



Molecular data show *Clinostomoides* Dollfus, 1950 is a junior synonym of *Clinostomum* Leidy, 1856, with redescription of metacercariae of *Clinostomum brienii* n. comb. (Caffara, Monica; Locke, Sean A.; Halajian, Ali; Luus-Powell, Wilmien; Benini, Deborah; Tedesco, Perla; Kasembele, Gyrhaiss Kapepula; Fioravanti, Maria L. - In: PARASITOLOGY ISSN 0031-1820 - STAMPA - 146:6(2019), pp. 805-813. [10.1017/S0031182018002172]

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Published Version:

Availability:

This version is available at: <https://hdl.handle.net/11585/685346> since: 2020-11-03

Published:

DOI: <http://doi.org/10.1017/S0031182018002172>

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This is the final peer-reviewed accepted manuscript of:

Monica Caffara, Sean A. Locke, Ali Halajian, Wilmien J Luus-Powell, Deborah Benini, Perla Tedesco, Gyrhaiss Kapepula Kasembele, Maria L. Fioravanti 2019. 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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Journal:	<i>Parasitology</i>
Manuscript ID	PAR-2018-0318.R2
Manuscript Type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Caffara, Monica; University of Bologna, Veterinary Medical Sciences Locke, Sean; University of Puerto Rico, Department of Biology Halajian, Ali; University of Limpopo, Department of Biodiversity Luus-Powell, Wilmien; University of Limpopo, Department of Biodiversity Benini, Deborah; University of Bologna, Veterinary Medical Sciences Tedesco, Perla; University of Bologna, Veterinary Medical Sciences Kasembele, Gyrhaiss; Universite de Lubumbashi, Unité de recherché en Biodiversité et Exploitation durable des Zones Humides (BEZHU), Faculté des Sciences Agronomiques Fioravanti, Maria; University of Bologna, Dept. Veterinary Medical Sciences
Key Words:	Yellow grub, halzoun, molecular prospecting, hybridization, incomplete lineage sorting, DNA barcode, heron, catfish

SCHOLARONE™
Manuscripts

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.

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

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

3

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23

24 **SUMMARY**

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

41

42

43

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

46

47

48 KEY FINDINGS

49 - Genus *Clinostomoides* Dollfus, 1950 originally distinguished from other clinostomids by
50 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).

53 - Phylogenetic analysis of three markers (18S, ITS rDNA and CO1 mtDNA) shows
54 *Clinostomoides brieni* belongs within *Clinostomum* Leidy, 1856.

55 - Amended diagnosis for *Clinostomum* to accommodate inclusion of *C. brieni*.

56 - 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
69 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 *Clinostomoides* have been described in Central India, the first being *C.*
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 *Clinostomoides* are currently available for comparison. Athokpam *et al*

98 (2014) provided rDNA sequences from *C. brienii*, but without supporting morphological
99 information. Moreover, the identification of *C. brienii* by these authors was questioned by
100 Brioso-Aguilar *et al* (2018) because of the close relationship of its 28S sequence to data from
101 *Clinostomum*. It is also relevant that the material sequenced by Athokpam *et al* (2014) was
102 from *Heteropneustes* in Northeastern India, rather than the region or hosts (central and
103 southern Africa, *Clarias*, *Ardea*) where the genus originated and is better known.

104 The aim of this work was to provide a redescription of *Clinostomoides brienii* metacercariae
105 based on morphological and molecular analyses, following Matthews and Cribb (1998), and
106 to provide an updated critical analysis of previously described species. To this end, we
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
109 results led us to transfer *Clinostomoides brienii* to *Clinostomum* and to consider species of
110 *Clinostomoides* from India as *species inquirendae*.

111

112 MATERIALS AND METHODS

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

118 Total lengths of metacercariae were measured before cutting a small piece of the posterior end
119 for molecular analyses. Morphometrics of hologenophores (*sensu* Pleijel *et al.* 2008) were
120 taken after clarification with Amman's lactophenol and staining by Malzacher's method
121 (Pritchard and Kruse, 1982). Line drawings were made with the aid of a drawing tube, and
122 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 Moszczyńska *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× 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 Advance™ 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 *et al.* 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
151 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

155 Among five 18S rDNA sequences 1826-1877 bp in length obtained from African samples of
156 *C. brienii* in the present study, there were four variable sites, all transitions, i.e. mean
157 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. brienii* from *Heteropneustes fossilis* in Manipur differed at 32
160 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. brienii* sequences and the two Indian
163 isolates averaged 1.4% (range 0.5-2.6%). Phylogenetic analysis showed that 18S sequences
164 from Indian and African *C. brienii* form a well-supported clade nested within *Clinostomum*
165 species. The *Clinostomum* + *C. brienii* clade is also well supported, and comparatively deeply
166 divergent from *Euclinostomum*. Variation among 18S sequences of *Clinostomum* spp.
167 averaged 0.9% (range 0.2-1.5%) and in the *Clinostomum* + *C. brienii* clade, 18S variation
168 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. brienii* sequence KF811009. Variation between
170 *Euclinostomum* and members of the *Clinostomum* + *C. brienii* clade averaged 2.8% (range 2.4-
171 5.0%).

172 The five ITS rDNA sequences 1005-1028 bp in length from African *C. brienii* were identical
173 to each other and to the 300-bp ITS2 sequence of *C. brienii* (KF781298) of Athokpam *et al.*
174 (2014). The ITS of *C. brienii* varied by a mean of 5.7% (range 4.7-7.1%) from species of
175 *Clinostomum*. Variation in ITS among species of *Clinostomum s.s.* was of similar magnitude:
176 mean 4.9% (range 0.1-8.6%). In contrast, ITS variation among members of the genera
177 *Euclinostomum*, *Odhneriotrema*, and *Clinostomum* + *Clinostomoides* averaged 15.2% (range
178 13.9-16.3%).

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

194 Analyses of three molecular markers indicate *Clinostomoides* should be regarded as junior
195 synonym of *Clinostomum*, as amended below. Tree topologies (Figs. 1-3) show relatively
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

205 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
207 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

210 body. Ovary intertesticular, to right of medial line. Vitelline follicles in lateral fields

211 anteriorly, from level of intestinal bifurcation or ventral sucker to posterior extremity, may

212 remain lateral and extracaecal or become confluent posterior to genital complex. Uterus

213 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.

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

246 same regions (Hardman, 2005; Kushwaha *et al.* 2015; Froese and Pauly, 2018). The
247 phylogenetic association of *C. brienii* with a clade of Old-World *Clinostomum* species (Figs 1-
248 3) is consistent with a biogeographic pattern that continues to be observed as data accumulate
249 from more species of *Clinostomum* (Locke *et al.* 2015; Pérez-Ponce de León *et al.* 2016;
250 Rosser *et al.* 2018), which adds further evidence that *C. brienii* belongs to *Clinostomum*.

251 One specimen we collected was morphologically indistinguishable and shared identical rDNA
252 sequences with other *C. brienii*, but its CO1 (MH253045) was highly divergent, and nearly
253 identical to *Clinostomum* morphotype 3, which Caffara *et al.* (2017) obtained from mochokid
254 and amphiliid catfishes in South Africa. We believe this can be most plausibly explained by
255 hybridization. Both *C. brienii* and *Clinostomum* morphotype 3 infect siluriform second
256 intermediate hosts in the same region, which are preyed upon by local ardeid definitive hosts,
257 including the type host of *C. brienii*, *A. goliath* (Mock and Mock, 1980). Other than this
258 particular specimen, *C. brienii* and *Clinostomum* morphotype 3 appear to be distantly related
259 (Figs. 1, 2). This argues against another possible explanation for mitochondrial haplotype
260 sharing between *C. brienii* and *Clinostomum* morphotype 3, incomplete sorting among
261 recently separated species. In any event, when viewed together with the highly distinctive
262 morphology of *C. brienii*, both hybridization and incomplete lineage sorting suggest species
263 belonging to a single genus, which supports our main taxonomic conclusion.

264 The most distinctive characters of *Clinostomoides* were the size of the adult (30 mm)
265 described in *A. goliath* by Dollfus (1950), the extremely posterior position of the genital
266 complex, and the position of the cirrus sac and genital pore within the genital complex,
267 characters considered also in regional descriptions of metacercariae (Prudhoe, 1957; Manter
268 and Pritchard, 1969; Fischthal and Thomas, 1970; van Rensburg *et al.* 2013). The most
269 obvious distinction of *Clinostomoides*, its large size, is noteworthy in that the adult is known
270 only from the largest of ardeids, *A. goliath* (Mock and Mock, 1980). In light of the molecular

271 evidence that *Clinostomoides* belongs within *Clinostomum*, the other characters may be
272 considered as size-related allometric changes.

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

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

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

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

321 1.16 x 0.78 mm vs acetabulum at distance of 1.16 mm behind anterior end of body and 0.78
322 mm in diameter) and host tissues (muscle vs intestine) recorded by Rai (1970).

323 *Clinostomoides meerutensis* Pandey and Tyagi, 1986 and *C. pandeyii* Singh and Sharma,
324 1994, were both created for metacercariae from the body surface of *H. fossilis* in Meerut
325 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
329 above. In the descriptions of *C. meerutensis*, *C. pandeyii* and *C. baughi*, the ovary is
330 described as opposite to the cirrus sac. However, to our knowledge the ovary in the genus
331 *Clinostomoides* and/or *Clinostomum* is always on the same side as the cirrus pouch. Line
332 drawings in Pandey and Agrawal (2013) appear to show specimens of the latter three species
333 transposed, with the uteroduct in the right side of the body.

334 In our opinion, these species of *Clinostomoides* (*C. dollfusi*, *C. rai*, *C. chauhani*, *C.*
335 *meerutensis*, *C. pandeyii*, *C. baughi*) described from India are *species inquirendae* because the
336 morphological basis of each may be an artifact of development, beginning with the
337 comparison of larval *C. dollfusi* with adult *C. brienii* and cascading through later descriptions.
338 The characters considered in these studies vary with parasite development and may also be
339 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 brienii* to be valid. Essentially, this
343 view follows Manter and Pritchard (1969) and Fischthal and Thomas (1970). Further work
344 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.

346 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).

349 **ACKNOWLEDGEMENTS**

350 The authors are grateful for the support of Professor A. Chocha Manda (Université de
351 Lubumbashi, R.D. Congo) and for constructive suggestions made by two anonymous
352 reviewers.

353

354 **FINANCIAL SUPPORT**

355 Part of the research done in South Africa by AH was supported by the South African
356 Research Chairs Initiative of the Department of Science and Technology and National
357 Research Foundation (NRF) of South Africa (Grant No 101054). Any opinion, finding and
358 conclusion or recommendation expressed in this material is that of the author(s) and the NRF
359 does not accept any liability in this regard. SL was supported by the Puerto Rico Science
360 Technology & Research Trust. MC, DB, TP and MLF received no specific grant from any
361 funding agency, commercial or not-for-profit sectors.

362

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504

For Peer Review

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

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

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

522 Fig. 4. Line drawing of metacercaria of *Clinostomum brienii* n. comb. Scale bar = 1000 μ m.

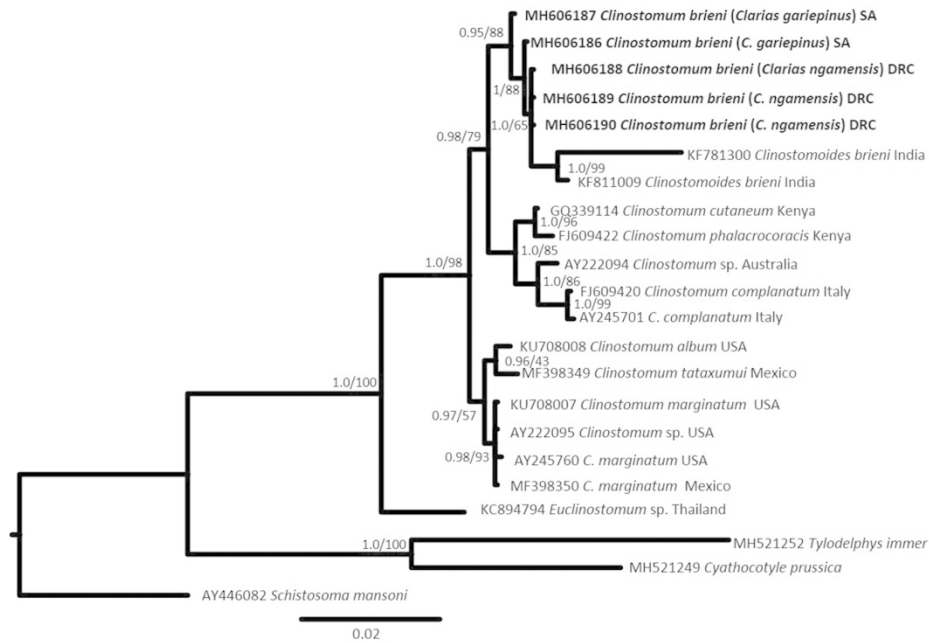


Fig. 1. Evolutionary history inferred using Bayesian Inference (nst=2+G) from 18S rDNA of *Clinostomum brienii* n. comb. generated in this study (in bold) with data from other studies (22 nucleotide sequences, 1663 positions). Nodes are labelled with posterior probability in Bayesian Inference analysis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

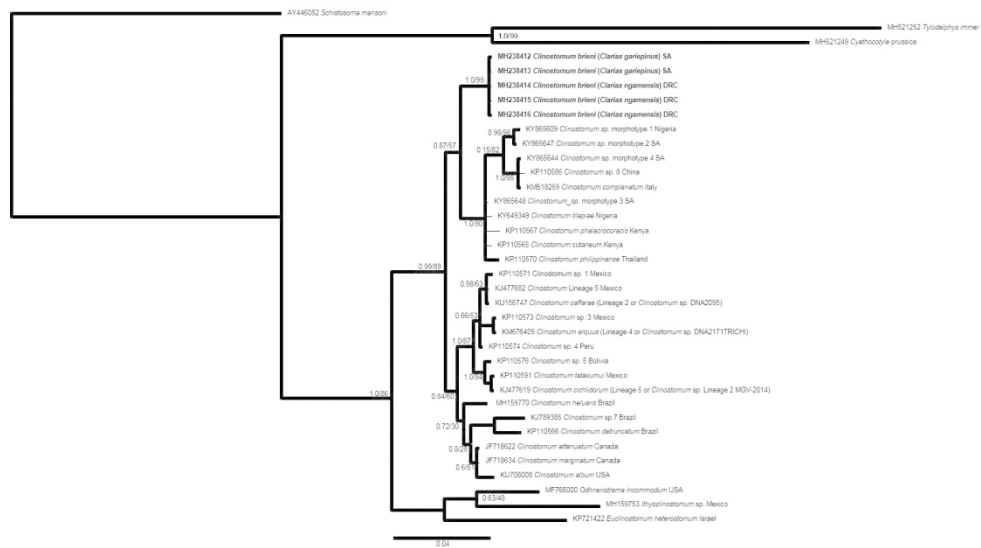


Fig. 2. Evolutionary history inferred using the Bayesian Inference analysis (nst=2+G) from ITS rDNA of *Clinostomum brieni* n. comb. generated in this study (in bold) with data from other studies (36 nucleotide sequences, 575 positions). Nodes are labelled posterior probability and, after the slash, 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)

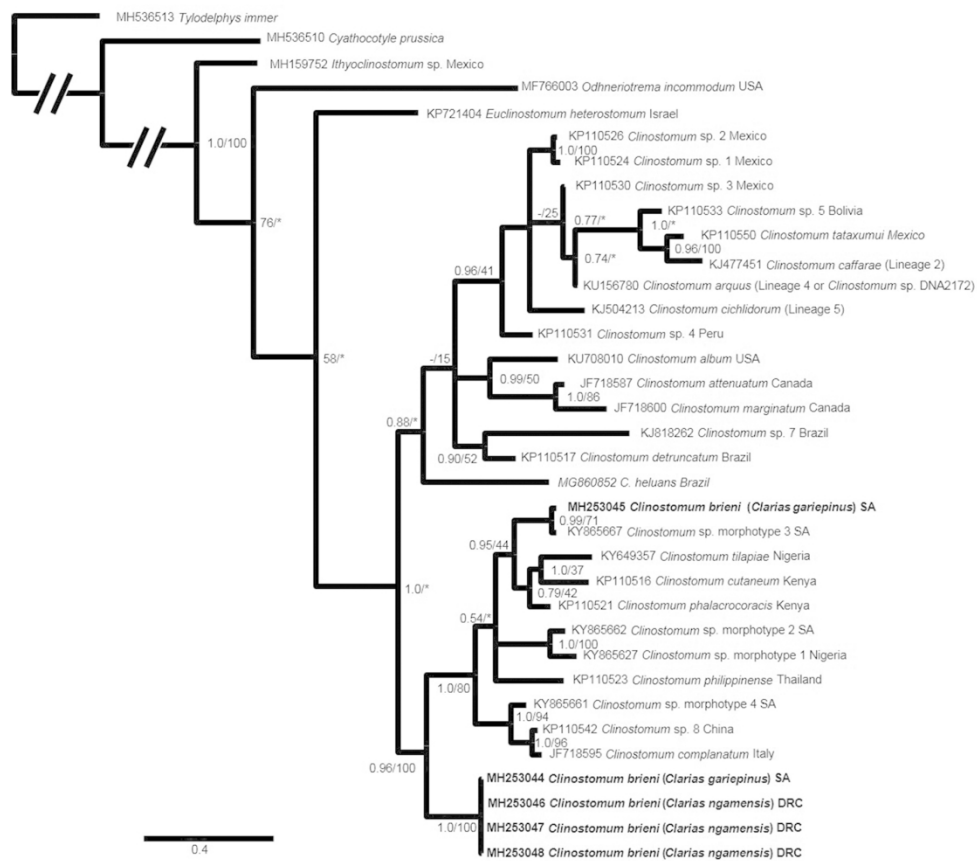


Fig. 3. Evolutionary history inferred in Bayesian Inference analysis (GTR+G+I) from CO1 mtDNA of *Clinostomum brienii* n. comb. generated in this study (in bold) with data from other studies (37 nucleotide sequences, 473 positions). Nodes are labelled with posterior probability and, after the slash, 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)

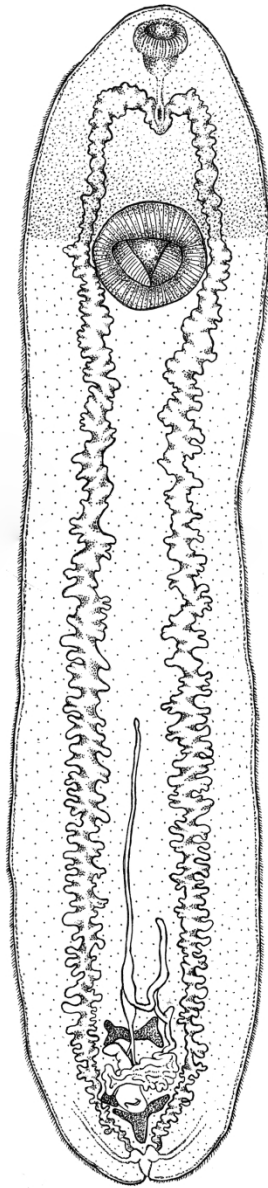







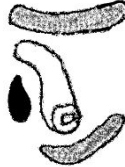





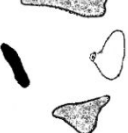
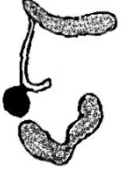
Fig. 4. Line drawing of metacercaria of *Clinostomum brieni* n. comb. Scale bar = 1000 μ m.

67x250mm (300 x 300 DPI)

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

Body Length	6762-10602 (8683 \pm 1425)
Body Width	1248-1527 (1392 \pm 118.3)
Body Length/Width	4.80-8.40 (6.27 \pm 1.17)
Oral Sucker (OS) Length	172-256 (218 \pm 37.91)
OS Width	172-282 (233 \pm 35.99)
OS Width/Body Width	0.11-0.19 (0.15 \pm 0.033)
Ventral Sucker (VS) Length	622-749 (676 \pm 54.08)
VS Width	678-794 (733 \pm 42.96)
VS Width/OS Width	2.91-4.61 (3.55 \pm 0.71)
VS Width/Body Width	0.48-0.57 (0.53 \pm 0.03)
Distance between OS and VS	531-1709 (1277 \pm 397.60)
Anterior Testis (AT) Length	104-128 (115 \pm 10.53)
AT Width	317-526 (426 \pm 76.8)
AT Width/Length	2.92-4.99 (3.74 \pm 0.87)
Posterior Testis (PT) Length	114-190 (145 \pm 30.57)
PT Width	254-444 (326 \pm 74.81)
PT Width/Length	1.62-3.03 (2.28 \pm 0.45)
Distance between Testes	426-650 (494 \pm 83.40)
Ovary Length	165-200 (178 \pm 12.17)
Ovary Width	35-108 (75 \pm 24.82)
Ovary Width/Length	0.20-0.60 (0.42 \pm 0.14)
Cirrus Sac (CS) Length	437-652 (577 \pm 76.76)
CS Width	112-223 (168 \pm 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 brieni</i> present study	<i>C. brieni</i> Dollfus, 1950 (A)	<i>C. brieni</i> Mirzoeva, 1981 (A)	<i>C. brieni</i> Prudhoe, 1957	<i>C. brieni</i> Manter and Pritchard, 1969	<i>C. brieni</i> Fischthal and Thomas, 1970	<i>C. brieni</i> van Rensburg <i>et al.</i> , 2013	<i>C. dollfusi</i> Agarwal, 1958	<i>C. chauhani</i> Pandey, 1970	<i>C. rai</i> Rai, 1970	<i>C. meerutensis</i> Pandey and Tyagi, 1986	<i>C. pandeyii</i> Singh and Sharma, 1994	<i>C. baughi</i> Pandey, 1998
													
Locality	SA, DRC	BC (DRC)	Azerbaijan	BC (DRC)	Rwanda	Ghana	Botswana	Jabalpur (India)	Lucknow (India)	Mathura (India)	Meerut (India)	Meerut (India)	Lucknow (India)
Host	<i>Clarias gariepinus</i> , <i>Clarias ngamensis</i>	<i>Ardea goliath</i>	<i>Ardea purpurea</i>	<i>Clarias lazera</i>	<i>Clarias</i> sp.	<i>Clarias senegalensis</i>	<i>C. gariepinus</i>	<i>Clarias</i> sp., <i>Heteropneustes</i> sp.	<i>Heteropneustes fossilis</i>	<i>Clarias batrachus</i>	<i>H. fossilis</i>	<i>H. fossilis</i>	<i>H. fossilis</i>
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		410	Ø 250-350	278	175-195		156-260	200	120	260	250-350	160-180
OSW	172-282	Ø 400	430		463	295-340		234-312	160	190	400	40-500	250-280
VSL	622-749		710	600-650	574	475-675		589-754	440	1160	520	500-600	500-530
VSW	678-794	Ø 1660	980	700-750	822	590-895		650-780	260	780	510	450-500	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.