophore 2, ventral view with vitellarium shown; (c) voucher 1, relaxed, lateral view; (d) voucher 2, contracted, lateral view with vitellarium omitted; (e) voucher 3, contracted, lateral view with vitellarium shown.

Site of infection: small intestine.
Type locality: Douglas Lake Michigan, USA.
Collection locality in this study: Grand Forks Co., North Dakota, USA (47°58′56.3″N 97°15′36.3″W).
Infection rate: numerous *C. bulboglossa* were found in single examined *M. alcyon* from North Dakota.
Type material: slides deposited in the National Museum of Natural History (NMNH), Washington DC, under accession number USNPC 071491.00.
New specimens deposited: 38 mature specimens deposited in the HWML. Vouchers: HWML 216888, labelled ex. *Megaceryle alcyon*, small intestine, Grand Forks Co., North Dakota, USA,

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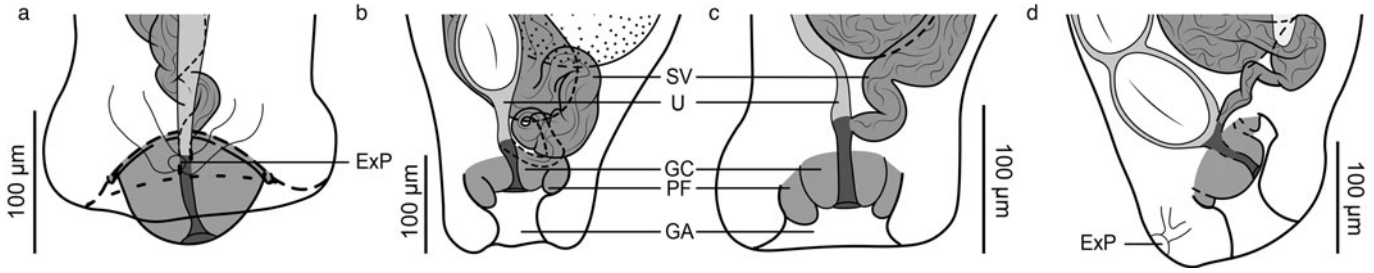

Fig. 3. Posterior end of opisthosoma of *Crassiphiala bulboglossa* with vitellarium omitted. (a) hologenophore 1, ventral view, genital cone everted; (b) hologenophore 2, ventral view; (c) voucher 1, lateral view; (d) hologenophore 3, lateral view. Abbreviations: ExP, excretory pore; GA, genital atrium; GC, genital cone; PF, prepucial fold; SV, seminal vesicle; U, uterus.

Table 2. Morphometric characters of diplostomids described in the present study.

Species	*Crassiphiala bulboglossa*	*Crassiphiala jeffreybelli* n. sp.	*Crassiphiala wecksteini* n. sp.	*Pseudocrassiphiala tulipifera* n. sp.
Body length	1,694–2,982 (2,649)	1,454–1,728 (1,585)	864–1,317 (1,164)	2,534–4,050 (3,373)
Prosoma length	356–545 (458)	315–374 (339)	230–335 (279)	473–612 (545)
Prosoma width	317–478 (389)	270–291 (281)	198–270 (236)	435–527 (480)
Opisthosoma length	1,252–2,562 (2,187)	1,139–1,511 (1,290)	589–1,020 (892)	2,024–3,502 (2,822)
Opisthosoma width	200–339 (288)	161–212 (187)	154–245 (202)	285–402 (345)
Oral sucker length	45–77 (57)	38–65 (47)	28–45 (38)	19–38 (28)
Oral sucker width	51–75 (63)	38–67 (59)	39–50 (44)	38–49 (45)
Pharynx length	37–59 (47)	40–50 (43)	31–43 (36)	40–50 (45)
Pharynx width	45–52 (48)	49–53 (51)	29–40 (35)	40–54 (49)
Oesophagus length	33–72 (52)	21–27 (24)	22–63 (39)	60–88 (73)
Holdfast organ length	194–336 (246)	110–157 (141)	94–191 (150)	205–285 (236)
Holdfast organ width	205–335 (252)	60–93 (72)	132–223 (174)	165–295 (225)
Anterior testis length	206–540 (384)	99–216 (173)	120–207 (175)	323–466 (394)
Anterior testis width	157–284 (221)	65–141 (117)	84–191 (154)	225–327 (271)
Posterior testis length	267–697 (547)	87–211 (164)	154–228 (192)	380–477 (420)
Posterior testis width	160–285 (244)	72–148 (120)	113–175 (151)	225–367 (286)
Ovary length	102–161 (123)	92–107 (99)	55–93 (79)	123–169 (146)
Ovary width	78–134 (107)	72–88 (82)	62–80 (74)	110–138 (129)
Number of eggs	0–32 (17)	2–3 (3)	0–3 (1)	0–20 (4)
Egg length	90–114 (102)	80–93 (88)	103–104 (104)	101–113 (107)
Egg width	48–67 (57)	45–62 (52)	56–63 (60)	46–64 (57)
Prosoma:opisthosoma length ratio	0.2–0.4 (0.2)	0.3 (0.3)	0.2–0.5 (0.3)	0.2–0.3 (0.2)
Opisthosoma length:width ratio	6.2–10.3 (7.8)	5.7–7.7 (7)	3.8–5.2 (4.5)	6.9–10.5 (8.2)
Holdfast organ:prosoma width ratio	0.5–0.9 (0.6)	0.2–0.3 (0.3)	0.6–1.0 (0.7)	0.3–0.5 (0.4)

Ranges provided followed by mean in parentheses.

June 07, 2018, coll. T. Achatz. Hologenophores (five slides): HWML 216889–216893, label identical to the vouchers.

Hologenophore DNA sequences: *cox*1: OP688075 (HWML 216889), OP688076 (HWML 216890), OP688077 (HWML 216891), OP688079 (HWML 216892), OP688081 (HWML 216893).

Previously published genetic lineage name: *Crassiphiala* sp. lineage 2 of Achatz *et al.* (2019b)

Description. Based on 38 adult specimens. Measurement ranges of series given in text and table 2. Body 1,694–2,982 long, consists of distinct prosoma and opisthosoma; prosoma oval, with shallow concavity, 356–545 long, widest at level of holdfast organ, 317–

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478; opisthosoma elongated, cylindrical, 1,252–2,562 × 200–339; opisthosoma length:width ratio 6.2–10.3. Prosoma:opisthosoma length ratio 0.2–0.4. Tegument unarmed. Oral sucker subterminal, 45–77 × 51–75. Pseudosuckers absent. Holdfast organ oval, with longitudinal aperture, armed with fine spines, proximal portion glandular, 194–336 × 205–335; holdfast organ:prosoma width ratio 0.5–0.9. Proteolytic gland consisting of diffuse gland cells. Prepharynx absent. Pharynx subspherical, 37–59 × 45–52. Oesophagus 33–72 long. Caecal bifurcation in anterior 40% of prosoma length. Caeca slender, extend to near level of seminal vesicle.

Testes two, tandem, rounded, entire, anterior testis 206–540 × 157–284, posterior testis 267–697 × 160–285. Seminal vesicle post-testicular, proximal portion pouch-like, followed by winding distal portion that joins distal part of metraterm to form short hermaphroditic duct. Hermaphroditic duct opens at apex of small, muscular genital cone. Genital cone surrounded by small prepucial fold, positioned within genital atrium. Prepucial fold small or not observable when genital cone everted (fig. 3a vs. fig. 3b–d). Genital atrium with terminal opening.

Ovary pretesticular, subspherical, 102–161 × 78–134. Oötype and Mehlis' gland intertesticular (not illustrated). Vitelline follicles limited to opisthosoma, distributed from near level of prosoma–opisthosoma junction to near posterior end of opisthosoma. Vitelline reservoir intertesticular. Uterus ventral to gonads, extends anteriorly beyond level of ovary before turning and extending posteriorly. Uterus in our specimens containing up to 32 eggs. Eggs 90–114 × 48–67.

Excretory pore subterminal, ventral.

Remarks

Historically, descriptions of many diplostomoideans were based on laterally oriented specimens: for example see the numerous illustrations in the monograph by Dubois (1968). The same is true in the case of the original description and illustrations of *C. bulboglossa* (Van Haitsma, 1925). Our newly collected adult specimens of *C. bulboglossa* allowed us to study these digeneans in both ventrodorsal and lateral orientations. Although the description by Van Haitsma (1925) lacked many essential measurements, the description and illustrations closely resemble our contracted, laterally positioned specimens (fig. 2d, e). Our newly collected digeneans demonstrated substantial morphological variation in appearance. Some of this variation may be the result of different ages of the diplostomids or the crowding effect, since the host studied was infected with many hundreds of these digeneans. The extent of body contraction and eversion of the genital cone are at least partly responsible for the observed morphological variation (figs 2 and 3). The partial *cox*1 sequences of *C. bulboglossa* obtained from specimens of different sizes and states of contraction were identical (table 1).

Crassiphiala jeffreybelli n. sp. Achatz, Von Holten, Fecchio et Tkach (figs 4 and 5)

Taxonomic summary

Type host: *Megaceryle torquata* (Linnaeus) (Coraciiformes: Alcedinidae).

Other host: *Chloroceryle americana* (Gmelin) (Coraciiformes: Alcedinidae).

Site of infection: small intestine.

Type locality: Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil (16°21′53″S 56°17′31″W).

Infection rate: one of four of *Megaceryle torquata* and one of three of *Chloroceryle americana* were infected by six and one specimens of *C. jeffreybelli* n. sp., respectively.

Type-material: the type series consists of six mature specimens deposited in the MPEG and HWML. Holotype: MPEG 000335, labelled ex. *Megaceryle torquata*, small intestine, Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil, June 07 2017, coll. A. Fecchio. Paratypes: MPEG 000336–000339 (lot of four slides). Hologenophore: HWML 216896, label identical to the holotype.

Hologenophore DNA sequences: *cox*1: OP688085 (HWML 216896).

Zoobank registration: urn:lsid:zoobank.org:act:153FC1A5-7E02-498D-8E71-F1BD95A24473.

Etymology: the species is named after Dr Jeffrey A. Bell for his contributions to our knowledge of parasites of South American birds and for being a terrific colleague and team member during field trips.

Description. Based on six adult specimens. Measurements of holotype in text; measurements of entire series given in table 2. Body 1,589 long, consists of distinct prosoma and opisthosoma; prosoma oval, flattened, posterior extremity with slight ventral concavity, 345 long, widest at level of holdfast organ, 291; opisthosoma elongated, cylindrical, 1,244 × 161; opisthosoma length:width ratio 7.7. Prosoma:opisthosoma length ratio 0.3. Tegument unarmed. Oral sucker subterminal, 38 × 61. Pseudosuckers absent. Holdfast organ oval, with longitudinal opening, proximal portion glandular, 143 × 60; holdfast organ:prosoma width ratio 0.2. Proteolytic gland consisting of diffuse gland cells. Prepharynx absent. Pharynx subspherical, 41 × 52. Oesophagus 21 long. Caecal bifurcation in anterior 40% of prosoma length. Caeca slender, extend to level of seminal vesicle.

Testes two, tandem, generally rounded, entire, anterior testis 142 × 102, posterior testis 141 × 104. Seminal vesicle post-testicular, proximal portion pouch-like, followed by winding distal portion connected to ejaculatory duct; ejaculatory duct strongly dilated, pouch-like, elongated, glandular, joining distal part of metraterm to form short hermaphroditic duct. Hermaphroditic duct opens at apex of small, muscular genital cone. Genital cone with prepucial fold, positioned within genital atrium; genital atrium with terminal opening.

Ovary pretesticular, subspherical, 98 × 77. Oötype and Mehlis' gland intertesticular (not illustrated). Vitelline follicles limited to opisthosoma, distributed from near level of prosoma to near posterior end of opisthosoma. Vitelline reservoir intertesticular. Uterus ventral to gonads, extends anteriorly beyond level of ovary before turning and extending posteriorly. Terminal part of uterus and proximal part of hermaphroditic duct muscular. Uterus contains three eggs in holotype (up to three eggs in paratypes). Eggs 80–93 × 45–62.

Excretory pore subterminal on ventral side.

Remarks

Crassiphiala jeffreybelli n. sp. belongs to *Crassiphiala* based on several morphological features, including a distinctly bipartite body, vitellarium confined to the opisthosoma, presence of a prepucial fold associated with the genital cone, unarmed tegument and the absence of pseudosuckers, ventral sucker and genital atrium sucker.

Unlike other *Crassiphiala* spp., except for *C. ceryliformis*, the ejaculatory duct is dilated and pouch-like. *Crassiphiala jeffreybelli* n. sp. and *C. ceryliformis* are similar in size (body length 1,454–1,728 in the new species vs. 1,440–1,648 in *C. ceryliformis*), but most organs/structures of C. *ceryliformis* are much smaller (e.g. oral sucker 38–65 × 38–67 in *C. jeffreybelli* n. sp. vs. 17.5–25 × 24–25 in *C. ceryliformis*; pharynx 40–50 × 49–53 in *C. jeffreybelli* n. sp. vs. pharynx 21 × 18–23 in *C. ceryliformis*).

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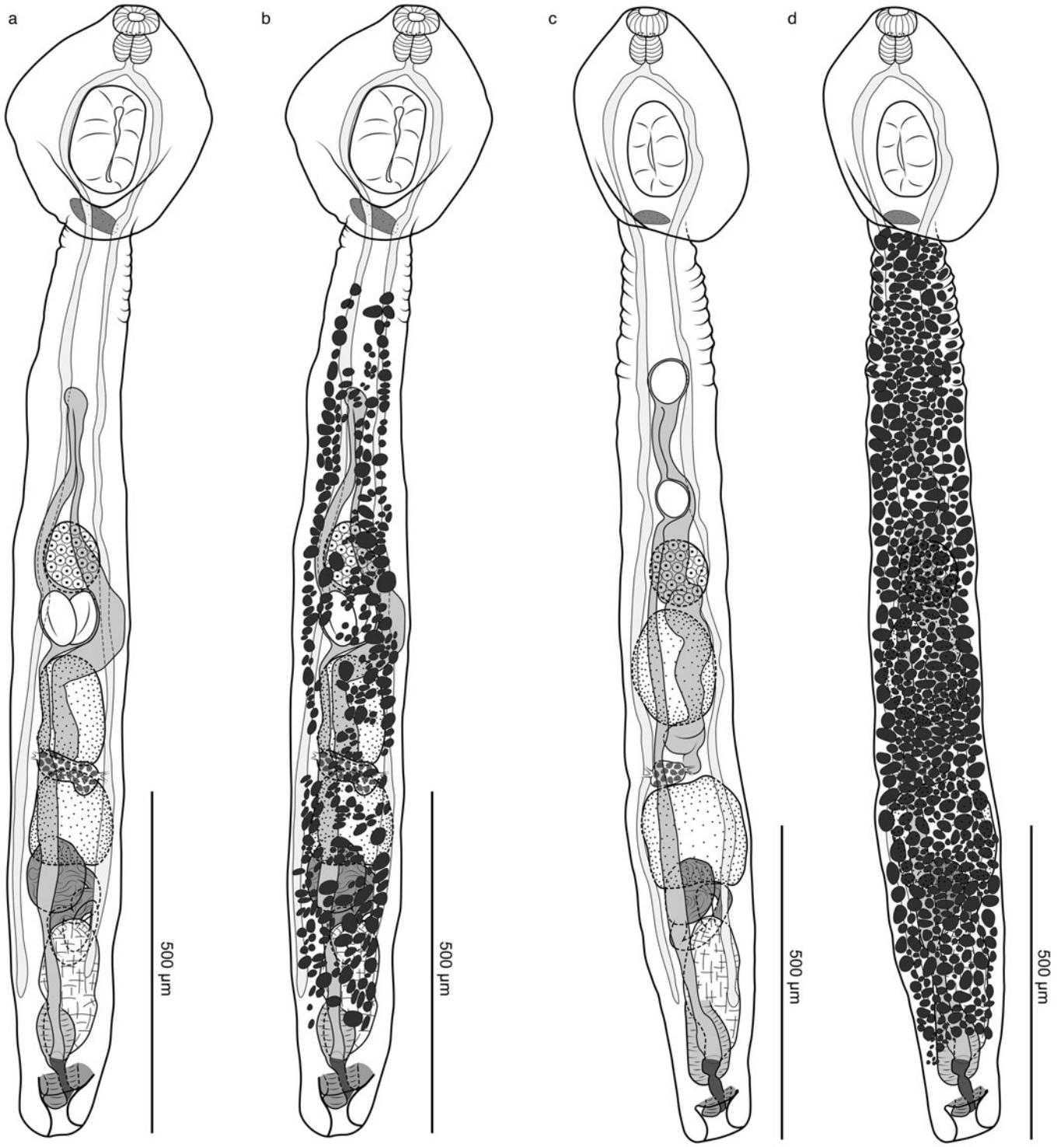

Fig. 4. *Crassiphiala jeffreybelli* n. sp. (a) holotype, ventral view with vitellarium omitted; (b) holotype, ventral view with vitellarium shown; (c) paratype, ventral view with vitellarium omitted; (d) paratype, ventral view with vitellarium shown.

The eggs of *C. ceryliformis* (40–80) are smaller than in *C. jeffreybelli* n. sp. (80–93), with the size ranges barely overlapping. The posterior testis of *C. ceryliformis* is bilobed, while the posterior testis of *C. jeffreybelli* n. sp. is not lobed. In addition, *C. ceryliformis* is only known from India, whereas the new species described herein was collected in Brazil. *Crassiphiala jeffreybelli* n. sp. differs from its congeners (except for *Crassiphiala* sp. lineage 1) by 0.9–1.1% in the partial sequences of 28S (table 3) and 10.4–17.9% in partial sequences of *cox*1 (table 4). No DNA sequences of *C. ceryliformis* are available. *Crassiphiala jeffreybelli* n. sp. and *Crassiphiala* sp. lineage 1 of Achatz *et al.* (2019b) have identical partial sequences of 28S (table 3), but differ by

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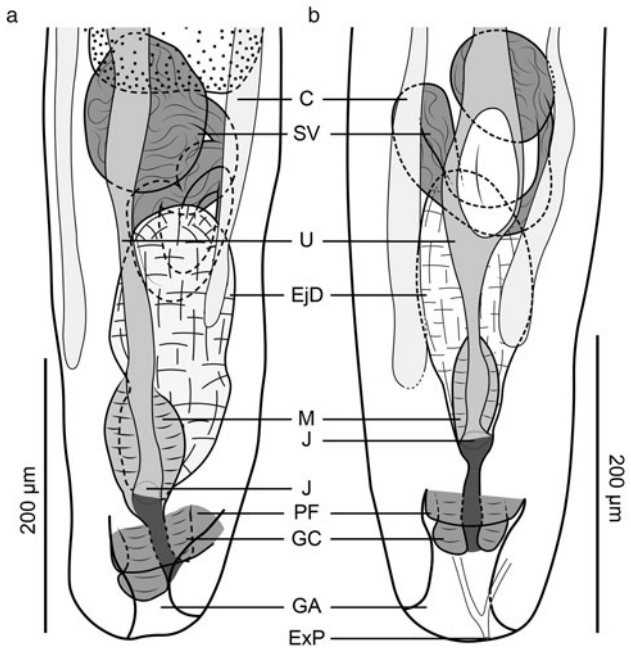

Fig. 5. Posterior end of opisthosoma of *Crassiphiala jeffreybelli* n. sp. with vitellarium omitted. (a) holotype, ventral view; (b) paratype, ventral view. Abbreviations: C, ceca; EjD, ejaculatory duct; ExP, excretory pore; GA, genital atrium; GC, genital cone; J, joining site of male and female ducts; M, metraterm; PF, prepucial fold; SV, seminal vesicle; U, uterus.

11.4% in partial sequences of *cox*1 (table 4). Unfortunately, the adult specimens of *Crassiphiala* sp. lineage 1 of Achatz *et al.* (2019b) from *M. alcyon* in Minnesota, USA are in too poor condition to describe or use for morphological differentiation.

Crassiphiala wecksteini n. sp. Achatz, Von Holten, Fecchio et Tkach (figs 6 and 7)

Taxonomic summary

Type host: *Megaceryle torquata* (Linnaeus) (Coraciiformes: Alcedinidae).

Site of infection: small intestine.

Type locality: Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil (16°21′53″S 56°17′31″W).

Infection rate: one of four *M. torquata* was infected with eight *C. wecksteini* n. sp.

Type-material: the type series consists of eight mature specimens deposited in the MPEG and HWML. Holotype: MPEG 000349, labelled ex. *Megaceryle torquata*, small intestine, Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil, June 07 2017, coll. A. Fecchio. Paratypes: MPEG 000350–000354 (five slides), HWML 216894 (one slide), labels identical to the holotype. Hologenophore: HWML 216895, label identical to the holotype.

Other specimens: HWML 216014.

Hologenophore DNA sequences: 28S: OP687979 (HWML 216895), *cox*1: OP688083 (HWML 216895).

Previously published genetic lineage name: *Crassiphiala* sp. lineage 5 of Achatz *et al.* (2019b).

Zoobank registration: urn:lsid:zoobank.org:act:689706E1-C42B-4021-90AA-E282BF2CED42.

Etymology: the species is named after Dr Jason D. Weckstein in recognition of his numerous contributions to knowledge of South American birds and their parasites.

Description. Based on eight adult specimens. Measurements of holotype in text; measurements of entire series given in table 2. Body 1,317 long, consists of distinct prosoma and opisthosoma; prosoma oval, generally flattened, posterior portion with slight concavity, 355 long, widest at level of holdfast organ, 270; opisthosoma elongated, cylindrical, 982 × 220; opisthosoma length:width ratio 4.5. Prosoma:opisthosoma length ratio 0.4. Tegument unarmed. Oral sucker subterminal, 45 × 44. Pseudosuckers absent. Holdfast organ oval, with longitudinal aperture, armed with fine spines, proximal portion glandular, 191 × 222; holdfast organ:prosoma width ratio 0.8. Proteolytic gland consisting of diffuse gland cells. Prepharynx absent. Pharynx subspherical, 38 × 40. Oesophagus 63 long. Caecal bifurcation in anterior half of prosoma length. Caeca slender, extend to level of seminal vesicle.

Testes two, tandem, rounded, entire; anterior testis 177 × 133, posterior testis 215 × 135. Seminal vesicle post-testicular, proximal portion pouch-like, followed by winding distal portion that joins distal part of metraterm to form short hermaphroditic duct. Hermaphroditic duct opens at apex of muscular genital cone. Genital cone surrounded by prepucial fold, positioned within genital atrium; genital atrium with terminal opening.

Ovary pretesticular, subspherical, 86 × 80. Oötype and Mehlis' gland intertesticular (not illustrated). Vitelline follicles limited to opisthosoma, distributed from near level of prosoma to near posterior end of opisthosoma. Vitelline reservoir intertesticular. Uterus ventral to gonads, extends anteriorly beyond level of ovary before turning and extending posteriorly. Terminal part of uterus and proximal part of hermaphroditic duct surrounded by distinct layer of muscles. Uterus contains no eggs in holotype (up to three in paratype). Eggs 103–104 × 56–63.

Table 3. Pairwise comparisons of partial 28S rDNA gene sequences among *Crassiphiala* spp.

		1. MN200255	**2.** OP687979	**3.** MN200257	**4.** OP687981	**5.** MN200252
1.	*Crassiphiala bulboglossa* MN200255[a]	–	0.2%	0.2%	0.9%	0.9%
2.	*Crassiphiala wecksteini* n. sp. OP687979	2	–	0.4%	1.1%	1.1%
3.	*Crassiphiala* sp. lineage 3 MN200257	2	4	–	1.1%	1.1%
4.	*Crassiphiala jeffreybelli* n. sp. OP687981	10	12	12	–	0%
5.	*Crassiphiala* sp. lineage 1 MN200252	10	12	12	0	–

Percentage differences are given above the diagonal, and number of variable nucleotide positions are given below the diagonal. Results based on a 1,108 bp long alignment.
[a]Previously published as *Crassiphiala* sp. lineage 2 of Achatz *et al.* (2019b).

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Table 4. Pairwise comparisons of partial *cox*1 mitochondrial DNA gene sequences among *Crassiphiala* spp.

		1. OP688077	**2**. MN193952	**3**. OP688083	**4**. OP688084	**5**. MN193959	**6**. MN193960	**7**. MN179323	**8**. MN193956	**9**. OP688086	**10**. OP688085	**11**. MN193951
1.	*Crassiphiala bulboglossa* OP688077	–	0.3%	11.7%	11.7%	11.9%	12.2%	12.4%	13.7%	14.2%	14.5%	12.2%
2.	*C. bulboglossa* MN193952[a]	1	–	11.9%	11.9%	12.2%	12.4%	12.7%	14.0%	14.5%	14.8%	12.4%
3.	*Crassiphiala wecksteini* n. sp. OP688083	45	46	–	1.0%	0.8%	0.5%	3.4%	13.0%	10.6%	10.6%	14.0%
4.	*C. wecksteini* n. sp. OP688084	45	46	4	–	0.8%	1.0%	2.3%	14.0%	10.6%	10.6%	13.7%
5.	*C. wecksteini* n. sp. MN193959[b]	46	47	3	3	–	0.8%	3.1%	13.2%	10.4%	10.4%	14.2%
6.	*C. wecksteini* n. sp. MN193960[b]	47	48	2	4	3	–	3.4%	13.5%	10.6%	10.6%	14.2%
7.	*Crassiphiala* sp. MN179323[c]	48	49	13	9	12	13	–	15.3%	10.9%	10.9%	14.8%
8.	*Crassiphiala* sp. lineage 3 MN193956	53	54	50	54	51	52	59	–	17.6%	17.9%	16.6%
9.	*Crassiphiala jeffreybelli* n. sp. OP688086	55	56	41	41	40	41	42	68	–	0.5%	11.4%
10.	*C. jeffreybelli* n. sp. OP688085	56	57	41	41	40	41	42	69	2	–	11.4%
11.	*Crassiphiala* sp. lineage 1 MN193951	47	48	54	53	55	55	57	64	44	44	–

Percentage differences are given above the diagonal, and number of variable nucleotide positions are given below the diagonal. Results based on a 386 bp long alignment.
[a]Previously published as *Crassiphiala* sp. lineage 2 of Achatz *et al.* (2019b).
[b]Previously published as *Crassiphiala* sp. lineage 5 of Achatz *et al.* (2019b).
[c]Previously published as Crassiphialinae gen. sp. of López-Hernández *et al.* (2019).

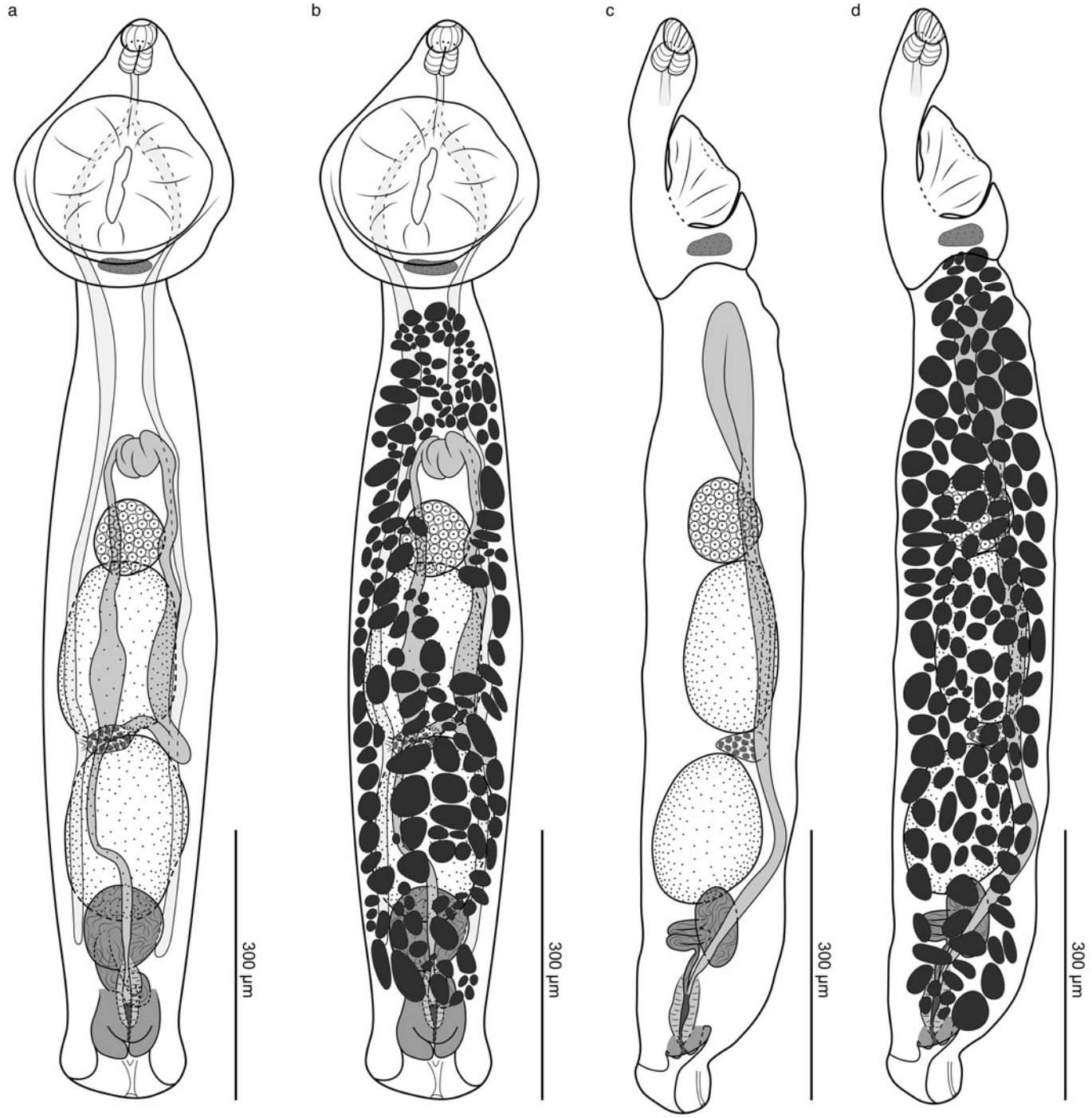

Fig. 6. *Crassiphiala wecksteini* n. sp. (a) holotype, ventral view with vitellarium omitted; (b) holotype, ventral view with vitellarium shown; (c) paratype, lateral view with vitellarium omitted; (d) paratype, lateral view with vitellarium shown.

Excretory pore subterminal on ventral side.

Remarks

The new species belongs to *Crassiphiala* based on morphological features, including a distinctly bipartite body, vitellarium confined to the opisthosoma, presence of a prepucial fold associated with the genital cone, unarmed tegument and the absence of pseudosuckers, ventral sucker and genital atrium sucker. The molecular phylogeny also positioned this species in a strongly supported clade (100% BI; 99% ML) with *C. bulboglossa* (fig. 1).

Crassiphiala wecksteini n. sp. can be easily distinguished from *C. jeffreybelli* n. sp. and *C. ceryliformis* by the absence of a dilated, pouch-like ejaculatory duct. In contrast, *C. wecksteini* n. sp. and *C. bulboglossa* are extremely similar morphologically. The majority of measurements of *C. wecksteini* n. sp. are smaller than those of *C. bulboglossa* (table 2) and the opisthosoma length:width ratio is somewhat smaller in *C. wecksteini* n. sp. (3.8–5.2; average 4.5) compared with *C. bulboglossa* (6.2–10.3; average 7.8). The most obvious difference is that the prepucial fold surrounding

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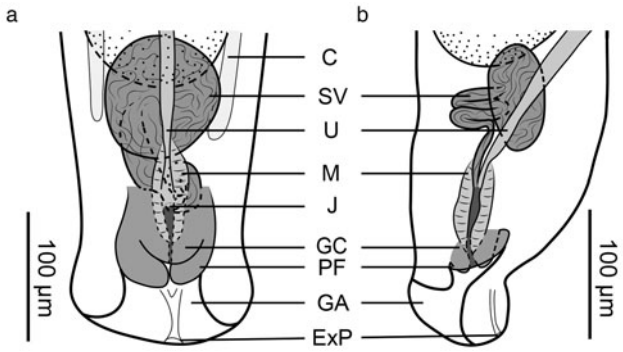

Fig. 7. Posterior end of opisthosoma of *Crassiphiala wecksteini* n. sp. with vitellarium omitted. (a) holotype, ventral view; (b) paratype, lateral view. Abbreviations: C, caeca; ExP, excretory pore; GA, genital atrium; GC, genital cone; J, joining site of male and female ducts; M, metraterm; PF, prepucial fold; SV, seminal vesicle; U, uterus.

the genital cone in *C. wecksteini* n. sp. (fig. 7) is more pronounced than in *C. bulboglossa* (fig. 3). The metraterm is well-developed in *C. wecksteini* n. sp., but not in *C. bulboglossa*. Based on molecular studies, *C. wecksteini* n. sp. has only been reported from the Neotropics, whereas *C. bulboglossa* is only known from the Nearctic, although there is a single report of *C. bulboglossa* from an unknown kingfisher in Brazil (Dubois, 1970). It is possible, however, that these digeneans may have broader distributions than currently recognized and may even occur in sympatry. *Megaceryle torquata* (the host of *C. wecksteini* n. sp.) is distributed between the southern USA and Tierra del Fuego, while *M. alcyon* (the host of *C. bulboglossa*) is distributed between the northern part of North America and northern edge of South America (Brush, 2020; Kelly *et al.*, 2020).

The partial 28S sequences of *C. wecksteini* n. sp. differ by at least 0.2% from its congeners (table 3), and the partial *cox*1 sequences of this new species differ by at least 11.7% from *C. bulboglossa* and by at least 10.4% from *C. jeffreybelli* (table 4).

Family Diplostomidae Poirier, 1886

Genus *Pseudocrassiphiala* n. gen. Achatz, Von Holten, Fecchio et Tkach

Diagnosis. Body distinctly bipartite; prosoma strongly concave, cup-shaped, much shorter than cylindrical opisthosoma. Tegument of prosoma and anterior part of opisthosoma armed with fine spines. Oral sucker present; ventral sucker and pseudosuckers absent. Holdfast organ subspherical, with longitudinal aperture, most of holdfast organ positioned inside prosoma concavity. Pharynx present; caeca reach level of or posterior to level of seminal vesicle. Testes two, tandem. Seminal vesicle post-testicular, winding, with pouch-like expansion at proximal end. Ejaculatory duct short, joins distal part of metraterm to form a short hermaphroditic duct. Hermaphroditic duct opens at apex of short genital cone. Genital cone with ventrolateral prepucial fold, opens into genital atrium. Genital atrium with terminal opening. Ovary pretesticular; oötype intertesticular. Vitellarium distributed throughout opisthosoma; vitelline reservoir intertesticular. Excretory pore subterminal on ventral side. In kingfishers. Neotropics.

Type species: Pseudocrassiphiala tulipifera n. sp. Achatz, Von Holten, Fecchio et Tkach.

Zoobank registration: urn:lsid:zoobank.org:act:F8E6CFEB-7F32-4499-9677-6AC4045E36CF.

Etymology: the name of the new genus refers to its morphological similarity to *Crassiphiala*.

Remarks

While molecular comparisons revealed the presence of two members in the new genus (fig. 1; 0.4% and 7.5–7.8% divergence between lineages in 28S and *cox*1 sequences, respectively), we only had quality adult specimens of the type-species. The differentiation below relies only on the type-species and may require amendment once morphological data from the second member become available.

Pseudocrassiphiala n. gen. belongs to the Diplostomidae based on the presence of a sucker-like holdfast organ and absence of a paraprostate. The new genus can be distinguished from most other diplostomids, with the exceptions of *Cercocotyla* and *Crassiphiala*, based on the absence of pseudosuckers and ventral sucker. It is worth noting that members of these genera were included in our 28S phylogenetic analyses (fig. 1). Members of *Cercocotyla* possess a genital atrium sucker which is absent in the type-species of *Pseudocrassiphiala* n. gen.

The new genus and *Crassiphiala* are morphologically similar and their reliable differentiation requires quality adult specimens. Based on light microscopy, in *Pseudocrassiphiala* n. gen. the tegument of the prosoma and anterior part of opisthosoma are armed with fine spines, while in *Crassiphiala* the tegument is unarmed. The type-species of *Pseudocrassiphiala* n. gen. has a cup-like prosoma with a deep concavity that opens anteriorly (fig. 8), while the prosoma of *Crassiphiala* is generally flattened or has only a shallow concavity that opens ventrally (figs 2, 4 and 6). In the type-species of *Pseudocrassiphiala* n. gen., most of the holdfast organ is positioned within the deep prosoma concavity, while most of the holdfast organ in *Crassiphiala* spp. is not within prosoma concavity.

Pseudocrassiphiala tulipifera n. sp. Achatz, Von Holten, Fecchio et Tkach (figs 8 and 9)

Taxonomic summary

Type host: *Megaceryle torquata* (Linnaeus) (Coraciiformes: Alcedinidae).

Other host: *Chloroceryle americana* (Gmelin) (Coraciiformes: Alcedinidae).

Site of infection: small intestine.

Type locality: Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil (16°21′53″S 56°17′31″W).

Other locality: Lábrea municipality, Amazonas State, Brazil.

Infection rate: one in four *M. torquata* was infected with 12 *P. tulipifera* n. sp. in Pantanal and a single examined specimen of *M. torquata* from Lábrea was infected with two *P. tulipifera* n. sp.

Type-material: the type series consists of 12 mature specimens deposited in the MPEG and HWML. Holotype: MPEG 000340, labelled ex. *Megaceryle torquata*, small intestine, Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil, June 07 2017, coll. A. Fecchio. Paratypes: MPEG 000341–000348 (lot of eight slides), HWML 216897 (lot of two slides), labels identical to the holotype. Hologenophore: HWML 216898, label identical to the holotype.

Other specimens: HWML 216013.

Hologenophore DNA sequences: 28S: MN200258, *cox*1: MN193957.

Previously published genetic lineage name: *Crassiphiala* sp. lineage 4 of Achatz *et al.* (2019b).

Zoobank registration: urn:lsid:zoobank.org:act:0CECC018-8A0A-4B5F-88E0-88951A62986A.

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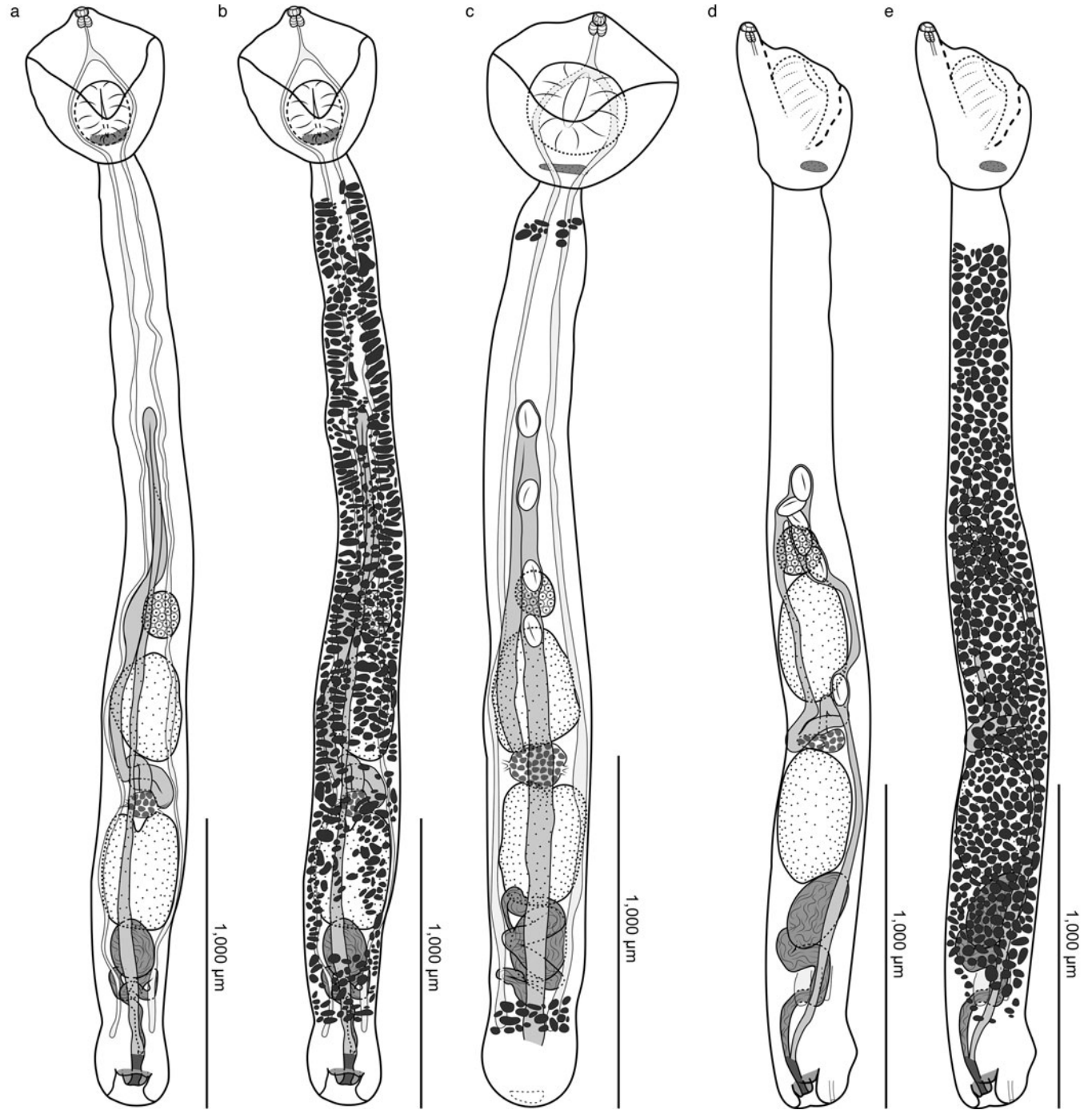

Fig. 8. *Pseudocrassiphiala tulipifera* n. sp. (a) holotype, ventral view with vitellarium omitted; (b) holotype, ventral view with vitellarium shown; (c) paratype 1, ventral view with vitellarium omitted; (d) paratype 2, lateral view with vitellarium omitted; (e) paratype 2, lateral view with vitellarium shown.

Etymology: the specific epithet refers to the tulip-shaped prosoma in the new species.

Description. Based on 12 adult specimens. Measurements of holotype in text; measurements of entire series given in table 2. Body 3,945 long, consists of distinct prosoma and opisthosoma; prosoma deeply concave, cup-like, 547 long, widest at level of holdfast organ, 489; opisthosoma elongated, cylindrical, 3,398 × 324; opisthosoma length:width ratio 10.5. Prosoma:opisthosoma length ratio 0.2. Tegument of prosoma and anterior part of opisthosoma armed with fine spines. Oral sucker subterminal, 32 × 48. Pseudosuckers absent. Holdfast organ subspherical, with longitudinal aperture, most of holdfast organ positioned within prosoma concavity, proximal portion glandular, 235 × 185; holdfast organ:prosoma width ratio 0.4. Proteolytic gland dorsal to holdfast organ, consists of diffuse gland cells. Prepharynx absent. Pharynx subspherical, 47 × 52. Oesophagus 76 long. Caecal bifurcation in anterior-most third of prosoma length. Caeca slender, reach level of, or posterior to, level of seminal vesicle.

Testes two, tandem, rounded, entire, anterior testis 403 × 260, posterior testis with shallow invagination on anterior margin,

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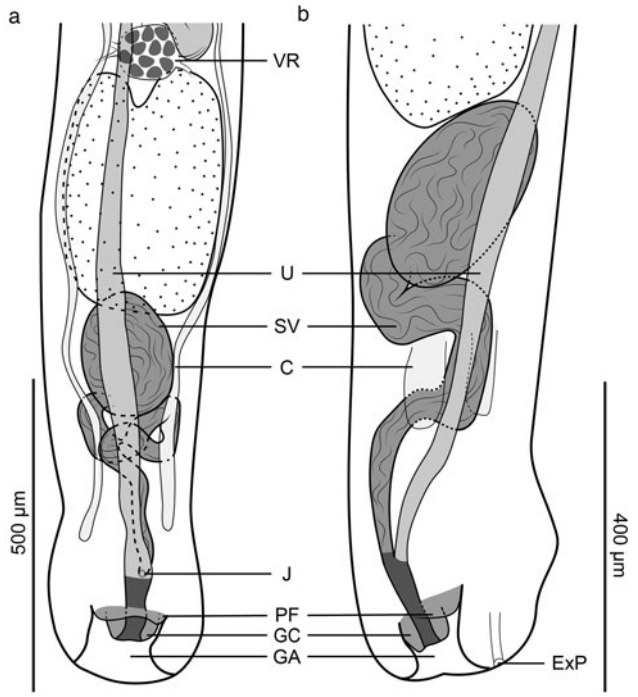

Fig. 9. Posterior end of opisthosoma of *Pseudocrassiphiala tulipifera* n. sp. with vitellarium omitted. (a) holotype, ventral view; (b) paratype 2, lateral view. Abbreviations: C, caeca; ExP, excretory pore; GA, genital atrium; GC, genital cone; J, joining site of male and female ducts; PF, prepucial fold; SV, seminal vesicle; U, uterus; VR, vitelline reservoir.

440 × 275. Seminal vesicle post-testicular, proximal portion expansive, pouch-like, followed by winding distal portion that joins distal part of metraterm to form short hermaphroditic duct. Hermaphroditic duct opens at apex of small genital cone. Genital cone with ventrolateral prepucial fold, positioned within genital atrium; genital atrium with terminal opening.

Ovary pretesticular, subspherical, 160 × 136. Oötype and Mehlis' gland intertesticular (not illustrated). Vitelline follicles limited to opisthosoma, distributed from near level of prosoma to near posterior end of opisthosoma. Vitelline reservoir intertesticular. Uterus ventral to gonads, extends anteriorly to beyond level of ovary before turning and extending posteriorly. Uterus in holotype does not contain eggs (up to 20 in paratype). Eggs 101–113 × 46–64.

Excretory pore subterminal, ventral.

Discussion

Our phylogenetic analyses (fig. 1) demonstrated that members of *Subuvulifer*, *Pseudocrassiphiala*, *Crassiphiala*, *Posthodiplostomoides* and *Uvulifer* form a strongly supported monophyletic group. Although the interrelationships among genera have not been resolved, all genus-level clades were strongly supported and revealed the presence of at least two unknown species-level lineages of *Crassiphiala* and one additional species of *Pseudocrassiphiala*. Unfortunately, specimens representing these three additional species were either adults in poor condition or metacercariae and thus not suitable for descriptions.

Van Haitsma (1925) erected *Crassiphiala* for diplostomids collected from the intestine of *M. alcyon* in Michigan, USA. Until recently, the genus was viewed as monotypic and limited in its distribution to the Nearctic (Preble & Harwood, 1944; Dubois & Rausch, 1948 Hoffman, 1956; Dubois, 1968; Boyd & Fry, 1971; Scott, 1984; Niewiadomska, 2002; Muzzall *et al.*, 2011), except for a single report by Dubois (1970) who identified *C. bulboglossa* among specimens from an unknown species of kingfisher in Brazil collected by A. Lutz. Based on DNA sequences, Achatz *et al.* (2019b) demonstrated the presence of at least five species-level lineages of *Crassiphiala* throughout the New World (three in North America and two in South America). The present data reveals the presence of at least two additional closely related species-level lineages in the New World. We have provided morphological descriptions for four of these seven species-level lineages, which include representatives of a new genus (*Pseudocrassiphiala* n. gen.) as well as *Crassiphiala* (tables 1 and 2). It is worth noting that Crassiphialinae gen. sp. collected from *Biomphalaria straminea* (Dunker) in Belo Horizonte, State of Minais Gerais, Brazil by López-Hernández *et al.* (2019), is potentially conspecific with *C. wecksteini* n. sp. based on the low level of genetic divergence. The two forms differ by only 2.3–3.4% in partial sequences of *cox*1 (table 4). However, previous studies (Achatz *et al.*, 2021b and references therein) have demonstrated that interspecific variation between diplostomid species may be as low as 3.4% in this fragment of *cox*1. Intraspecific variability of *cox*1 sequences of *Crassiphiala* spp. in our study showed minimal variation (0.5% in *C. jeffreybelli*, up to 0.3% in *C. bulboglossa*, and up to 1% in *C. wecksteini*). The two partial *cox*1 sequences of *P. tulipifera* differed by only 1.3%, despite originating from distant (about 1,400 km) geographic locations in Brazil.

In the original description of *C. bulboglossa*, Van Haitsma (1925) inaccurately referred to an expanded portion of the seminal vesicle as an ejaculatory pouch. An ejaculatory pouch in diplostomids is a muscular/glandular structure that surrounds at least part of the ejaculatory duct (Achatz *et al.*, 2022b). This structure is absent in *Crassiphiala* spp., but present in members of other diplostomid genera, including *Uvulifer* (Niewiadomska, 2002; Achatz *et al.*, 2019a, 2022b).

Vidyarthi (1938) described *C. ceryliformis* based on specimens collected from the intestine of the pied kingfisher *Ceryle rudis* (Linnaeus) in India. *Crassiphiala ceryliformis* was described as lacking a ventral sucker, but having an ejaculatory pouch (Vidyarthi, 1938). Bhalerao (1942) transferred this species into *Uvulifer* based on the smaller holdfast organ compared with *C. bulboglossa* and stated that the relative holdfast organ size held more taxonomic importance than the presence/absence of the ventral sucker. It is clear that *Uvulifer ceryliformis* (Vidyarthi, 1938) is more morphologically similar to *C. jeffreybelli* n. sp. than to any member of *Uvulifer*. Based on the illustration by Vidyarthi (1938), the ejaculatory pouch of *U. ceryliformis* is probably a dilated ejaculatory duct, similar to the condition in *C. jeffreybelli* n. sp. Both *U. ceryliformis* and *C. jeffreybelli* n. sp. lack a ventral sucker and have a holdfast organ that does not occupy much of the prosoma width. Based on morphological comparisons, we return *U. ceryliformis* to *Crassiphiala* as *Crassiphiala ceryliformis* (Vidyarthi, 1938).

The fauna of diplostomids parasitic in New World kingfishers is likely much richer than previously known. Until 2018, only a single species of *Crassiphiala* and five species of *Uvulifer* were known from kingfishers in the New World. The present study and recent publications (López-Jiménez *et al.*, 2018; Achatz *et al.*, 2019a, b) have revealed four additional species/species-level lineages of *Crassiphiala* and seven additional species/species-level

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lineages of *Uvulifer* in the New World. The diversity of these diplostomids from kingfishers in the New World is further expanded by the members of *Pseudocrassiphiala* n. gen. (two species/species-level lineages), *Sphincterodiplostomum* Dubois, 1936 (one species) and *Posthodiplostomum* Dubois, 1936 (one species) (Achatz *et al.*, 2021a, b). Based on the current knowledge, it is certain that at least four species of *Uvulifer* as well as additional *Crassiphiala* (two species) and *Pseudocrassiphiala* n. gen. (one species) require description when suitable specimens are available. Future parasitological surveys should attempt to collect quality adult specimens of these diplostomids for morphological study.

We have provided the first DNA sequence data from a member of *Subuvulifer* (*S. glandulaxiculus*) and *U. semicircumcisus*. *Subuvulifer* is a small genus with only three nominal species: *Subuvulifer halcyonae* (Gogate, 1940), *Subuvulifer sabahensis* (Fischthal et Kuntz, 1973) and *S. glandulaxiculus*. Members of this genus are only known to parasitize kingfishers and have only been reported in Southeastern Asia and India. It would not be surprising if *Subuvulifer* also contains several currently undescribed species. More research is required to further explore the diversity and relationships of diplostomids, and other digeneans, parasitic in kingfishers.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of animals.

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