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Molecular data show Clinostomoides Dollfus, 1950 is a junior synonym of Clinostomum Leidy, 1856, with redescription of metacercariae of Clinostomum brieni n. comb

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## Molecular data show Clinostomoides Dollfus, 1950 is a junior synonym of Clinostomum Leidy, 1856, with redescription of metacercariae of Clinostomum brieni n. comb.

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Key Words:	Yellow grub, halzoun, molecular prospecting, hybridization, incomplete lineage sorting, DNA barcode, heron, catfish



## We appreciate the time and effort the reviewer has spent on our manuscript.

Referee: 1

Comments to the Author

Line 221 Intestinal ceca bifurcation in preacetabular region, forming caecal shoulders before running Is it the caeca that bifurcate? 'Intestinal bifurcation' is a more usual descriptor.

## • Changed

Perhaps 'preacetabular' might confuse some readers as there is no mention of an acetabulum (It is referred to as a ventral sucker in the description and acetabulum in parts of the discussion). It could be replaced by 'in forebody'.

• Although we don't think readers of *Parasitology* will be confused by the term 'acetabulum', we changed it as the reviewer requests / to 'anterior to the ventral sucker'.

The American spelling 'ceca' and the British spelling 'caecal' are used in the same phrase. Use just one spelling consistently.

• Changed

Line 223. sucker, muscular, trilobed structure well visible – easily visible

• Changed

Line 284. The posterior testis in most descriptions is moon-shaped. The moon is a sphere! Use a more precise descriptor.

Review

• We changed this to 'crescent shaped', the term we use in morphological description

1	Molecular data show <i>Clinostomoides</i> Dollfus, 1950 is a junior synonym of <i>Clinostomum</i>
2	Leidy, 1856, with redescription of metacercariae of <i>Clinostomum brieni</i> n. comb.
3	
4	MONICA CAFFARA <sup>1*</sup> , SEAN A. LOCKE <sup>2</sup> , ALI HALAJIAN <sup>3</sup> , WILMIEN J LUUS-
5	POWELL <sup>3</sup> , DEBORAH BENINI <sup>1</sup> , PERLA TEDESCO <sup>1</sup> , GYRHAISS KAPEPULA
6	KASEMBELE <sup>4</sup> , MARIA L. FIORAVANTI <sup>1</sup>
7	
8	<sup>1</sup> Department of Veterinary Medical Sciences, <i>Alma Mater Studiorum</i> University of Bologna,
9	Via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy.
10	<sup>2</sup> Department of Biology, University of Puerto Rico, Box 9000, Mayagüez, Puerto Rico
11	00681-9000.
12	<sup>3</sup> Department of Biodiversity, University of Limpopo, Private Bag X1106, Sovenga 0727,
13	South Africa.
14	<sup>4</sup> Unité de recherché en Biodiversité et Exploitation durable des Zones Humides (BEZHU),
15	Faculté des Sciences Agronomiques, Université de Lubumbashi, Haut-Katanga, R.D. Congo.
16	
17	
18	*Corresponding author: Department of Veterinary Medical Sciences, Alma Mater Studiorum
19	University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia (BO) E-mail:
20	monica.caffara@unibo.it
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#### 24 SUMMARY

The genus *Clinostomoides* Dollfus, 1950 was erected to accommodate a single worm from 25 Ardea goliath sampled in the Belgian Congo. The specimen was distinguished from other 26 clinostomids by its large size and posterior genitalia. In the following years, metacercariae of 27 Clinostomoides brieni, have been described in Clarias spp. in southern and western Africa. A 28 few authors have referred to *Clinostomum brieni*, but all such usages appear to be *lapsus* 29 calami, and the validity of Clinostomoides remains widely accepted. In this study our aim 30 was: position *Clinostomoides brieni* among the growing clinostomids molecular database, and 31 redescribe the species with emphasis on characters that have emerged as important in recent 32 33 work. We sequenced two nuclear (partial 18S and ITS) and one mitochondrial marker (partial cytochrome c oxidase I) and studied morphology in metacercariae from hosts and localities 34 likely to harbor the type species (*Clarias* spp., Democratic Republic of the Congo, South 35 Africa). Phylogenetic analysis shows *Clinostomoides brieni* belongs within *Clinostomum* 36 Leidy, 1856. We therefore transfer Clinostomoides brieni to Clinostomum, amend the 37 diagnosis for the genus *Clinostomum*, and provide a critical analysis of other species in 38 *Clinostomoides*, all of which we consider *species inquirendae*, as they rest on comparisons of 39 different developmental stages. 40

- 41
- 42
- 43

Key words: Yellow grub, halzoun, molecular prospecting, hybridization, incomplete lineage
sorting, DNA barcode, heron, catfish

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47

#### 48 **KEY FINDINGS**

- Genus *Clinostomoides* Dollfus, 1950 originally distinguished from other clinostomids by
  large size and posterior genitalia.
- 51 Morphological and molecular study of metacercariae from hosts and localities likely to
- 52 harbor the type species *Clinostomoides brieni* (*Clarias* spp., southern Africa).
- Phylogenetic analysis of three markers (18S, ITS rDNA and CO1 mtDNA) shows
- 54 *Clinostomoides brieni* belongs within *Clinostomum* Leidy, 1856.
- Amended diagnosis for *Clinostomum* to accommodate inclusion of *C. brieni*.
- Critical analysis of other species in *Clinostomoides*, all considered species inquirendae.
- 57

## 58 INTRODUCTION

- 59 The genus *Clinostomoides* was erected by Dollfus (1950) to accommodate a single adult
- 60 collected from the esophagus of *Ardea goliath* sampled in the Belgian Congo (now
- 61 Democratic Republic of the Congo DRC). The type species, *C. brieni* Dollfus, 1950, was
- 62 distinguished from *Clinostomum* based on its large body size (30 mm), the genital complex in
- 63 the posterior (rather than middle or across middle and posterior) third of body, and the genital
- 64 pore located ventral to the posterior testis (rather than lateral to the anterior testis). In the
- 65 following years, metacercariae of *C. brieni* were described by Prudhoe (1957) from *Clarias*
- 66 *lazera* collected in Belgian Congo, by Manter and Pritchard (1969) from *Clarias* sp. from
- 67 Rwanda, by Fischthal and Thomas (1970) from *C. senegalensis* in Ghana, by Barson *et al.*
- 68 (2008) and by Jansen van Rensburg *et al.* (2013) from *C. gariepinus* sampled in Zimbabwe
- and Botswana, respectively. Outside the African continent, Mirzoeva (1981) described one
- 70 adult of C. brieni collected from the esophagus of Ardea purpurea in Azerbaijan, and
- 71 metacercariae of *C. brieni* were also reported in the Philippines (Arthur and Lumanlan-Mayo,
- 72 1997).

73	Additional species of <i>Clinostomoides</i> have been described in Central India, the first being <i>C</i> .
74	dollfusi Agarwal 1958, followed by C. ophicephali (Tubangui and Masiluñgan, 1935)
75	Agarwal 1958 (transferred from genus Clinostomum), C. chauhani Pandey 1971, C. rai Rai
76	1970, C. meerutensis Pandey and Tyagi 1986, C. pandeyii Singh and Sharma 1994, and C.
77	baughi Pandey 1998. However, all were erected based either on comparisons among these
78	regional species or with the earliest species in the region, C. dollfusi. Most importantly, all,
79	including C. dollfusi, were based on metacercariae. This is problematic because in erecting C.
80	dollfusi, Agarwal (1958) compared metacercariae with the adult described by Dollfus (1950),
81	not with metacercariae described by Prudhoe (1957). Manter and Pritchard (1969)
82	synonymized C. dollfusi with C. brieni, and their doubts about the morphological characters
83	used to erect the junior species were confirmed by Fischthal and Thomas (1970). However,
84	subsequent work in India has not taken the latter studies into account and continues to treat as
85	valid C. dollfusi and other species erected in comparison to it (e.g. Pandey and Agrawal,
86	2013). Finally, the species described by Dollfus has occasionally been reported as
87	Clinostomum brieni, in lapsus calami. Prudhoe (1957) and Douellou (1992) used both
88	"Clinostomum brieni" and "Clinostomoides brieni" as names for the same species, and Lio-Po
89	et al (1983) listed "Clinostomum brieni," but without taxonomic comment or support. Other
90	than these isolated cases, the genus Clinostomoides and its type species Clinostomoides brieni
91	are widely considered valid (e.g., Kanev et al. 2002).
92	A combination of molecular and morphological approaches is a useful way to resolve
93	situations like this, as has already been shown in other clinostomids, i.e., Clinostomum Leidy,
94	1856 (Caffara et al. 2011; Sereno-Uribe et al. 2013), Euclinostomum Travassos, 1928
95	(Senapin et al. 2014; Caffara et al. 2016), Odhneriotrema Travassos, 1928 (Woodyard et al.
96	2017) and Ithyoclinostomum Witenberg, 1925 (Briosio-Aguilar et al. 2018). However, few
97	DNA sequences from <i>Clinostomoides</i> are currently available for comparison. Athokpam <i>et al</i>

(2014) provided rDNA sequences from C. brieni, but without supporting morphological 98 information. Moreover, the identification of C. brieni by these authors was questioned by 99 Brioso-Aguilar et al (2018) because of the close relationship of its 28S sequence to data from 100 *Clinostomum*. It is also relevant that the material sequenced by Athokpam *et al* (2014) was 101 102 from *Heteropneustes* in Northeastern India, rather than the region or hosts (central and southern Africa, *Clarias*, *Ardea*) where the genus originated and is better known. 103 The aim of this work was to provide a redescription of Clinostomoides brieni metacercariae 104 based on morphological and molecular analyses, following Matthews and Cribb (1998), and 105 to provide an updated critical analysis of previously described species. To this end, we 106 107 collected in localities and hosts in which we were likely to encounter the same species as 108 Dollfus (1950) and Prudhoe (1957), with the aim of characterizing the type species. Our results led us to transfer Clinostomoides brieni to Clinostomum and to consider species of 109 *Clinostomoides* from India as *species inquirendae*. 110

111

## 112 MATERIALS AND METHODS

Two metacercariae of *Clinostomoides* sp. were collected from *Clarias gariepinus* sampled at Phalaborwa barrage, Limpopo province (South Africa) and four from *C. ngamensis* sampled in the Democratic Republic of the Congo (1 from Lake Tshangalele, Kapolowe Mission and 3 from Kiswishi River near Futuka Farm). All were recovered from the body cavity, cleaned in saline and preserved in 70% ethanol.

Total lengths of metacercariae were measured before cutting a small piece of the posterior end for molecular analyses. Morphometrics of hologenophores (*sensu* Pleijel *et al.* 2008) were taken after clarification with Amman's lactophenol and staining by Malzacher's method (Pritchard and Kruse, 1982). Line drawings were made with the aid of a drawing tube, and measurements are given in micrometers following Matthews and Cribb (1998). DNA was

123	extracted from hologenophore subsamples using a PureLink Genomic DNA Kit (Invitrogen)
124	following the manufacturer's protocol. Amplification of 18S and Internal transcribed Spacer
125	1-5.8S-Internal Transcribed Spacer 2 (ITS) rDNA employed protocols and primers of
126	Gustinelli et al. (2010), cytochrome c oxidase I (CO1) mtDNA those of Moszczynska et al.
127	(2009).
128	Amplified products were resolved on a 1% agarose gel stained with SYBR Safe DNA Gel
129	Stain in $0.5 \times$ TBE (Molecular Probes – Life Technologies). For sequencing of 18S, ITS and
130	CO1, bands were excised and purified by NucleoSpin Gel and PCR Cleanup (Mackerey-
131	Nagel) and sequenced with an ABI 3730 DNA analyser at StarSEQ GmbH (Mainz,
132	Germany). Contigs were assembled with Vector NTI AdvanceTM 11 software (Invitrogen)
133	and sequences are published in GenBank under the following accession numbers:
134	MH606186-90 (18S), MH238412-16 (ITS) and MH253044-48 (CO1).
135	Pairwise p-distances and models of nucleotide evolution (BIC) were calculated using MEGA
136	6.06 (Tamura et al. 2013). For trees constructed with Bayesian Inference, in MrBayes 3.2.6
137	(Ronquist <i>et al.</i> 2012), nst=2+G was used for ITS and 18S, and GTR+G+I was used for CO1.
138	The K2P+G-model was used for Maximum Likelihood analysis in MEGA of 18S and ITS
139	rDNA while GTR+G+I was used for ML analysis of CO1 mtDNA.
140	The newly generated sequences of 18S, ITS and CO1 were aligned along with one or two
141	representative sequences of Clinostomum species (C. complanatum, C. cutaneum, C.
142	phalacrocoracis, C. tilapiae, C. philippinensis, C. marginatum, C. tataxumui, C. album, C.
143	poteae, C. heluans, C. attenuatum, C. detruncatum, C. arquus, C. caffarae, C. cichlidorum)
144	plus undescribed or unidentified species of Clinostomum (Locke et al. 2015; Caffara et al.
145	2017). Euclinostomum heterostomum (ITS: KP721422, CO1: KP721404), Odhneriotrema
146	incommodum (ITS: MF766000, CO1: MF766003) and Ithyoclinostomum (ITS: MH159753,
147	CO1: MH159752) were used as outgroup for the subfamily Clinostomatinae, while

148 *Tylodelphys immer* (18S and ITS: MH521252; CO1: MH536513), *Cyathocotyle prussica* 

- 149 (18S and ITS: MH521249; CO1: MH536510), Schistosoma mansoni (18S: U65657; ITS:
- 150 AY446082) as outgroup for Clinostomidae. All codon positions in the CO1 alignment were
- used in the analysis because of lack of evidence of nucleotide saturation (Iss =0.237,
- 152 Iss.c=0.697, df=472, P=0, Xia *et al.* 2003; Xia and Lemey, 2009).
- 153
- 154 **RESULTS**
- Among five 18S rDNA sequences 1826-1877 bp in length obtained from African samples of
- 156 *C. brieni* in the present study, there were four variable sites, all transitions, i.e. mean
- divergence 0.1%, range 0-0.2%. All variation was in two sequences from South Africa; three
- 158 18S sequences from Congo were identical. An 18S sequence (KF781300, 1907 bp) of
- 159 Athokpam *et al.* (2014) from *C. brieni* from *Heteropneustes fossilis* in Manipur differed at 32
- positions (1.7%) from another 18S sequence by the same authors (KF811009, 1859 bp) from
- 161 the same host in Meghalaya (the latter sequence is not mentioned in the paper of Athokpam *et*
- 162 *al.* 2014). Variation in 18S between the five African *C. brieni* sequences and the two Indian
- isolates averaged 1.4% (range 0.5-2.6%). Phylogenetic analysis showed that 18S sequences
- 164 from Indian and African *C. brieni* form a well-supported clade nested within *Clinostomum*
- species. The *Clinostomum* + *C. brieni* clade is also well supported, and comparatively deeply
- 166 divergent from *Euclinostomum*. Variation among 18S sequences of *Clinostomum* spp.
- averaged 0.9% (range 0.2-1.5%) and in the *Clinostomum* + *C. brieni* clade, 18S variation
- averaged 1.1% (range 0-3.6%). All the highest divergence values ( $\geq 1.8\%$ ) in the latter clade
- 169 were associated with the unpublished *C. brieni* sequence KF811009. Variation between
- 170 *Euclinostomum* and members of the *Clinostomum* + *C. brieni* clade averaged 2.8% (range 2.4-
- 171 5.0%).

The five ITS rDNA sequences 1005-1028 bp in length from African *C. brieni* were identical
to each other and to the 300-bp ITS2 sequence of *C. brieni* (KF781298) of Athokpam *et al.*(2014). The ITS of *C. brieni* varied by a mean of 5.7% (range 4.7-7.1%) from species of *Clinostomum.* Variation in ITS among species of *Clinostomum s.s.* was of similar magnitude:
mean 4.9% (range 0.1-8.6%). In contrast, ITS variation among members of the genera *Euclinostomum, Odhneriotrema*, and *Clinostomum + Clinostomoides* averaged 15.2% (range 13.9-16.3%).

The CO1 sequences of four specimens of C. brieni were identical but that of one specimen 179 (MH253045, from C. gariepinus in South Africa) differed by 11% from the other four. This 180 specimen did not differ morphologically from the other five C. brieni examined, and its 18S 181 (MH606187) and ITS (MH238413) sequences were not similarly divergent (Figs 1, 2). The 182 CO1 of this specimen differed by 0.2% from *Clinostomum* morphotype 3 (KY865667, from 183 the Amphilius uranoscopus in South Africa). The DNA from this specimen was amplified and 184 sequenced an additional four times with the same results. The BI and ML trees were based on 185 186 a 473 bp CO1 alignment and had little statistical support at deeper nodes, but both showed C. brieni within Clinostomum. The C. brieni specimen MH253045 grouped with Clinostomum 187 sp. morphotype 3, while the other four C. brieni sequences form a monophyletic clade within 188 the Old-World clade of *Clinostomum* species. The four monophyletic C. brieni sequences 189 differ by mean 15.3% (range 13-19.5%) from other *Clinostomum* species. Interspecific CO1 190 variation in *Clinostomum* is similar, with mean 16.3% (range 3.5-22.1%). The mean 191 intergeneric CO1 distances between members of Odhneriotrema, Clinostomoides + 192 Clinostomum, Euclinostomum and Ithyoclinostomum is 19.8% (range 17.3-23.5%). 193 Analyses of three molecular markers indicate Clinostomoides should be regarded as junior 194 synonym of *Clinostomum*, as amended below. Tree topologies (Figs. 1-3) show relatively 195

196 deep divergence between Odhneriotrema, Euclinostomum and Clinostomum whereas

- 197 *Clinostomoides* falls within a clade of *Clinostomum* species. Genetic distances between *C*.
- 198 *brieni* and *Clinostomum* species are comparable to those within *Clinostomum* s.s. and inferior
- 199 to distances among other clinostomid genera.
- 200
- 201 *Clinostomum* Leidy, 1856
- 202 (Synonym *Clinostomoides* Dollfus, 1950)
- 203 Family Clinostomidae Lühe, 1901
- 204 Subfamily Clinostominae Lühe, 1901
- Body medium to very large, linguiform, stout, convex dorsally and concave ventrally.
- 206 Tegument smooth or with spines. Oral sucker may or may not be surrounded by collar-like
- fold when retracted. Ventral sucker muscular, well developed, always larger than oral sucker.
- 208 Caeca long, simple, with more or less sinuous wall, particularly in anterior half of body, but
- 209 lacking lateral branches or diverticula. Testes smooth or irregular in shape, in posterior half of
- body. Ovary intertesticular, to right of medial line. Vitelline follicles in lateral fields
- anteriorly, from level of intestinal bifurcation or ventral sucker to posterior extremity, may
- remain lateral and extracaecal or become confluent posterior to genital complex. Uterus
- intercaecal, extending from Mehlis' gland to fill part of total distance to ventral sucker.
- 214 Genital pore anterior, lateral or posterior to testicular-ovary complex. Cosmopolitan. Type
- 215 species *Clinostomum complanatum* (Rudolphi, 1819).
- 216 Morphological Description of Clinostomum brieni (Dollfus, 1950) n. comb. (Fig. 4, Table 1)
- 217 (based on 5 hologenophores and 1 paragenophore)
- 218 (Synonym *Clinostomoides brieni* Dollfus, 1950)
- 219 Body regularly elongated, narrow, tongue-shaped. Oral sucker small, with indistinct marginal
- 220 limits. Pre-pharynx cup-shaped elongated, thick, muscular. Pharynx visible, muscular.

Intestinal bifurcation anterior to ventral sucker, forming caecal shoulders before running 221 222 laterally to ventral sucker to posterior end of body. Ventral sucker robust, larger than oral sucker, muscular, trilobed structure easily visible. Caeca provided with small lateral pockets 223 becoming more digitated posteriorly to ventral sucker. Whole genital complex in posterior 224 part of posterior third of body. Testes two, tandem, intercaecal, transversely elongated. 225 Anterior testis bow-tie shaped. Posterior testis Y- to crescent shaped with anterior margin 226 concave. Cirrus sac comma-shaped, thick walled, intertesticular dextral, from right posterior 227 margin of anterior testis to anterior margin of posterior testis, genital pore opening in concave 228 margin of posterior testis. Ovary small, intertesticular dextrally to cirrus sac, close to right 229 230 margin of posterior testis. Uteroduct emerging from ootype complex runs around left margin of anterior testis, 231 ascending sinistrally with some undulation to slightly above metraterm before looping 232 posteriorly on itself entering directly into the proximal part of uteroduct and opening into 233 uterine sac. Uterine sac median elongate narrow, tip reaching the posterior part of middle 234 third of body. Tegument armed with spines (8-11 µm) from posterior part of oral sucker to 235 posterior end of body. Excretory bladder Y-shaped, postcaecal, arms extending anteriorly in 236 237 extracaecal position. Excretory pore terminal.

238

#### 239 **DISCUSSION**

240 Our original aim was redescribe metacercariae of *Clinostomoides brieni* and assess the

241 relationship of the species with other clinostomids using DNA. Unexpectedly, the molecular

242 data strongly indicate the species belongs within *Clinostomum*, which led us to amend the

243 diagnosis of the genus to accommodate Clinostomum brieni n. comb. The genus

244 *Clinostomoides* was limited to Afrotropic and Indo-Malayan regions, and most reports are

from *Clarias* or *Heteropneustes*, which are closely related siluriform genera occurring in the

same regions (Hardman, 2005; Kushwaha et al. 2015; Froese and Pauly, 2018). The 246 phylogenetic association of C. brieni with a clade of Old-World Clinostomum species (Figs 1-247 3) is consistent with a biogeographic pattern that continues to be observed as data accumulate 248 from more species of Clinostomum (Locke et al. 2015; Pérez-Ponce de León et al. 2016; 249 Rosser et al. 2018), which adds further evidence that C. brieni belongs to Clinostomum. 250 One specimen we collected was morphologically indistinguishable and shared identical rDNA 251 sequences with other C. brieni, but its CO1 (MH253045) was highly divergent, and nearly 252 253 identical to Clinostomum morphotype 3, which Caffara et al. (2017) obtained from mochokid and amphiliid catfishes in South Africa. We believe this can be most plausibly explained by 254 255 hybridization. Both C. brieni and Clinostomum morphotype 3 infect siluriform second 256 intermediate hosts in the same region, which are preved upon by local ardeid definitive hosts, including the type host of C. brieni, A. goliath (Mock and Mock, 1980). Other than this 257 particular specimen, C. brieni and Clinostomum morphotype 3 appear to be distantly related 258 259 (Figs. 1, 2). This argues against another possible explanation for mitochondrial haplotype sharing between C. brieni and Clinostomum morphotype 3, incomplete sorting among 260 recently separated species. In any event, when viewed together with the highly distinctive 261 morphology of C. brieni, both hybridization and incomplete lineage sorting suggest species 262 belonging to a single genus, which supports our main taxonomic conclusion. 263 The most distinctive characters of *Clinostomoides* were the size of the adult (30 mm) 264 265 described in A. goliath by Dollfus (1950), the extremely posterior position of the genital complex, and the position of the cirrus sac and genital pore within the genital complex, 266 characters considered also in regional descriptions of metacercariae (Prudhoe, 1957; Manter 267 268 and Pritchard, 1969; Fischthal and Thomas, 1970; van Rensburg et al. 2013). The most obvious distinction of *Clinostomoides*, its large size, is noteworthy in that the adult is known 269 270 only from the largest of ardeids, A. goliath (Mock and Mock, 1980). In light of the molecular

evidence that *Clinostomoides* belongs within *Clinostomum*, the other characters may beconsidered as size-related allometric changes.

273 The clinostomid metacercariae we collected from Clarias species in South Africa and Democratic Republic of the Congo were morphologically consistent with previous 274 descriptions of C. brieni in the same host and region (Prudhoe, 1957; Manter and Pritchard, 275 1969; Fischthal and Thomas, 1970; van Rensburg et al. 2013). The only inconsistency was 276 the pharyngeal morphology. Fischthal and Thomas (1970) reported a thick-walled, muscular 277 278 pre-pharynx and very muscular pharynx (as observed in our specimens), while Prudhoe (1957) reported the pharynx absent, Manter and Pritchard (1969) did not mention it, and van 279 280 Rensburg et al. (2013) reported a short pre-pharynx and muscular pharynx. In our opinion, these structures are probably always present but not always visible. 281

As the genital complex provides reliable features for discriminating species of *Clinostomum* 282 (i.e. Caffara et al. 2017, Sereno-Uribe et al. 2018, see Table 2), it can also shed light on prior 283 records of *Clinostomum brieni*. The posterior testis in most descriptions is crescent-shaped, 284 285 and showed this form in two of six subjects we examined, but in four worms the posterior testis was Y-shaped, as also reported by Fischthal and Thomas (1970). In metacercariae of 286 *Clinostomum*, testes are more digitated than in pre-adults/adults (Ukoli, 1966). The only 287 description of the adult of C. brieni is that of Dollfus (1950), who reported testes similar to 288 289 those later described in metacercariae, except for small marginal lobules in the adult organs. 290 Thus, in this species of *Clinostomum*, developmental variation in the morphology of the testicular margin appears to be reversed (going from smooth to more digitated), although data 291 are needed from additional adults to confirm this. The cirrus pouch (CP) in C. brieni is well 292 293 developed and lies between the testes, at a variable distance from the posterior border of the anterior testis and touching the concave part of the posterior testis where the genital pore 294 opens. This pattern has been observed in all previous descriptions of C. brieni except that of 295

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Fischthal and Thomas (1970), in which the CP did not touch the posterior testis. Finally, in all
metacercarial descriptions the uteroduct forms a similar loop devoid of eggs, which becomes
filled with eggs in the adult (Dollfus, 1950).

Molecular data indicate a single species (albeit with potential capacity to hybridize) of 299 *Clinostomum brieni* among samples spanning approximately 1500 km of the known 300 geographic range of this species, including the type region. Morphological differences among 301 our specimens and those of prior regional accounts (Dollfus, 1950; Prudhoe, 1957; Manter 302 303 and Pritchard, 1969; Jansen van Rensburg et al. 2013) therefore likely represent intraspecific variation. This can provide a useful perspective for considering species of *Clinostomoides* 304 305 described from the Indian subcontinent, the first of which was C. dollfusi, which Agarwal 306 (1958) described from metacercariae from *Clarias* sp. and *Heteropneustes* (=*Saccobranchus*) 307 sp. collected in Jabalpur. Agarwal (1958) emphasized body length (7.8-9.8 mm in the Indian metacercariae vs the 30 mm in adult holotype of C. brieni), the absence of lateral sacculations 308 309 of the uterus, and distance between suckers. However, all these characters are in fact typical of metacercariae of C. brieni (Manter and Pritchard, 1969; Fischthal and Thomas, 1970; Van 310 Rensburg et al. 2013; present study), if not of the larger adult holotype. All other species of 311 *Clinostomoides* in this region are from *Heteropneustes*. *Clinostomoides chauhani* Pandey, 312 1971, was described from the body cavity and viscera of *H. fossilis* collected in Lucknow, 313 314 based on comparison with C. dollfusi and C. ophicephali, which were both synonymized with C. brieni by Manter and Pritchard (1969) and Fischthal and Thomas (1970). The species C. 315 chauhani is said to possess an aspinose body but spinose cuticle (Pandey and Agrawal, 2013). 316 317 Clinostomoides rai was proposed by Rai (1970) (later revised by Pandey, 1974) for metacercariae from the intestine of Clarias batrachus collected in Mathura, on the basis of 318 319 distance between suckers and limbs of uterus (Pandey and Agrawal, 2013). However, Pandey and Agrawal (2013) appear to have mistranscribed morphological values (i.e. acetabulum 320

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1.16 x 0.78 mm *vs* acetabulum at distance of 1.16 mm behind anterior end of body and 0.78
mm in diameter) and host tissues (muscle *vs* intestine) recorded by Rai (1970). *Clinostomoides meerutensis* Pandey and Tyagi, 1986 and *C. pandeyii* Singh and Sharma,

1994, were both created for metacercariae from the body surface of *H. fossilis* in Meerut

based on a spinose tegument and ovary opposite to cirrus sac. Pandey and Kiran (2002)

326 synonymized C. rai, C. meerutensis and C. pandeyii with C. dollfusi. Clinostomoides baughi

327 Pandey, 1998 was described from metacercariae in skin near the operculum of *H. fossilis* in

328 Lucknow, based only on comparisons with the species of Indian *Clinostomoides* mentioned

above. In the descriptions of *C. meerutensis, C. pandeyii* and *C. baughi*, the ovary is

described as opposite to the cirrus sac. However, to our knowledge the ovary in the genus

*Clinostomoides* and/or *Clinostomum* is always on the same side as the cirrus pouch. Line
 drawings in Pandey and Agrawal (2013) appear to show specimens of the latter three species
 transposed, with the uteroduct in the right side of the body.

In our opinion, these species of *Clinostomoides* (*C. dollfusi, C. rai, C. chauhani, C.* 

*meerutensis, C. pandeyii, C baughi*) described from India are *species inquirendae* because the
 morphological basis of each may be an artifact of development, beginning with the

337 comparison of larval *C. dollfusi* with adult *C. brieni* and cascading through later descriptions.

338 The characters considered in these studies vary with parasite development and may also be

influenced by fixation and mounting (Manter and Pritchard, 1969; Fischthal and Thomas,

340 1970). Until such species can be verified through morphological comparisons at equivalent

341 stages of development, preferably with detailed accounts of the genital complex and with

342 supporting molecular data, we consider only *Clinostomum brieni* to be valid. Essentially, this

- view follows Manter and Pritchard (1969) and Fischthal and Thomas (1970). Further work
- may reveal additional species of *Clinostomum* with the *Clinostomoides*-morphotype on the
- 345 Indian subcontinent or elsewhere, but currently this is supported with neither molecular (Figs.

- 1, 2) nor morphological data. Connectivity between Indian and African populations of *C*.
- 347 *brieni* could be maintained by species of *Ardea* which occur in both regions (e.g., *A. cinerea*,
- 348 *A. purpurea*, *A. goliath*, BirdLife International, 2018).

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to per period

Fig. 1. Evolutionary history inferred using Bayesian Inference (nst=2+G) from 18S rDNA of 505 Clinostomum brieni n. comb. generated in this study (in bold) with data from other studies (22 506 nucleotide sequences, 1663 positions). Nodes are labelled with posterior probability in 507 Bayesian Inference analysis and, after the slash, percent of bootstrap support in 1000 508 replicates in Maximum Likelihood. SA=South Africa, DRC=Democratic Republic of the 509 Congo. 510 Fig. 2. Evolutionary history inferred using the Bayesian Inference analysis (nst=2+G) from 511 512 ITS rDNA of Clinostomum brieni n. comb. generated in this study (in bold) with data from other studies (36 nucleotide sequences, 575 positions). Nodes are labelled posterior 513 probability and, after the slash, with percent of bootstrap support in 1000 replicates in 514 Maximum Likelihood. SA=South Africa, DRC=Democratic Republic of the Congo. 515 Fig. 3. Evolutionary history inferred in Bayesian Inference analysis (GTR+G+I) from CO1 516 mtDNA of *Clinostomum brieni* n. comb. generated in this study (in bold) with data from other 517 studies (37 nucleotide sequences, 473 positions). Nodes are labelled with posterior probability 518 and, after the slash, percent of bootstrap support in 1000 replicates in separate Maximum 519 Likelihood analysis; an asterisk indicates a clade that was not recovered with Maximum 520 Likelihood. SA=South Africa, DRC=Democratic Republic of the Congo. 521

522 Fig. 4. Line drawing of metacercaria of *Clinostomum brieni* n. comb. Scale bar =  $1000 \mu m$ .

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Fig. 1. Evolutionary history inferred using Bayesian Inference (nst=2+G) from 18S rDNA of Clinostomum brieni n. comb. generated in this study (in bold) with data from other studies (22 nucleotide sequences, 1663 positions). Nodes are labelled with posterior probability in Bayesian Inference analysis and, after the slash, percent of bootstrap support in 1000 replicates in Maximum Likelihood. SA=South Africa, DRC=Democratic Republic of the Congo.



Fig. 2. Evolutionary history inferred using the Bayesian Inference analysis (nst=2+G) from ITS rDNA of Clinostomum brieni n. comb. generated in this study (in bold) with data from other studies (36 nucleotide sequences, 575 positions). Nodes are labelled posterior probability and, after the slash, with percent of bootstrap support in 1000 replicates in Maximum Likelihood. SA=South Africa, DRC=Democratic Republic of the Congo.

157x93mm (300 x 300 DPI)



Fig. 3. Evolutionary history inferred in Bayesian Inference analysis (GTR+G+I) from CO1 mtDNA of Clinostomum brieni n. comb. generated in this study (in bold) with data from other studies (37 nucleotide sequences, 473 positions). Nodes are labelled with posterior probability and, after the slash, percent of bootstrap support in 1000 replicates in separate Maximum Likelihood analysis; an asterisk indicates a clade that was not recovered with Maximum Likelihood. SA=South Africa, DRC=Democratic Republic of the Congo.

166x146mm (300 x 300 DPI)



Fig. 4. Line drawing of metacercaria of Clinostomum brieni n. comb. Scale bar = 1000  $\mu m.$ 

67x250mm (300 x 300 DPI)

Table. 1. Morphological data of Clinostomum brieni [range (mean  $\pm$  SD)]

Body Length	6762-10602 (8683 ± 1425)
Body Width	$1248\text{-}1527\ (1392\pm118.3)$
Body Length/Width	$4.80\text{-}8.40~(6.27\pm1.17)$
Oral Sucker (OS) Length	172-256 (218 ± 37.91)
OS Width	172-282 (233 ± 35.99)
OS Width/Body Width	$0.11 - 0.19 \ (0.15 \pm 0.033)$
Ventral Sucker (VS) Length	622-749 (676 ± 54.08)
VS Width	678-794 (733 ± 42.96)
VS Width/OS Width	$2.91\text{-}4.61\;(3.55\pm0.71)$
VS Width/Body Width	$0.48-0.57 \ (0.53 \pm 0.03)$
Distance between OS and VS	531-1709 (1277 ± 397.60)
Anterior Testis (AT) Length	104-128 (115 ± 10.53)
AT Width	317-526 (426 ± 76.8)
AT Width/Length	$2.92\text{-}4.99~(3.74\pm0.87)$
Posterior Testis (PT) Length	114-190 (145 ± 30.57)
PT Width	254-444 (326 ± 74.81)
PT Width/Length	$1.62-3.03 \ (2.28 \pm 0.45)$
Distance between Testes	426-650 (494 ± 83.40)
Ovary Length	165-200 (178 ± 12.17)
Ovary Width	35-108 (75 ± 24.82)
Ovary Width/Length	0.20-0.60 (0.42 ± 0.14)
Cirrus Sac (CS) Length	437-652 (577 ± 76.76)
CS Width	112-223 (168 ± 39.14)
CS Length/Body Length	$0.06 - 0.075 \ (0.06 \pm 0.005)$

Table 2. Morphological data and line drawings of the genital complex of *Clinostomum brieni* n. comb. and in species described from the genus *Clinostomoides*.

	<i>Clinostomum</i> <i>brieni</i> present study	C. brieni Dollfus, 1950 (A)	C. brieni Mirzoeva, 1981 (A)	C. brieni Prudhoe, 1957	C. brieni Manter and Pritchard, 1969	C. brieni Fischthal and Thomas, 1970	C. brieni van Rensburg et al., 2013	C. dollfusi Agarwal, 1958	<i>C. chauhani</i> Pandey, 1970	<i>C. rai</i> Rai, 1970	C. meerutensis Pandey and Tyagi, 1986	<i>C. pandeyii</i> Singh and Sharma, 1994	C. baughi Pandey, 1998
	A start		R				5						
Locality	SA, DRC	BC (DRC)	Azerbaijan	BC (DRC)	Rwanda	Ghana	Botswana	Jabalpur (India)	Lucknow (India)	Mathura (India)	Meerut (India)	Meerut (India)	Lucknow (India)
Host	Clarias gariepinus, Clarias ngamensis	Ardea goliath	Ardea purpurea	Clarias lazera	Clarias sp.	Clarias senegalensis	C. gariepinus	Clarias sp., Heteropneustes sp.	Heteropneustes fossilis	Clarias batrachus	H. fossilis	H. fossilis	H. fossilis
Location	Body cavity	Esophagus	Esophagus	Gills	Gills	Pharyngeal region	Gill chamber/ branchial region		Body cavity and viscera	Intestine	Body surface	Body surface	Skin near operculum
BL	6762-10602	30700	17000	6000-9000	6198-10138	5980-9085		7800-9800	5470	7410	5670	8500-10200	5050-5850
BW	1248-1527	3700	4000	1250-1650	1443-1850	1075-1670		1220-1990	1200	1370	1350	1500-2300	1450-1500
OSL	172-256	<i>(</i> 7, 400	410	Ø 250 250	278	175-195		156-260	200	120	260	250-350	160-180
OSW	172-282	0 400	430	0 250-350	463	295-340		234-312	160	190	400	40-500	250-280
VSL	622-749	Ø 1660	710	600-650	574	475-675		589-754	440	1160	520	500-600	500-530
VSW	678-794	0 1000	980	700-750	822	590-895		650-780	260	780	510		450-500
ATL	104-128		510	60		75-150		130-150	20	86	70	350-440	90-100
ATW	317-526		1060	150		375-620		280-620	150	430	300	150-220	350-400
PTL	114-190		310			55-110		70-130	220	68	60	210-230	100-400
PTW	254-444		670			345-500		230-330	160	340	230	350-440	420
OL	165-200	1060	950	250		200-287		150-220	100	65	120	220-260	120-130
OW	35-108	540	330	50		92-144		70-130	40	170	110	110-130	90-100
CSL	437-652			300-500		380-600		390-620	390		200	500-600	180-200
CSW	112-223			120-150		105-260		100-180	50		70	200-300	90

Abbreviations: BL=Body Length, BW=Body, Oral Sucker Length=OSL, Oral Sucker Width=OSW, Ventral Sucker Length=VSL, Ventral Sucker Width=VSW, Anterior Testis Length=ATL, Anterior Testis Width=ATW, Posterior Testis Length=PTL, Posterior Testis Width=PTW, Ovary Length=OL, Ovary Width=OW, Cirrus Pouch Length=CPL, Cirrus Pouch Width=CPW, SA=South Africa, DRC=Democratic Republic of Congo, BC=Belgian Congo, (A)=adult.. The line drawings were based on figures in the original publications.