Parasitology Research (2019) 118:2781–2787
https://doi.org/10.1007/s00436-019-06439-y

FISH PARASITOLOGY - ORIGINAL PAPER

Phylogenetic relationships, expanded diversity and distribution of *Crassiphiala* spp. (Digenea, Diplostomidae), agents of black spot disease in fish

Tyler J. Achatz[1] **· Eric E. Pulis**[2] **· Alan Fecchio**[3] **· Isaac J. Schlosser**[1] **· Vasyl V. Tkach**[1]

Received: 2 June 2019 / Accepted: 22 August 2019 / Published online: 6 September 2019

Abstract

Crassiphiala is a monotypic genus of diplostomid digeneans and is the type genus of the subfamily Crassiphialinae. The type species *Crassiphiala bulboglossa* parasitizes kingfishers in the Nearctic and has a *Neascus*-type metacercaria that encysts on fish intermediate hosts, often causing black spot disease. While recent molecular phylogenetic studies included some members of the Crassiphialinae, no DNA sequence data of *Crassiphiala* is currently available. Our molecular and morphological study of adult and larval crassiphialines from the Americas revealed the presence of at least three lineages of *Crassiphiala* from the Nearctic and two lineages from the Neotropics. This is the first record of *Crassiphiala* from the Neotropics. Herein, we provide the first molecular phylogeny of the Diplostomoidea that includes *Crassiphiala*. Our data revealed 0.2–2.4% divergence among 28S sequences and 11–19.8% among *CO1* sequences of lineages of *Crassiphiala*. The results of our analyses did not support the monophyly of Crassiphialinae. Our results clearly demonstrated that the diversity of *Crassiphiala* has been underestimated.

Keywords Diplostomidae · *Crassiphiala* · Molecular phylogeny · Diversity · Black spot disease

Introduction

Crassiphiala Van Haitsma, 1925 (Diplostomidae: Crassiphialinae) is a monotypic genus of diplostomid digeneans parasitic in kingfishers (Alecedinidae Rafinesque) (Dubois 1968). The type species *Crassiphiala bulboglossa* Van Haitsma, 1925 was described from the intestine of the belted kingfisher *Megaceryle alcyon* (Linnaeus) from Michigan, USA (Van Haitsma 1925) and since then reported only in the Nearctic (Preble and Harwood 1944; Dubois and Rausch 1948; Hoffman 1956; Dubois 1969; Boyd and Fry 1971; Scott 1984; Niewiadomska 2002; Muzzall et al. 2011). The life cycle of *C. bulboglossa* is similar to that of the members of the genus *Uvulifer* Yamaguti, 1934 and includes planorbid snails and fishes as intermediate hosts (Hoffman 1956). Notably, *C. bulboglossa* has a *Neascus*-type metacercaria that normally encysts in fish skin and is often melanized by the fish host. This infection is often referred to as the "black spot disease" (Hunter 1933; Hoffman 1956; Niewiadomska 2002; McAllister et al. 2013). Adult *Crassiphiala* are characterized, among other features, by a large holdfast organ, rudimentary or absent ventral sucker and the absence of an ejaculatory pouch (Niewiadomska 2002).

Crassiphiala is the type genus of the subfamily Crassiphialinae Sudarikov, 1960. The most recent revision of the Crassiphialinae by Niewiadomska (2002) included 15 genera; however, adults of only 4 of these genera have been included in prior molecular phylogenetic analyses based on the 28S rDNA gene. The molecular phylogenetic studies that included more than three genera of the Crassiphialinae have shown mixed support for the monophyly of the subfamily (see Blasco-Costa and Locke 2017; Hernández-Mena et al. 2017; López-Jiménez et al. 2018; Achatz et al. 2019). Despite very weak support or the lack of support evident from their phylogenetic trees, some authors have repeatedly suggested that this subfamily may warrant elevation to family (Blasco-Costa and

---

Section Editor: Stephen A. Bullard

✉ Vasyl V. Tkach
vasyl.tkach@und.edu

[1] Department of Biology, University of North Dakota, Starcher Hall, 10 Cornell Street Stop 9019, Grand Forks, ND 58202, USA

[2] Department of Science and Mathematics, Northern State University, Aberdeen, SD 57401, USA

[3] Programa de Pós-Graduação em Ecologia e Conservação da Biodiversidade, Universidade Federal de Mato Grosso, Cuiabá, MT, Brazil

Locke 2017; Hernández-Mena et al. 2017; Locke et al. 2018). Moreover, no prior molecular phylogenetic study included representatives of *Crassiphiala*, the type genus of the Crassiphialinae. The purpose of this study is the demonstration of the phylogenetic placement of *Crassiphiala* using DNA sequence data for the first time as well as the presence of this genus in South America. Formal morphological and taxonomic descriptions of the genetic lineages presented herein will be published separately.

Materials and methods

We obtained adult specimens of *Crassiphiala* from intestines of *M. alcyon* collected in Clearwater (one specimen) and St. Louis (one specimen) Counties in Minnesota, USA (collecting permit MB072162-0) and a single ringed kingfisher *Megaceryle torquata* (Linnaeus) collected in Pantanal, Fazenda Retiro Novo, Municipality of Poconé, Mato Grosso State, Brazil (collecting permit 10698 approved by the Instituto Chico Mendes de Conservação da Biodiversidade), using corresponding federal and state collecting permits. In addition, metacercaria of *Crassiphiala* were collected from the skin and fins of yellow perch *Perca flavescens* Mitchill from Cass County, Minnesota USA, central mudminnow *Umbra limi* Kirtland from Hubbard County, Minnesota and *Phoxinus eos* Cope from St. Louis County, Minnesota, USA. Live digeneans removed from the hosts were briefly rinsed in saline, killed with hot 10% ethanol, and preserved in 70% ethanol. One sample was obtained from a frozen carcass of *M. alcyon* that died after flying into a glass window. These specimens were directly fixed in 70% ethanol. In total, we obtained five adult specimens of *Crassiphiala* lineage 1 from *M. alcyon* collected in the St. Louis Co., MN; a single adult specimen of *Crassiphiala* lineage 2 from *M. alcyon* collected in the Clearwater Co., MN; one metacercaria of *Crassiphiala* lineage 3 from *P. eos* in St. Louis County; several dozen adult specimens of *Crassiphiala* lineage 4 and 52 specimens of *Crassiphiala* lineage 5 from *M. torquata* in Pantanal.

Specimens for light microscopy were stained with aqueous alum carmine according to Lutz et al. (2017) and studied using a DIC-equipped Olympus BX51 compound microscope (Tokyo, Japan) with a digital imaging system. Vouchers of adult specimens of genetic lineages 1, 4, and 5 are deposited in the collection of the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum, Lincoln, NE, USA (Table 1). The lineage 2 was represented in our material by a single adult specimen which was used for DNA extract and the lineage 3 was a metacercaria sequenced as a part of an unrelated study.

Genomic DNA was extracted according to the protocol described by Tkach and Pawlowski (1999). A fragment of the 5′ end of 28S was amplified by polymerase chain reactions (PCR) as described in Tkach et al. (2003). A fragment of the cytochrome *c* oxidase (*CO1*) gene was amplified using the previously published forward primer Cox1_Schist_5’ and reverse primers acox650R and JB5 (Lockyer et al. 2003; Derycke et al. 2005; Kudlai et al. 2015). In some cases, *CO1* was amplified in two overlapping fragments using a combination of published primers and new internal primers designed for this study by TJA. The forward primer Cox1_Schist_5′ was used with new reverse primer BS_CO1_IntR (5′-TAA TAC GAC TCA CTA TAA AAA AAA MAM AGA AGA RAA MAC MGT AGT AAT-3′); the new forward primer BS_CO1_IntF (5′-ATT AAC CCT CAC TAA ATG ATT TTT TTY TTT YTR ATG CC-3′) was used with the reverse primer acox650R. The underlined portions indicate a shortened T3 and T7 tail sequences. PCRs of *CO1* were performed in a total volume of 25 μl using New England Biolabs (Ipswich, MA, USA) One-Taq quick load PCR mix according to the manufacturer’s protocol. The thermocycling protocol for *CO1* was as follows: 30 s denaturation hold at 94 °C; 40 cycles of 25 s at 94 °C, 30 s at 45 °C, 1 min at 68 °C; and 5 min extension hold at 68 °C.

Table 1 List of *Crassiphiala* samples used in the phylogenetic analyses of 28S rDNA and *COI* mtDNA including sample size (*n*), Harold W. Manter Laboratory (HWML) voucher numbers, their host species, geographical origin of material, and GenBank accession numbers

Digenean taxa	Host species	Geographic origin	Museum No.	Accession numbers	
				28S	*COI*
Crassiphiala lineage 1 ($n = 2$)	*Megaceryle alcyon*	USA	HWML-216012	MN200252, MN200253	MN193951
Crassiphiala lineage 2 ($n = 1$)	*M. alcyon*	USA	–	MN200254	MN193952
Crassiphiala lineage 2 ($n = 1$)	*Phoxinus eos*	USA	–	MN200255	MN193953
Crassiphiala lineage 2 ($n = 2$)	*Umbra limi*	USA	–	MN200256	MN193954, MN193955
Crassiphiala lineage 3 ($n = 1$)	*Perca flavescens*	USA	–	MN200257	MN193956
Crassiphiala lineage 4 ($n = 3$)	*Megaceryle torquata*	Brazil	HWML-216013	MN200258-MN200260	MN193957, MN193958
Crassiphiala lineage 5 ($n = 2$)	*M. torquata*	Brazil	HWML-216014	MN200261	MN193959, MN193960

PCR products were purified using ExoSap PCR clean-up enzymatic kit from Affymetrix (Santa Clara, CA, USA) following the manufacturer's protocol. PCR products were cycle-sequenced directly using BrightDye® Terminator Cycle Sequencing Kit (MCLAB, California, USA) chemistry, alcohol precipitated, and run on an ABI 3130 automated capillary sequencer (Thermo Fisher Scientific, Waltham, MA, USA).

Sequencing reactions of 28S were carried out as described in Achatz et al. (2019). The PCR primers were used for sequencing of *CO1* PCR reactions. In addition, the shortened T3 tail (5′-ATT AAC CCT CAC TAA A-3′) and shortened T7 tail (5′-TAA TAC GAC TCA CTA TA-3′) primers from Van Steenkiste et al. (2015) were used for sequencing of the PCR reactions prepared with BS_CO1_IntF and BS_CO1_IntR primers. Contiguous sequences were assembled using Sequencher version 4.2 software (GeneCodes Corp., Ann Arbor, Michigan, USA). Newly generated sequences are deposited in the GenBank (Table 1).

Phylogenetic relationships of *Crassiphiala* were analyzed using 28S and *CO1* datasets as separate alignments. Three *CO1* sequences (two from the lineage 2 and one from the lineage 4) were much shorter than the rest and therefore not included in the alignment, although they were submitted to the

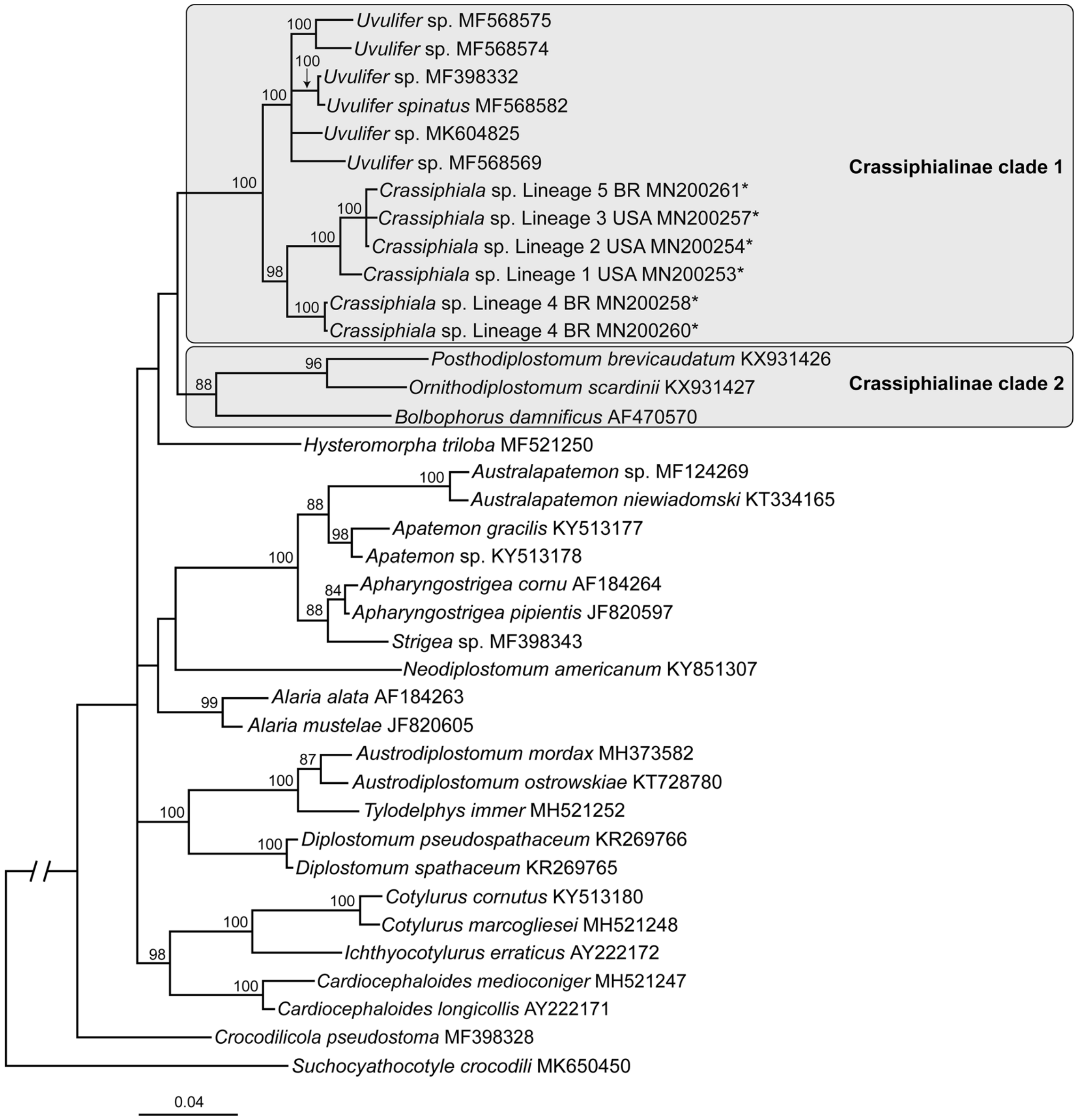


Fig. 1 Phylogenetic interrelationships among 38 diplostomoidean taxa including *Crassiphiala* based on Bayesian inference (BI) analysis of partial 28S rRNA gene sequences. Members of the subfamily Crassiphialinae as currently recognized are indicated by the shaded rectangles. Bayesian inference posterior probability values lower than 80% are not shown. New sequences obtained in this study are marked by an asterisk. Scale bar indicates number of substitutions per site. Abbreviations: BR–Brazil, USA–United States of America

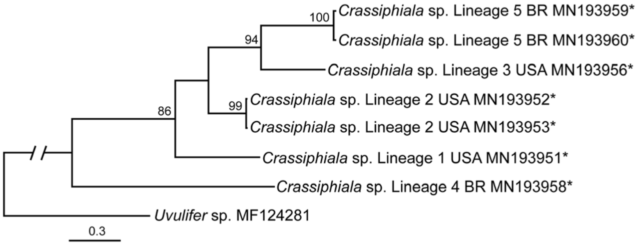


Fig. 2 Phylogenetic interrelationships among *Crassiphiala* lineages based on Bayesian inference (BI) analysis of partial *CO1* mtDNA sequences. Bayesian inference posterior probability values lower than 80% are not shown. All sequences of *Crassiphiala* included in our analysis are new and marked by an asterisk. Scale bar indicates number of substitutions per site. Abbreviations: BR–Brazil, USA–United States of America

GenBank. These shorter sequences were identical to their longer counterparts.

Newly obtained and previously published sequences were aligned using ClustalW implemented in Mega7 (Kumar et al. 2016); both alignments were trimmed to the length of the shortest sequence. The cyathocotylid *Suchocyathocotyle crocodili* (Yamaguti, 1954) (GenBank accession MK650450) was selected as outgroup in the 28S analysis based on the topology presented by Achatz et al. (2019). *Uvulifer* sp. (GenBank accession MF124281; Blasco-Costa and Locke 2017) was selected as outgroup in the *CO1* analysis based on the results of our 28S analysis and genetic distances.

The 28S alignment included newly generated sequences of 6 taxa of *Crassiphiala* and previously published sequences of 18 members of the Diplostomidae Poirier, 1886, 1 member of the Proterodiplostomidae Dubois, 1936, and 12 members of the Strigeidae Railliet, 1919. The *CO1* alignment included newly generated sequences of 7 taxa of *Crassiphiala*. Phylogenetic analyses were conducted as described by Achatz et al. (2019). The trees were visualized in FigTree ver. 1.4 software (Rambaut 2016) and annotated in Adobe Illustrator®.

Results

Upon trimming to the length of the shortest sequence the 28S alignment was 1137 bp long. Similar to the results of several previous studies (e.g., Blasco-Costa and Locke 2017; Hernández-Mena et al. 2017; Achatz et al. 2019), the phylogenetic tree resulting from the BI analysis demonstrated the non-monophyletic nature of the Diplostomidae and Strigeidae. Members of the Diplostomidae formed 6 clades: (1) Crassiphialinae clade 1 which included *Crassiphiala* + *Uvulifer* (100%), (2) Crassiphialinae clade 2 which included *Bolbophorus* Dubois, 1935 + *Ornithodiplostomum* Dubois, 1936 + *Posthodiplostomum* Dubois, 1936 (88%), (3) *Hysteromorpha* Lutz, 1931, (4) *Austrodiplostomum* Szidat & Nani, 1951 + *Diplostomum* von Nordmann, 1832 + *Tylodelphys* Diesing, 1850 (100%), (5) *Neodiplostomum* Railliet, 1919, and (6) *Alaria* Schrank, 1788 (99%). All sequenced lineages of *Crassiphiala* formed a strongly supported clade (98%); *Crassiphiala* lineage 4 from Brazil formed a sister group to all other *Crassiphiala* isolates (Fig. 1). Members of the Strigeidae formed 2 strongly supported clades. The first clade (100%) included *Apharyngostrigea* Ciurea, 1927 + *Strigea* Abildgaard, 1790 + *Apatemon* Szidat, 1928 + *Australapatemon* Sudarikov, 1959; the second clade (98%) included *Cardiocephaloides* Sudarikov, 1959 + *Cotylurus* Szidat, 1928 + *Ichthyocotylurus* Odening, 1969.

The internal interrelationships among available isolates of *Crassiphiala* were studied using the 392-bp-long *CO1* alignment. While the overall topology in the *CO1* tree was similar to that of the *Crassiphiala* clade in the 28S tree, the much more variable *CO1* sequences provided added resolution in form of the well-supported cluster of *Crassiphiala* lineages 3 and 5 which was unresolved in the 28S tree. *Crassiphiala* lineage 4 formed a sister group with all other *Crassiphiala* isolates, although with a somewhat lower support than in the 28S gene tree (Fig. 2).

Pairwise nucleotide comparisons of 28S sequences among all unique *Crassiphiala* isolates are provided in Table 2. The divergence in 28S sequences of *Crassiphiala* lineages was generally low (0.2–2.4%). One of the 28S sequences of *Crassiphiala* lineage 4 (MN200258) had a single mixed base (adenine or guanine), while the two other isolates of *Crassiphiala* lineage 4 (MN200259, MN200260) had only guanine in this position. No other variation within lineages was detected in sequences of 28S.

Table 2 Pairwise comparisons of partial sequences of the 28S rRNA gene between lineages of *Crassiphiala* included in this study. Percentage differences are given above diagonal and the number of variable nucleotide positions is given below the diagonal. The 28S results are based on a 1132-bp-long alignment

	***Cr.* 1** MN200253	***Cr.* 2** MN200254	***Cr.* 3** MN200257	***Cr.* 4** MN200260	***Cr.* 5** MN200261
Crassiphiala lineage 1 MN200253 USA	–	0.9%	1.1%	2.2%	1.1%
Crassiphiala lineage 2 MN200254 USA	10	–	0.2%	2.2%	0.2%
Crassiphiala lineage 3 MN200257 USA	12	2	–	2.4%	0.4%
Crassiphiala lineage 4 MN200260 Brazil	25	25	27	–	2.4%
Crassiphiala lineage 5 MN200261 Brazil	12	2	4	27	–

Table 3 Pairwise comparisons of partial sequences of the *COI* mtDNA gene between lineages of *Crassiphiala* included in this study. Percentage differences are given above diagonal and the number of variable nucleotide positions is given below the diagonal. Results are based on a 435-bp-long alignment

	***Cr.* 1** MN193951	***Cr.* 2** MN193952	***Cr.* 2** MN193953	***Cr.* 3** MN193956	***Cr.* 4** MN193958	***Cr.* 5** MN193959	***Cr.* 5** MN193960
Crassiphiala lineage 1 MN193951 USA	–	12.4%	12.2%	16.3%	15.6%	14.3%	14.3%
Crassiphiala lineage 2 MN193952 USA	54	–	0.2%	13.1%	16.1%	11.3%	11.5%
Crassiphiala lineage 2 MN193953 USA	53	1	–	12.9%	15.9%	11.0%	11.3%
Crassiphiala lineage 3 MN193956 USA	71	57	56	–	19.8%	12.9%	13.1%
Crassiphiala lineage 4 MN193958 Brazil	68	70	69	86	–	17.0%	17.2%
Crassiphiala lineage 5 MN193959 Brazil	62	49	48	56	74	–	0.7%
Crassiphiala lineage 5 MN193960 Brazil	62	50	49	57	75	3	–

Pairwise nucleotide comparisons of *CO1* sequences among all unique *Crassiphiala* isolates are provided in Table 3. The *CO1* sequences showed a much greater divergence among lineages (11–19.8%) than in 28S. There were no differences between the *CO1* sequences of *Crassiphiala* lineage 4.

Discussion

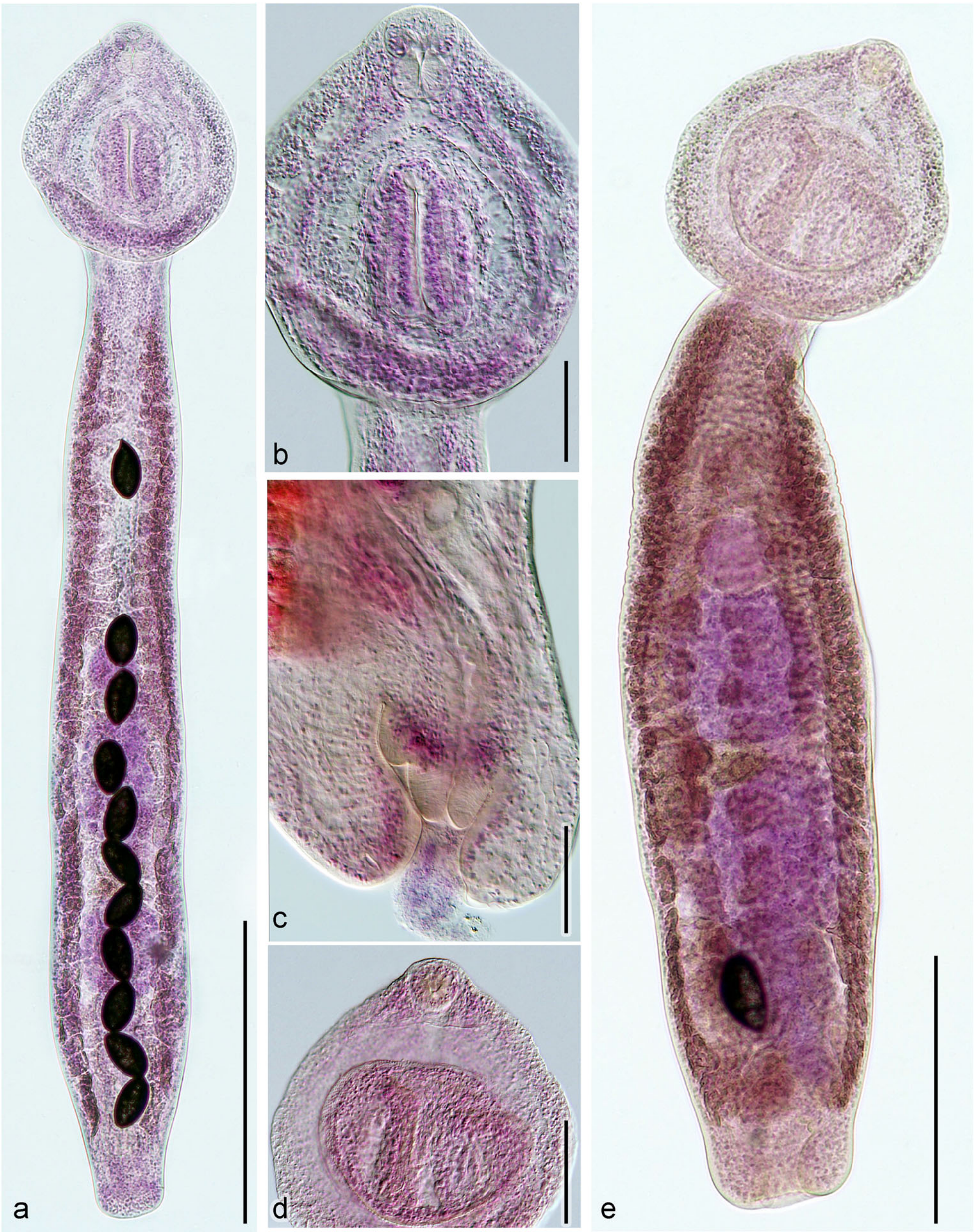
The morphology of the adult *Crassiphiala* specimens included in our study conforms closely to the diagnosis of the genus provided by Niewiadomska (2002) (Fig. 3).

While some authors (Blasco-Costa and Locke 2017; Hernández-Mena et al. 2017; Locke et al. 2018) have noted that the Crassiphialinae may be elevated to the family level, our analysis of 28S did not support the monophyly of the Crassiphialinae with two clades comprising members of this subfamily being branches of a large polytomy. This is in concordance with the phylogenetic data and branch support of corresponding clades in some of the recent works, e.g., Blasco-Costa and Locke (2017) and Hernández-Mena et al. (2017) which reported a low level of support for the Crassiphialinae. Although the authors of those publications suggested the monophyly of the Crassiphialinae, their phylogenetic trees did not provide a sufficient evidence for such conclusions. The content and systematics of the Crassiphialinae need to be carefully re-evaluated based on a detailed morphological study and additional phylogenetic analyses of its constituent taxa which is outside of the scope of this work. Based on the data obtained in the present work, particularly the demonstrated non-monophyly of the Crassiphialinae, we do not see a sufficient ground for elevating its status to the family level. Moreover, the content of the Crassiphialinae as currently recognized (Niewiadomska 2002) needs to be revised; likely, only *Crassiphiala* and *Uvulifer* should remain in the subfamily. However, a more detailed analysis involving a greater diversity of crassiphialine taxa and a thorough morphological study is needed to adequately address this question.

Despite the relatively low number of *Crassiphiala* lineages, our analyses (Figs. 1 and 2) allowed for an interesting observation that the phylogenetic relationships within the genus do not follow the geographic origin of the samples. One of the lineages from Brazil (*Crassiphiala* lineage 4) appeared on the trees as the sister group to all other members of the genus while the branch that included the other Brazilian lineage (*Crassiphiala* lineage 5) was nested among North American isolates. The reasons for this pattern are not clear at this time. One explanation may be the relatively old evolutionary origin of *Crassiphiala* which allowed for transcontinental spread (in both directions). Another explanation could be based on the partial overlap of the geographic distribution of the typically North American *M. alcyon* with several species of kingfishers broadly distributed in the Central and South America.

This study is the first to generate DNA sequence data of adult specimens of *Crassiphiala* and the first to report *Crassiphiala* in the Neotropics. Our results demonstrated the presence of at least five lineages of *Crassiphiala* in the Nearctic and Neotropics. This indicates that the diversity of *Crassiphiala* was seriously underestimated and allows us to hypothesize that additional species belonging to this genus are likely to be discovered in future studies. Central and South America hold a greater potential in this respect due to the more diverse fauna of kingfishers.

Fig. 3 Adult specimens of *Crassiphiala* from *Megaceryle torquata* from Pantanal, Brazil. **a** Ventral view of whole mount of *Crassiphiala* lineage 4. **b** Ventral view of the prosoma of *Crassiphiala* lineage 4. **c** Lateral view of posterior body end of *Crassiphiala* lineage 4. **d** Ventral view of the prosoma of *Crassiphiala* lineage 5. **e** Ventral view of whole mount of *Crassiphiala* lineage 5. Scale bars: **a** = 500 μm; **b** = 100 μm; **c** = 100 μm; **d** = 100 μm; **e** = 300 μm



Acknowledgments We are grateful to Dr. João B. Pinho (Universidade Federal de Mato Grosso, Cuiabá, Mato Grosso, Brazil), Dr. Francisco Tiago de Melo (Federal University of Pará, Belém, Pará, Brazil), and Dr. Jeffrey A. Bell (University of North Dakota) for their invaluable help with obtaining permits and field collecting. We are grateful to Mary Jaros-Gourneau (University of North Dakota) for her assistance with processing of some of the samples.

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

Funding information Collecting and processing of the specimens were supported by the grant DEB-1120734 from the National Science Foundation and grant R15AI092622 from the National Institutes of Health, USA to VVT, and the Joe K. Neel Memorial Award from the University of North Dakota and Willis A. Reid, Jr. Student Research Grant from the American Society of Parasitologists to TJA. AF was supported by a postdoctoral fellowship (PNPD scholarship) from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards (University of North Dakota IACUC protocol 0610-1). This article does not contain any studies with human participants performed by any of the authors.

References

Achatz TJ, Pulis EE, Junker K, Binh TT, Snyder SD, Tkach VV (2019) Molecular phylogeny of the Cyathocotylidae (Digenea, Diplostomoidea) necessitates systematic changes and reveals a history of host and environment switches. Zool Scr 48:545–556

Blasco-Costa I, Locke SA (2017) Life history, systematics and evolution of the Diplostomoidea Poirier, 1886: progress, promises and challenges emerging from molecular studies. Adv Parasitol 98:167–225. https://doi.org/10.1016/bs.apar.2017.05.001

Boyd EM, Fry AE (1971) Metazoan parasites of the eastern belted kingfisher, *Megaceryle alcyon alcyon*. J Parasitol 57:150–156. https://doi.org/10.2307/3277771

Derycke S, Remerie T, Vierstraete A, Backeljau T, Vanfleteren J, Vincx M, Moens T (2005) Mitochondrial DNA variation and cryptic speciation within the free-living marine nematode *Pellioditis marina*. Mar Ecol Prog Ser 300:91–103. https://doi.org/10.3354/MEPS300091

Dubois G (1968) Synopsis des Strigeidae et des Diplostomatidae (Trematoda). Premiere Partie. Mem Soc Sci Nat Neuchâtel 10:1–727 [In French]

Dubois G (1969) Les *Strigeata* (Trematoda) de la collection Elizabeth M. Boyd. Bull Soc Neuchl Sci Nat 92:5–12 [In French]

Dubois G, Rausch R (1948) Seconde contribution a l'etude des strigeides (Trematoda) Nord-Américains. Bull Soc Neuchl Sci Nat 71:29–61 [In French]

Hernández-Mena DI, García-Varela M, Pérez-Ponce de León G (2017) Filling the gaps in the classification of the Digenea Carus, 1863: systematic position of the Proterodiplostomidae Dubois, 1936 within the superfamily Diplostomoidea Poirier, 1886, inferred from nuclear and mitochondrial DNA sequences. Syst Parasitol 94:833–848. https://doi.org/10.1007/s11230-017-9745-1

Hoffman GL (1956) The life cycle of *Crassiphiala bulboglossa* (Trematoda: Strigeida): development of the metacercaria and cyst, and effect on the fish hosts. J Parasitol 42:435–444. https://doi.org/10.2307/3274528

Hunter GW III (1933) The strigeid trematode, *Crassiphiala ambloplitis* (Hughes, 1927). Parasitology 25:510–517. https://doi.org/10.1017/S0031182000019752

Kudlai O, Kostadinova A, Pulis EE, Tkach VV (2015) A new species of *Drepanocephalus* Dietz, 1909 (Digenea: Echinostomatidae) from the double-crested cormorant *Phalacrocorax auritus* (lesson) (Aves: Phalacrocoracidae) in North America. Syst Parasitol 90:221–230. https://doi.org/10.1007/s11230-015-9550-7

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054

Locke SA, Van Dam AR, Caffara M, Pinto HA, López-Hernández D, Blanar CA (2018) Validity of the Diplostomoidea and Diplostomida (Digenea, Platyhelminthes) upheld in phylogenomic analysis. Int J Parasitol 48:1043–1059. https://doi.org/10.1016/j.ijpara.2018.07.001

Lockyer AE, Olson PD, Østergaard P, Rollinson D, Johnston DA, Attwood SW, Southgate VR et al (2003) The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858. Parasitology 126:203–224. https://doi.org/10.1017/S0031182002002792

López-Jiménez A, Pérez-Ponce de León G, García-Varela M (2018) Molecular data reveal high diversity of *Uvulifer* (Trematoda: Diplostomidae) in middle America, with the description of a new species. J Helminthol 92:725–739. https://doi.org/10.1017/S0022149X17000888

Lutz HL, Tkach VV, Weckstein JD (2017) Methods for specimen-based studies of avian symbionts. In: Webster M (ed) The role of collections in ornithology: the extended specimen. Studies in avian biology. CRC Press, Boca Raton, pp 127–183. https://doi.org/10.1201/9781315120454

McAllister CT, Tumlison R, Robison HW, Trauth SE (2013) Initial survey on black-spot disease (Digenea: Strigeoidea: Diplostomidae) in select Arkansas fishes. J Ark Acad Sci 67:200–203

Muzzall PM, Cook V, Sweet DJ (2011) Helminths of belted kingfishers, *Megaceryle alcyon* Linnaeus, 1758, from a fish hatchery in Ohio, U.S.a. Comp Parasitol 78:367–372. https://doi.org/10.1654/4492.1

Niewiadomska K (2002) Family Diplostomidae Poirier, 1886. In: Gibson DI, Jones A, Bray RA (eds) Keys to the Trematoda, vol 1. CAB international and the Natural History Museum, Wallingford—London, pp 167–196. https://doi.org/10.1017/S0031182002222830

Preble NA, Harwood PD (1944) A heavy infection of strigeids in a kingfisher (*Megaceryle alcyon alcyon*). Trans Am Microsc Soc 63:340–341. https://doi.org/10.2307/3223306

Rambaut A (2016) Figtree (Version 1.4.3). Available at http://tree.bio.ed.ac.uk/software/figtree/. Accessed 14 Mar 2019

Scott ME (1984) Helminth community in the belted kingfisher, *Ceryle alcyon* (L.) in southern Québec. Can J Zool 62:2670–2681. https://doi.org/10.1139/z84-391

Tkach VV, Littlewood DTJ, Olson PD, Kinsella JM, Swiderski Z (2003) Molecular phylogenetic analysis of the Microphalloidea Ward, 1901 (Trematoda: Digenea). Syst Parasitol 56:1–15. https://doi.org/10.1023/A:1025546001611

Tkach VV, Pawlowski J (1999) A new method of DNA extraction from the ethanol-fixed parasitic worms. Acta Parasitol 44:147–148

Van Haitsma JP (1925) *Crassiphiala bulboglossa* nov. gen., nov. spec., a holostomatid trematode from the belted kingfisher, *Ceryle alcyon* Linn. Trans Am Microsc Soc 44:121–131. https://doi.org/10.2307/3221460

Van Steenkiste N, Locke SA, Castelin M, Marcogliese DJ, Abbott C (2015) New primers for DNA barcoding of digeneans and cestodes (Platyhelminthes). Mol Ecol Resour 15:945–952. https://doi.org/10.1111/1755-0998.12358