# A morphological and molecular study of Clinostomid metacercariae from African fish with a redescription of *Clinostomum tilapiae*

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SUMMARY

The genus *Clinostomum* Leidy, 1856 (Digenea: Clinostomidae) has been reported in all ecozones of the world and a clear separation between the species of the 'Old World' and 'New World' has been recognized based on molecular studies. Recent works on Afrotropical species include redescriptions of *C. cutaneum* and *C. phalacrocoracis*, while *C. tilapiae* has yet to be studied using modern taxonomic approaches. In the present research, morphological redescription of *C. tilapiae* metacercariae from a new host, *Synodontis batensoda* sampled at Anambra River Basin, Nigeria, together with molecular analysis of nuclear internal transcribed spacer rDNA and cytochrome *c* oxidase 1 mtDNA are reported. We also provide morphological and molecular data from four further putative species of *Clinostomum* (morphotypes 1–4) from different areas of Africa, as well as the first report of *C. phalacrocoracis* in South Africa.

Key words: species delineation, cryptic diversity, helminths, parasites, molecular prospecting, freshwater fish, larvae, DNA barcode.

## INTRODUCTION

The genus *Clinostomum* Leidy, 1856 (Digenea: Clinostomidae) has been reported in all ecozones of the world and a clear separation between the species of the 'Old World' and 'New World' has been recognized based on molecular studies (Caffara *et al*. 2011; Locke *et al*. 2015*b*; Pérez-Ponce de León *et al*. 2016; Rosser *et al*. 2017). As stated by several authors (Matthews and Cribb, 1998; Gustinelli *et al*. 2010; Caffara *et al*. 2011; Sereno-Uribe *et al*. 2013; Rosser *et al*. 2017), the taxonomy of this genus remains in need of revision with morphological and molecular data. Ukoli (1966) recognized 13 species, of which seven have been supported in studies using both molecular and morphological approaches, namely *C. complanatum* Rudolphi, 1814, *C. cutaneum* Paperna, 1964, *C. phalacrocoracis* Dubois, 1930, *C. marginatum* Rudolphi, 1819, *C. attenuatum* Cort, 1913, *C. detruncatum* Braun, 1899 *and C. philippinensis* Velazquez, 1959 (Gustinelli *et al*. 2010; Caffara *et al*. 2011, 2014*b*; Locke *et al*. 2015*b*; Acosta *et al*. 2016), while two more *C. tataxumui* Sereno-Uribe *et al*. 2013 and *C. album* Rosser *et al*. 2017, have recently been described (Sereno-Uribe *et al*. 2013; Rosser *et al*. 2017).

Since the 1930s, five *Clinostomum* spp. have been described or reported from the African continent, but few studies include complete morphological description (Dubois, 1930; Ukoli, 1966), and reports of unidentified metacercariae of *Clinostomum* are numerous (see Table 1). Recent work on Afrotropical species includes redescriptions of *C. cutaneum* (Gustinelli *et al*. 2010) and *C. phalacrocoracis* (Caffara *et al*. 2014*b*). However, *Clinostomum tilapiae* has yet to be studied using the molecular and morphological methods of Matthews and Cribb (1998) and later authors. This species was erected by Ukoli (1966), who described metacercariae encysted in the branchial region and eye sockets of naturally infected *Tilapia* spp. (Perciformes: Cichlidae) sampled in Ghana, as well as adults from experimentally infected cattle egret (*Bubulcus ibis*). *Clinostomum tilapiae* was subsequently reported again from *Tilapia* spp. in the type locality in Ghana (Fischthal and Thomas, 1970), as well as from the oesophagus and crop of *Ardea goliath* in Congo (Manter and Pritchard, 1969). Fischthal and Thomas (1970) also considered that unidentified metacercariae described and drawn by Williams and

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Table 1. Reports of *Clinostomum* spp. from Africa

| Country | Species | Host | Reference |
|---|---|---|---|
| Angola | *C. phalacrocoracis* | *Phalacrocorax levaillanti* | Dubois (1930) |
| Benin | *Clinostomum* sp. | *Synodontis schall*, *S. nigrita* | Dougnon *et al.* (2012) |
| Botswana | *Clinostomum* sp. | *Clarias gariepinus* | Jansen van Rensburg *et al.* (2003) |
| Burkina Faso | *Clinostomum* sp. | *Oreochromis niloticus*, *Sarotherodon galilaeus*, *Hemicromis fasciatus* | Coulibaly *et al.* (1995) |
| Congo | *C. tilapiae*. | *Ardea goliath* | Manter and Pritchard (1969) |
| | *Clinostomum* sp. | *A. goliath* | Dollfus (1950) |
| Ivory Coast | *Clinostomum* sp. | *Tilapia melanopleura* | Dollfus (1950) |
| Egypt | *Clinostomum* sp. | *O. niloticus* | Marwan and Mohammed (2003) |
| | *C. tilapiae* | *O. niloticus* | Taher (2009) |
| Ethiopia | *Clinostomum* sp. | *O. niloticus*, *Tilapia zilli*, *C. gariepinus; Varincorhinus beso* | Yimer (2000); Yimer and Enyew (2003) |
| Gabon | *Clinostomum* sp. | *V. beso* | Manter and Pritchard (1969) |
| Ghana | *C. tilapiae* | *T. zilli*, *T. heudeloti*, *T. galilaea* | Ukoli (1966); Fischthal and Thomas (1970) |
| | *C. phalacrocoracis* | *Anhinga rufa rufa* | Ukoli (1966) |
| Kenya | *Clinostomum* sp. | *O. leucostictus* | Aloo (2002) |
| | *C. tilapiae* | *O. niloticus* | Ochieng *et al.* (2012) |
| Mozambique | *C. tilapiae* | *Cyprinus carpio* | Boane *et al.* (2008) |
| Nigeria | *C. tilapiae* | *Chromidotilapia guntheri*, *T. mariae* *T. zilli*, *Hemichromis fasciatus*, *O. niloticus* | Okaka and Akhigbe (1999); Agbede *et al.* (2004); Olurin and Somorin (2006); Olurin *et al.* (2012); Echi *et al.* (2012); Okoye *et al.* (2014) |
| | *Clinostomum* sp | *Synodontis eupterus*, *Ctenopoma kingsleye*, *T. zilli*, *Citharinus citharius*, *Lebeo cubie* | Okaka and Akhigbe (1999); Ayotunde *et al.* (2007); Onyedineke *et al.* (2010); Okoye *et al.* (2014) |
| Ruanda | *C. macrosomum* | *T. nilotica* | Manter and Pritchard (1969) |
| Sierra Leone | *Clinostomum* sp. | *Epiplatys* sp. | Williams and Chaytor (1966) |
| South Africa | *C. tilapiae* | *O. mossambicus* | Britz *et al.* (1984, 1985) |
| | *C. van der horsti* | *Gnathonemus macrolepidotus*, *A. melanocephale* | Ortlepp (1935); Lombard (1968) |
| | *Clinostomum* sp. | *O. mossambicus*, *Schilbe intermedius*, *C. gariepinus*, *T. mossambica*, *Chiloglanis pretoriae*, *Amphilius uranoscopus* | Lombard (1968); Ramollo *et al.* (2006); Madanire-Moyo *et al.* (2012); Smit and Luus-Powell (2012); Luus-Powell *et al.* (2015) |
| Sudan | *Clinostomum* sp. | *Tilapia* sp. | Paperna (1980) |
| Uganda | *Clinostomum* sp. | *Haplochromis obliquindes* | Khalil and Thurston (1973) |
| Zaire | *Clinostomum* sp. | *Oreochromis* sp. | Kabunda and Sommerville (1984) |
| Zambia | *C. complanatum* | Cichlids | Batra (1984) |
| Zimbabwe | *C. complanatum* | *C. gariepinus* | Barson *et al.* (2008) |

Chaytor (1966), from *Epiplatys* spp. (Cyprinodontiformes: Aplocheilidae) from Sierra Leone, belonged to *C. tilapiae*. Later Britz *et al.* (1984) described adults of *C. tilapiae* obtained from experimentally infected *A. cinerea*, in Transvaal (South Africa), and in 1985, the same authors reported *C. tilapiae* encysted in the gills of *Oreochromis mossambicus* (Cichlidae). Finkelman (1988) described *C. tilapiae* from *Pelecanus onocrotalus* (Aves: Pelicanidae) and *Sarotherodon galilaeus* (Cichlidae) and *O. aureus* (Cichlidae) sampled in Lake Kinneret, Israel, with a full description of all developmental stages. A number of other studies have reported *Clinostomum* spp. or *C. tilapiae* from various African localities without morphological data (Table 1).

In the present study, we redescribe the metacercaria of *C. tilapiae* from a new host, *Synodontis batensoda* (Siluriformes: Mochokidae), sampled at Anambra River Basin, Nigeria, supporting this with sequences of nuclear internal transcribed spacer (ITS) DNA and cytochrome *c* oxidase 1 (COI) from the mitochondrion. We compare the morphological and molecular data to other *Clinostomum* spp. metacercariae collected in the same sampling site and other areas/hosts in South Africa.

## MATERIALS AND METHODS

Twenty-six metacercariae of *Clinostomum* spp., of which eight were *C. tilapiae*, were removed from fresh skin tissue of three *S. batensoda* collected in the Anambra River Basin, Nigeria, and 33 were taken from the abdominal cavity or gill chambers of fishes sampled in different areas of Limpopo and Mpumalanga provinces, South Africa, and the Bubiana River, Zimbabwe, namely *Barbus trimaculatus* and *B. unitaeniatus* (Cypriniformes: Cyprinidae);

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*Marcusenius macrolepidotus* and *M. pongolensis* (Osteoglossiformes: Mormyridae); *O. mossambicus* (Perciformes: Cichlidae), *Amphilius uranoscopus* (Siluriformes: Amphiliidae), *Clarias gariepinus* (Siluriformes: Clariidae), *Chiloglanis pretoriae* (Siluriformes: Mochokidae) and *Schilbe intermedius* (Siluriformes: Schilbeidae). The parasites were excysted, washed in saline and preserved in 70% ethanol for morphological and molecular analysis. Morphometrics 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). In 40 of these specimens, morphometric variation was visualized with principal components analysis (PCA) of 14 morphometrics normalized to range from −1 to 1. The ordination was overlaid with a minimum spanning tree (MST) based on Euclidean distances among the specimens.

The posterior end of 59 specimens was removed for DNA extraction using a PureLink Genomic DNA Kit (Invitrogen) following the manufacturer's protocol. Amplification of ITS rDNA employed protocols and primers of Gustinelli *et al*. (2010); COI mtDNA those of Moszczynska *et al*. (2009). Amplified products were resolved on a 1% agarose gel stained with SYBR Safe DNA Gel Stain in 0·5× TBE (Molecular Probes – Life Technologies). For sequencing of both ITS and COI, bands were excised and purified by NucleoSpin Gel and PCR Clean-up (Mackerey-Nagel) and sequenced with an ABI 3730 DNA analyser at StarSEQ GmbH (Mainz, Germany). Contigs were assembled with Vector NTI AdvanceTM 11 software (Invitrogen) and sequences published in GenBank (COI: KJ786967-74, KY649357-64, KY865627-43, KY865661-81, KY906227-38; ITS: KJ786975-82, KY649349-56, KY865609-26, KY865644-60).

Three alignments were generated using default parameters with MAFFT (Katoh *et al*. 2002) including a subset of previously published data from clinostomids (Supplementary Table S1). One consisted of COI sequences, one of ITS sequences, and one was based on a concatenated subset of these COI and ITS sequences. Pairwise distances and models of nucleotide evolution were calculated using MEGA (Tamura *et al*. 2013). Bayesian inference (Ronquist *et al*. 2012) was used to construct evolutionary trees in MrBayes. The Bayesian Information Criterion indicated the Kimura-2-parameter model with gamma-distributed rates of variation to be the best for nucleotide evolution in ITS sequences (in MrBayes, nst = 2), and a binary model was used to model evolution of gaps in the ITS alignment (lset coding = variable) that were coded using FastGap (Borchsenius, 2009). For the three codon positions of COI, the best models (and corresponding settings in MrBayes) were Tamura–Nei 93 + Gamma (nst = 6 rates = gamma), Hasegawa–Kishino–Yano (nst = 2) and Tamura-Nei 93 +G+I (nst = 6 rates = invgamma) with two random perturbations to starting trees and four search chains sampled every 100 generations, with the initial 25% of trees discarded.

## RESULTS

### *Morphological description*

*Clinostomum tilapiae* Ukoli, 1966 (Fig. 1, Table 2) eight specimens, Anambra River Basin, Nigeria.

Body stout, widest in gonadic region. Oral sucker small, surrounded by oral collar (not always visible). Pharynx small, opening into pharyngeal bulb. Ventral sucker larger than oral sucker. Intestinal caeca provided with small lateral pouches lateral to ventral sucker, run to posterior end of body. Testes strongly digitated. Anterior testis in middle third of body, irregularly lobed, slightly displaced to left. Posterior testis in posterior part of middle third of body with posterior lobe protruding in anterior part of posterior third, symmetrical, triangular, with two lateral and one posterior lobes, each subdivided into smaller lobes. Cirrus pouch oval with slight cleft, in intertesticular space on right, between left margin of anterior testis and right caecum. Genital pore position medial to cirrus sac, close to right anterior margin of anterior testis. Ovary small, ovoid, not median, in intertesticular space dextrally alongside cirrus pouch. Uterus runs straight from ventral sucker to anterior testis. Uteroduct runs around left margin of anterior testis, forming knee-like folding before opening into uterine sac close to anterior testis. Metraterm muscular and connecting uterus to genital atrium. Tegument armed by numerous spines.

Morphological study of metacercariae other than *C. tilapiae* allowed recognition of four morphotypes, distinguished based on the structure of the genital complex and DNA sequences, as described below.

*Clinostomum* sp. morphotype 1 (Table 2): 18 metacercariae from Nigeria (*S. batensoda*) and six from South Africa (*S. intermedius*). Genital complex between middle and posterior third of body. Cirrus pouch bean-shaped near right margin of anterior testis, overlapping it. Testes slightly lobed. Posterior testis more digitated. Ovary at left side of cirrus pouch. Caeca clearly digitated. Tegument completely covered with minute spines.

*Clinostomum* sp. morphotype 2 (Table 2): Five metacercariae from South Africa (four from *M. macrolepidotus* and one from *M. pongolensis*). Genital complex between middle and posterior third; cirrus pouch reniform, at right lateral side of anterior testis. Testes strongly digitated. Ovary posterior to cirrus pouch. Caeca slightly digitated. Tegument completely covered with thin papillae.

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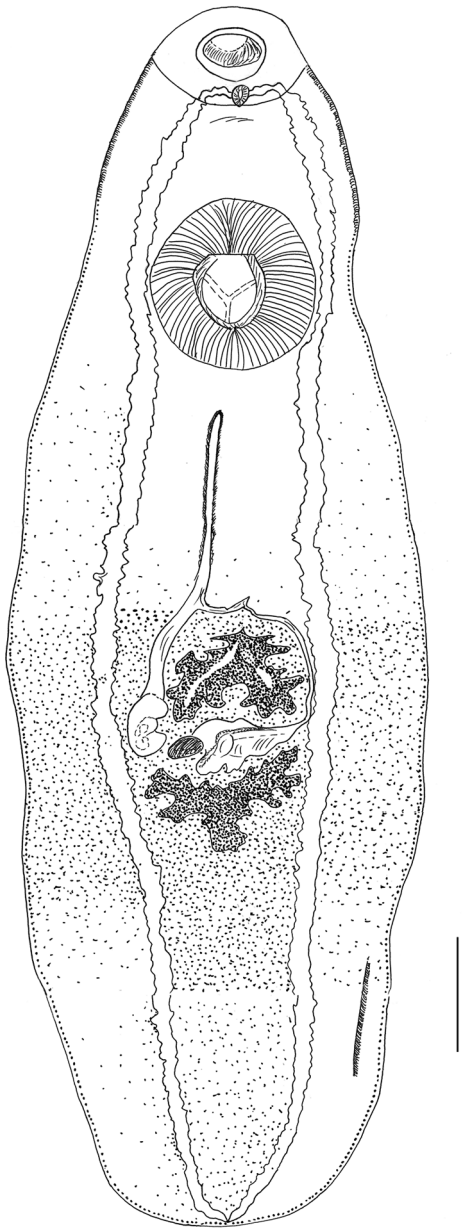

Fig. 1. Line drawing of *Clinostomum tilapiae* metacercaria. Scale bar = 300 $\mu$m.

*Clinostomum* sp. morphotype 3 (Table 2): Nine metacercariae from South Africa (seven from *A. uranoscopus* and two from *C. pretoriae*). Genital complex in middle third of body. Cirrus pouch oval, at right margin of anterior testis. Testes lobed. Ovary in intertesticular space at left side of cirrus pouch. Caeca digitated. Tegument completely covered with minute spines and papillae.

*Clinostomum* sp. morphotype 4 (Table 2): One metacercariae from South Africa (*B. trimaculatus*). Genital complex between middle and posterior third of body. Cirrus pouch reniform, close to right posterior margin of anterior testis. Testes strongly digitated. Ovary posterior to cirrus pouch. Caeca strongly digitated. Tegument completely covered with minute spines.

The first two axes of PCA explained 64% of variation among 14 measurements in 40 specimens, but yielded incomplete separation among *C. tilapiae* and the four morphotypes (Fig. 2). The strongest distinction was between morphotypes 2 and 3, in which there was little overlap along both axes. Morphotype 2 was also mostly distinct from *C. tilapiae* along PC1. The MST indicated morphometrically similar specimens were usually close together in the ordination, with some exceptions. Notably, specimens of *Clinostomum* morphotype sp. 2 were always joined by the MST, including the individual placed next to *C. tilapiae* and *Clinostomum* morphotype sp. 3 in the ordination. In other words, even the small overlap along PC1 between morphotype 2 *vs* morphotype 3 and *C. tilapiae* was a result of distortion in the ordination. This first axis, PC1, explained 49·5% of morphometric variation, and was defined by – 0·326 (body length) – 0·316 (posterior testis width) – 0·314 (ventral sucker length) – 0·311 (distance between suckers) – 0·305 (ventral sucker width) – 0·289 (body width) – 0·278 (oral sucker width) – 0·261 (anterior testis length) – 0·256 (anterior testis width) – 0·251 (posterior testis length) – 0·245 (distance between testes) – 0·242 (oral sucker length) – 0·149 (cirrus sac length) – 0·086 (cirrus sac width). The results of the analysis were essentially the same if an outlier (a specimen in morphotype 1, see square at the top of Fig. 2) was excluded.

### *Molecular results*

In both ITS rDNA and COI, intraspecific divergences generally did not exceed interspecific divergences (Table 3), except for *Clinostomum* morphotypes 1 and 3, for which variation in ITS overlapped within and between species. Table 3 summarizes genetic distances among species in the present study as well as representatives of major clades, species and putative species in prior studies. BLAST searches of public data on GenBank yielded essentially the same results. For example, ITS rDNA sequences from eight *C. tilapiae* differ by >1% and COI mtDNA were >10% different from other published sequences from *Clinostomum*.

In phylogenetic analysis of ITS, *Clinostomum* morphotypes 1 and 2 clustered with *C. complanatum* and morphotype 4 in a well-supported clade, and *Clinostomum* morphotype 1 was paraphyletic with respect to morphotype 2 (Fig. 3A). This clade was in turn nested within a clade including *C. tilapiae*, morphotype 3, *C. cutaneum* and *C. phalacrocoracis*, among which relationships were unresolved. In the latter, more basal and inclusive clade, *Clinostomum* morphotype 3 was paraphyletic or unresolved with respect to other clade members. The COI sequences of *C. tilapiae* and all morphotypes formed well-supported monophyletic groups (Fig. 3B), and this

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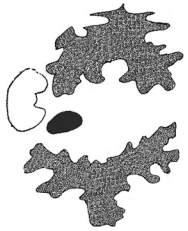
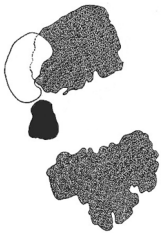
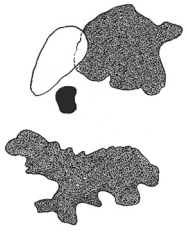
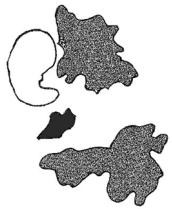
Table 2. Morphological data and line drawings of the genital complex of *C. tilapiae* and the four *Clinostomum* sp. morphotypes (1–4) [Min–Max (Mean ± S.D.)] described in this study

| | *Clinostomum tilapiae* (eight specimens) | *Clinostomum* sp. morphotype 1 (24 specimens) | *Clinostomum* sp. morphotype 2 (five specimens) | *Clinostomum* sp. morphotype 3 (nine specimens) | *Clinostomum* sp. morphotype 4 (one specimen) |
|---|---|---|---|---|---|
| |  |  |  |  |  |
| BL | 2495–7339 (5651 ± 1575) | 4649–8804 (6176 ± 1152·12) | 6385–7546 (7064 ± 514) | 4116–8847 (6895 ± 1761) | 3666 |
| BW | 1488–2237 (1961 ± 268·3) | 1290–3546 (2083 ± 554·40) | 2130–2672 (2356 ± 216) | 1478–2201 (1875 ± 244) | 1176 |
| BL/BW | 1·49–3·28 (2·84 ± 0·572) | 2·06–4·20 (3·05 ± 0·48) | 2·79–3·53 (3·01 ± 0·30) | 2·78–4·62 (3·65 ± 0·70) | 3·11 |
| OSL | 142–385 (301 ± 76·76) | 169–599 (317 ± 118·81) | 384–565 (453 ± 75) | 195–431 (340 ± 75) | 256 |
| OSW | 171–509 (381 ± 95·77) | 286–796 (500 ± 116) | 626–757 (665 ± 53) | 333–547 (460 ± 81) | 287 |
| OSW/BW | 0·10–0·34 (0·20 ± 0·06) | 0·14–0·36 (0·25 ± 0·05) | 0·24–0·36 (0·29 ± 0·04) | 0·17–0·31 (0·25 ± 0·04) | 0·24 |
| VSL | 360–1145 (881 ± 239) | 693–1058 (913 ± 94·21) | 929–982 (951 ± 23) | 722–1046 (911 ± 118) | 773 |
| VSW | 360–1148 (870 ± 246) | 697–1125 (921 ± 121·34) | 935–1073 (995 ± 50) | 659–1138 (950 ± 174) | 811 |
| VSW/OSW | 1·40–2·69 (2·3 ± 0·41) | 1·34–2·74 (1·90 ± 0·42) | 1·31–1·70 (1·50 ± 0·14) | 1·52–2·93 (2·11 ± 0·47) | 2·82 |
| VSW/BW | 0·21–0·52 (0·44 ± 0·098) | 0·31–0·70 (0·46 ± 0·10) | 0·37–0·47 (0·42 ± 0·04) | 0·45–0·61 (0·50 ± 0·05) | 0·68 |
| DBS | 240–1641 (1244 ± 444·33) | 916–2085 (1312 ± 324·16) | 1125–1329 (1234 ± 74) | 991–1441 (1179 ± 190) | 719 |
| ATL | 195–537 (432 ± 118·47) | 349–870 (535 ± 130·87) | 610–790 (666 ± 78) | 248–436 (355 ± 67) | 447 |
| ATW | 234–734 (537 ± 148·01) | 263–865 (609 ± 131·32) | 500–685 (608 ± 67) | 286–760 (470 ± 145) | 489 |
| ATW/ATL | 0·97–1·54 (1·26 ± 0·22) | 0·60–1·69 (1·17 ± 0·25) | 0·79–1·02 (0·92 ± 0·10) | 1·13–1·74 (1·31 ± 0·22) | 1·09 |
| PTL | 220–583 (427 ± 118·53) | 214–857 (560 ± 159·08) | 410–643 (546 ± 92) | 207–512 (382 ± 98) | 305 |
| PTW | 215–836 (604 ± 186·34) | 459–985 (718 ± 145·09) | 519–910 (761 ± 149) | 451–764 (617 ± 116) | 534 |
| PTW/PTL | 0·97–1·76 (1·40 ± 0·26) | 0·37–1·21 (0·78 ± 0·19) | 1·25–1·58 (1·39 ± 0·16) | 1·26–2·18 (1·68 ± 0·35) | 1·75 |
| DBT | 246–628 (502 ± 131·37) | 361–874 (609 ± 131·79) | 380–795 (647 ± 157) | 316–515 (420 ± 67) | 155 |
| OL | 132–212 (170 ± 31·09) | 142–417 (249 ± 70·23) | 157–245 (196 ± 37) | 104–164 (126 ± 20) | – |
| OW | 77–124 (105 ± 17·91) | 161–480 (254 ± 71·98) | 120–206 (161 ± 35) | 78–180 (114 ± 37) | – |
| OW/OL | 0·50–0·86 (0·63 ± 0·12) | 0·50–1·98 (1·09 ± 0·39) | 0·64–1·12 (0·84 ± 0·20) | 0·65–1·40 (0·91 ± 0·28) | – |
| CPL | 245–356 (322 ± 37·98) | 317–923 (487 ± 122·03) | 393–492 (457 ± 38) | 236–395 (322 ± 57) | 374 |
| CPW | 141–334 (214 ± 58·87) | 172–598 (246 ± 85·39 | 202–321 (243 ± 48) | 163–215 (184 ± 20) | 193 |
| CPL/BL | 0·04–0·09 (0·06 ± 0·01) | 0·04–0·15 (0·08 ± 0·02) | 0·06–0·07 (0·06 ± 0·01) | 0·03–0·06 (0·05 ± 0·01) | 0·10 |

BL = Body Length, BW = Body Width, Body length/width = BL/BW, Oral Sucker length = OSL, Oral Sucker Width = OSW, OS width/body width = OSW/OSW, Ventral Sucker length = VSL, Ventral Sucker width = VSW, VS width/OS width = VSW/OSW, VS width/body width = VSW/BW, Distance between Sucker = DBS, Anterior Testis Length = ATL, Anterior Testis Width = ATW, AT width/length = ATW/ATL, Posterior Testis Length = PTL, Posterior Testis Width = PTW, PT width/length = PTW/PTL, Distance between Testes = DBT, Ovary Length = OL, Ovary Width = OW, Ovary width/length = OW/OL, Cirrus Pouch Length = CPL, Cirrus Pouch Width = CPW, CP length/Body length = CPL/BL.

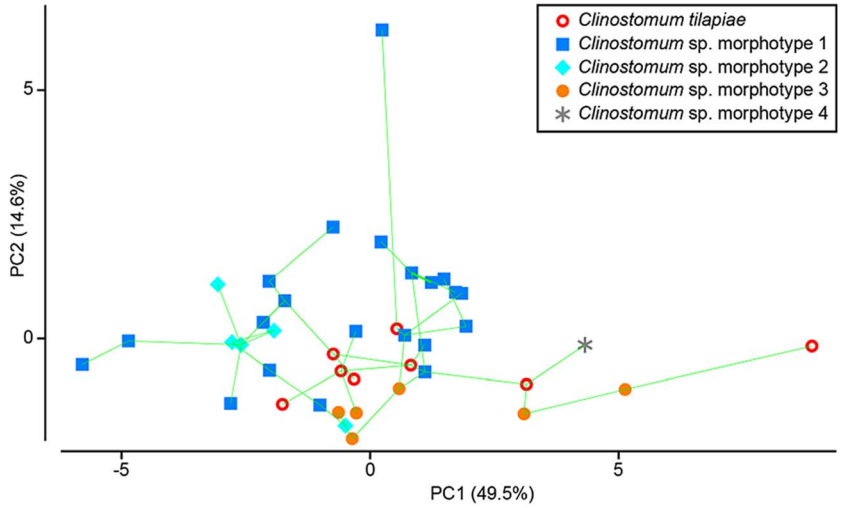



Fig. 2. PCA. Principal components analysis of 14 morphometrics from 40 metacercariae of Clinostomum spp. from Nigeria and South Africa (body length and width, oral and ventral sucker lengths and widths, distance between suckers, lengths and widths and distance between testes, and cirrus sac length and width). As indicated by the axis labels, the first principal component explained 49·5% of morphometric variation, the second, 14·6%. The MST is based on Euclidean distances among the 14 normalized morphometrics.

Table 3. Mean uncorrected p-distances (range) (%) within and among *Clinostomum tilapiae*, *C. phalacrocoracis* and four putative species collected in Nigeria and South Africa, including comparisons with data from other studies (79 ITS and 93 COI sequences; GB accessions in legends of Fig. 3)

| | Internal transcribed spacer | | | Cytochrome *c* oxidase 1 | | |
|---|---|---|---|---|---|---|
| | *n* | Intraspecific | Interspecific | *n* | Intraspecific | Interspecific |
| *Clinostomum* morphotype 1 | 23 | 0·07 (0–0·51) | 3·3 (0·11–8·92) | 23 | 0·52 (0–1·49) | 14·63 (7·06–21·44) |
| *Clinostomum* morphotype 2 | 3 | 0 (0–0) | 2·55 (0·11–8·79) | 5 | 0 (0–0) | 12·68 (7·06–21·84) |
| *Clinostomum* morphotype 3 | 8 | 0·03 (0–0·12) | 2·39 (0·12–7·98) | 9 | 0·16 (0–0·33) | 13·63 (10·43–20·03) |
| *Clinostomum* morphotype 4 | 1 | | 2·97 (0·2–8·34) | 1 | | 13·43 (6·01–18·57) |
| *C. tilapiae* | 8 | 0·17 (0–0·61) | 2·48 (0·12–8·02) | 8 | 0·27 (0–0·66) | 14·56 (9·97–20·66) |
| *C. phalacrocoracis* | 11 | 0 (0–0) | 2·9 (0·63–7·81) | 23 | 0·26 (0–0·87) | 13·36 (8·72–20·17) |

also occurred in analysis of concatenated markers (Fig. 3C). In addition, COI sequences of 12 South African specimens from *O. mossambicus* identified as *C. phalacrocoracis* based on morphology grouped with previously published data from this species (Fig. 3B). In all three phylogenetic analyses, the species of the New World and Old World fell into separate, strongly supported clades.

## DISCUSSION

Three *Clinostomum* species have been validated in Palearctic/Afrotropic areas using a combined morphological and molecular approach (Nolan and Cribb, 2005), namely *C. complanatum*, *C. cutaneum* and *C. phalacrocoracis* (Gustinelli *et al.* 2010; Caffara *et al.* 2011, 2014*b*). Here, based on metacercariae collected in Nigeria, we add *C. tilapiae* to the list of Old World species supported by this approach. We also provide morphological and molecular data from four unidentified species of *Clinostomum* from Africa, as well as the first report of *C. phalacrocoracis* in South Africa.

The specimens we here identify as *C. tilapiae* are similar to those described by Ukoli (1966), except that our specimens are slightly bigger. In *C. tilapiae*, the genital complex is in the posterior portion of the middle third of the body, with the posterior lobe of the posterior testis extending into the posterior third of body, while in *C. cutaneum* (Gustinelli *et al.* 2010) and *Clinostomum* sp. morphotype 3 it is entirely in the middle third, and in *C. phalacrocoracis* (Caffara *et al.* 2014*b*), *C. complanatum* (Caffara *et al.* 2011) and *Clinostomum* sp. morphotypes 1 and 2, it is between the middle and posterior third of the body. The anterior testis of *C. tilapiae* is irregularly lobed, and asymmetric to the longitudinal axis of the body, while the posterior testis has two main lateral lobes and one posterior lobe, almost completely filling the intracecal space. In

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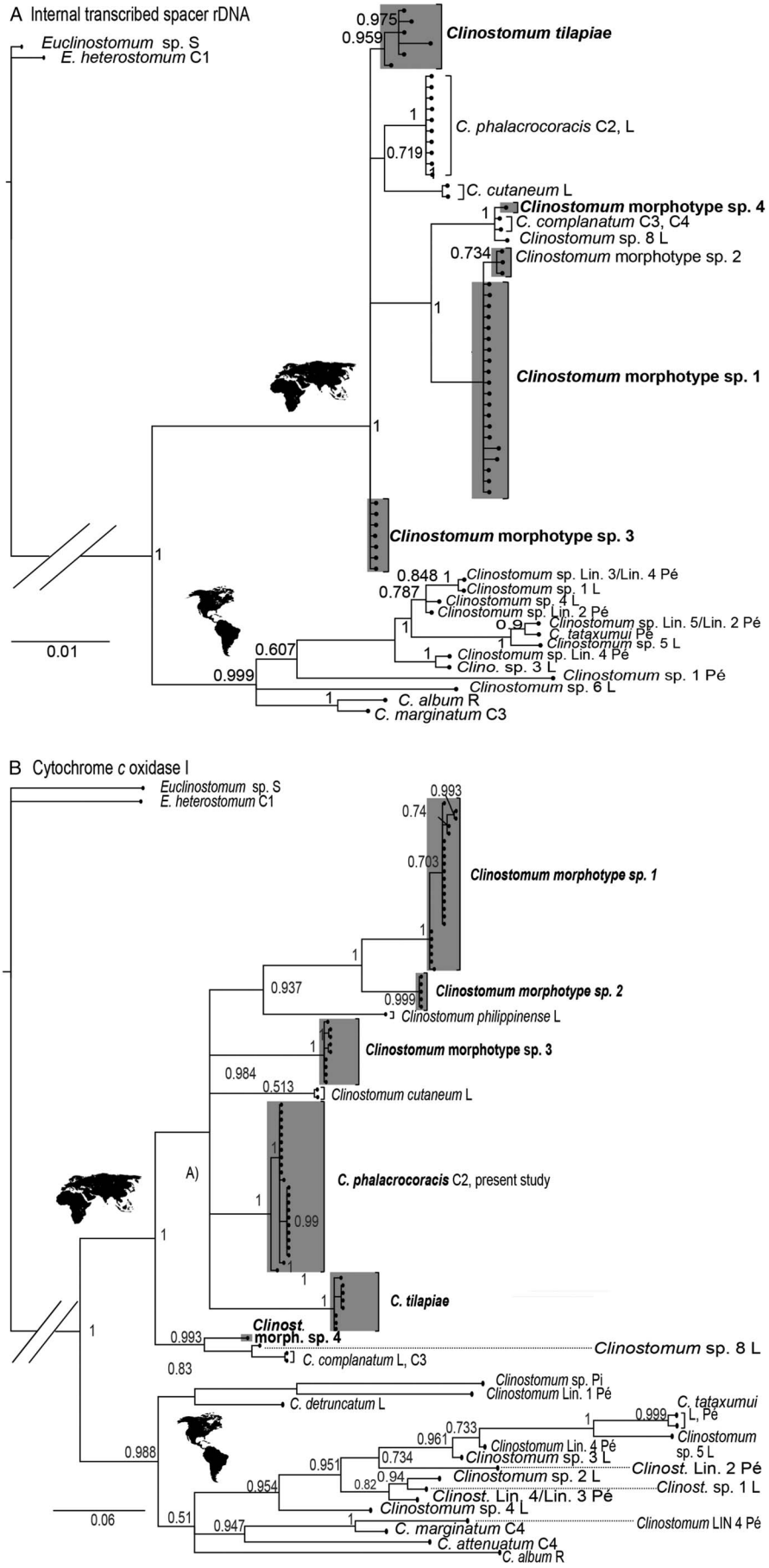


Fig. 3. Continued.

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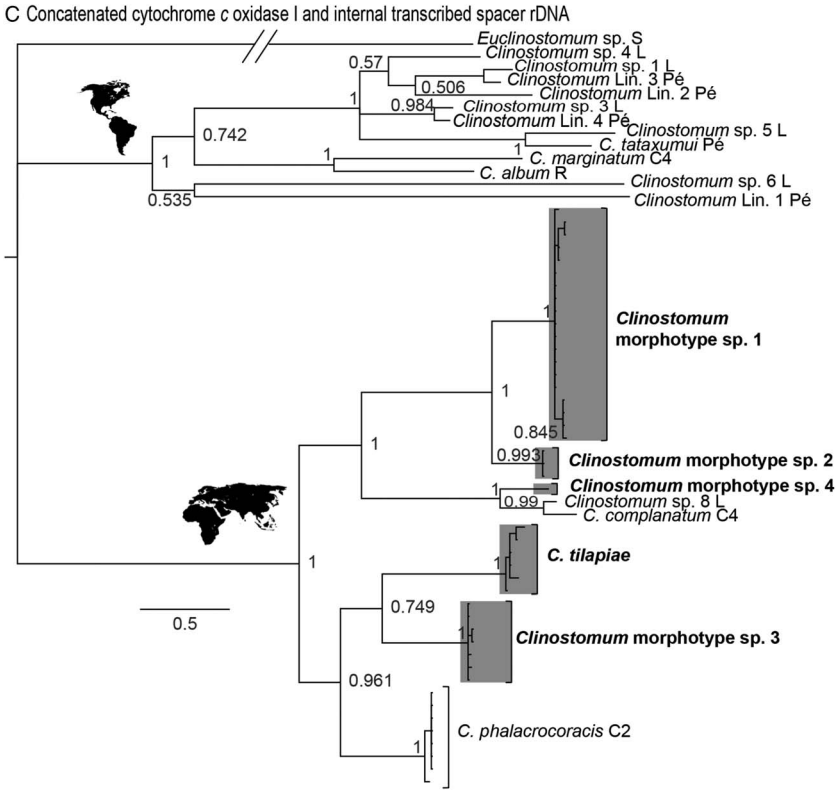


Fig. 3. Consensus topologies from Bayesian inference of mitochondrial and nuclear markers from clinostomids from Nigeria, South Africa and elsewhere. Data from the present study are indicated by shaded boxes and bold labels. Data from other studies are indicated by abbreviations: S = Senapin *et al*. (2014); C1 = Caffara *et al*. (2016); C2 = Caffara *et al*. (2014*b*); C3 = Caffara *et al*. (2014*a*); C4 = Caffara *et al*. (2011); L = Locke *et al*. (2015*b*); Pi = Pinto *et al*. (2015); Pé = Pérez-Ponce de León *et al*. (2016); R = Rosser *et al*. (2017). GenBank accessions are listed in Supplementary Table S1. Provisional names separated by forward slash indicate different names in paper/in GenBank record. Two clades containing only New World or Old World species are indicated. (A) Tree based on 861 common sites and 16 alignment gaps in 67 sequences of ITS rDNA. Posterior probabilities at nodes reflect 6122 trees. (B) Tree based on 474 common sites in 93 sequences of COI mtDNA. Posterior probabilities reflect 32 432 trees. (C) Tree based on 1271 common sites in 59 concatenated sequences of COI (468 bp), ITS (803 bp) and 13 gaps in the ITS alignment. Posterior probabilities reflect 16 638 trees.

contrast, in *C. complanatum*, the anterior testis is strongly left-dislocated by the cirrus pouch; in *C. phalacrocoracis*, it is fan-shaped; in *C. cutaneum* and *Clinostomum* sp. morphotype 2, testes are triangular and clearly digitated; and in *Clinostomum* sp. morphotypes 1 and 3, the testes are slightly lobed. The tegument of the metacercariae of *C. tilapiae* is completely covered with spines that are absent in African species except *Clinostomum* sp. morphotypes 1, 3 and 4. The cirrus pouch of *C. tilapiae* is oval, between the testes, and almost in contact with the right caeca, while in *C. phalacrocoracis* it is reniform in the dextral intertesticular space; in *C. cutaneum* it is round with a deep cleft forming two lobes, and in *C. complanatum*, it is wide, extending from the intertesticular space to the posterior right margin of the anterior testis. In *Clinostomum* sp. morphotypes 1–3, the cirrus pouch is in close contact with the right margin of the anterior testis, while in *Clinostomum* sp. morphotype 4, the cirrus pouch is in the intertesticular space close to the right posterior margin of anterior testis.

The validation of *C. tilapiae* as distinct species has relevance for prior records of this species and other regional reports. *Clinostomum tilapiae* was described by Ukoli (1966) from metacercariae in *Tilapia zilli*, *T. heudeloti* and *T. galilaea* from Ghana, and from adults in experimentally infected *B. ibis*. Subsequent epidemiological reports of *C. tilapiae* in Africa often lacked morphological support (Manter and Pritchard, 1969; Fischthal and Thomas, 1970; Okaka and Akhigbe, 1999; Olurin and Somorin, 2006; Boane *et al*. 2008; Taher, 2009; Echi *et al*. 2012; Ochieng *et al*. 2012; Olurin *et al*. 2012; see also Table 1). Agbede *et al*. (2004) used S.E.M. description to study metacercariae of *C. tilapiae* from *O. niloticus* but did not mention its spiny surface, although tegumental spines are visible with light microscopy in *C. tilapiae* (Ukoli, 1966) and were observed in our specimens. The identity of the species in many African reports of *Clinostomum* sp. cannot be determined (Khalil and Thurston, 1973; Paperna, 1980; Coulibaly *et al*. 1995; Yimer, 2000; Aloo, 2002; Yimer and Enyew, 2003; Jansen van Rensburg *et al*. 2003; Marwan and

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Mohammed, 2003; Ramollo *et al*. 2006; Ayotunde *et al*. 2007; Onyedineke *et al*. 2010; Madanire-Moyo *et al*. 2012 see also Table 1). However, in some studies in which specimens were not identified, authors provided drawings. In particular, Dollfus (1950) drew *Clinostomum* sp. from *A. goliath* (Fig. 57) and from *T. melanopleura* (Fig. 62) that Ukoli (1966) synonymized with *C. tilapiae*. In our opinion, the genital complex of the adult of *Clinostomum* sp. from *A. goliath* reported by Dollfus (1950, see Fig. 55) indicates that it does correspond to *C. tilapiae*. Onyedineke *et al*. (2010) and Dougnon *et al*. (2012) reported *Clinostomum* sp. from *Synodontis eupterus*, *S. schall* and *S. nigrita* collected, respectively in Nigeria and South Benin, but without any information relevant to species identification; we cannot identify these as belonging to *C. tilapiae*.

The genetic and morphological comparisons among the African species strongly support the validity of *C. tilapiae*. Among the evidence supporting four further putative species (morphotypes 1–4) in our samples are genetic distances that generally do not overlap within and between species and morphotypes. However, divergence values should be carefully evaluated to avoid over or underestimation of species diversity (Pérez-Ponce de León *et al*. 2016). Key aspects of genetic divergence within and between species are influenced by sampling effort (Locke *et al*. 2015*a* and references therein), which can make conclusions difficult during the initial stages of populating molecular databases. However, early errors are likely to be corrected with additional samples. In Mexico, for example, two putative species of *Clinostomum* were tentatively distinguished by Locke *et al*. (2015*b*) based on mitochondrial sequences from five specimens, and rDNA sequences from two, but this distinction was not maintained in the subsequent, expanded sampling in the same region by Pérez-Ponce de León *et al*. (2016). With this in mind, it is well to note that *C. tilapiae* and the four morphotypes distinguished herein are also supported by differences in the genital complex and by the monophyly of their mitochondrial and combined nuclear and mitochondrial sequences. Interestingly, the phylogenetic relationships of these five species are consistent with their morphometric similarity. The most strongly partitioned species in PCA (morphotype 2 *vs* morphotype 3 and *C. tilapiae*) are found in different clades in phylogenetic trees, and morphometrically overlapping species (morphotype 3 and *C. tilapiae*; morphotypes 1 and 2) occur in the same clades. The present phylogenetic analysis also reaffirms a previously observed separation of Old and New world species of *Clinostomum*, both in terms of the geographic ranges of the species, and a deep evolutionary division between species found in both regions. This was noted in an analysis of 16 species and putative species (Locke *et al*. 2015*b*); its emergence here is notable because the analysis includes eight additional lineages or species (morphotypes 1–4, *C. album*, and three lineages distinguished by Pérez-Ponce de León *et al*. (2016)).

## SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182017001068.

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## REFERENCES

**Acosta, A. A., Caffara, M., Fioravanti, M. L., Utsunomia, R., Zago, A. C., Franceschini, L. and Josè da Silva, R.** (2016). Morphological and molecular characterization of *Clinostomum detruncatum* (Trematoda: Clinostomidae) metacercaria infecting *Synbranchus marmoratus*. *Journal of Parasitology* **102**, 151–156.

**Agbede, S. A., Adeyemo, A. A. and Taiwo, V. O.** (2004). Scanning electron microscopic studies of *Clinostomum tilapiae* Ukoli 1960. *Tropical Veterinarian* **22**, 1–3.

**Aloo, P. A.** (2002). A comparative study of helminth parasites from the fish *Tilapia zilli* and *Oreochromis leucostictus* in Lake Naivasha and Oloidien Bay, Kenya. *Journal of Helminthology* **76**, 95–102.

**Ayotunde, E. O., Ochang, S. N. and Okey, I. B.** (2007). Parasitological examinations and food composition in the gut of feral African carp, *Labeo coubie* in the Cross River, Southeastern, Nigeria. *African Journal of Biotechnology* **6**, 625–630.

**Barson, M., Bray, R., Ollevier, F. and Huyse, T.** (2008). Taxonomy and faunistics of the helminth parasites of *Clarias gariepinus* (Burchell, 1822), and *Oreochromis mossambicus* (Peters, 1852) from temporary pans and pools in the Save-Runde River floodplain, Zimbabwe. *Comparative Parasitology* **75**, 228–240.

**Batra, V.** (1984). Prevalence of helminth parasites in three species of cichlids from a man-made lake in Zambia. *Zoological Journal of the Linnean Society* **82**, 319–333.

**Boane, C., Cruz, C. and Saraiva, A.** (2008). Metazoan parasites of *Cyprinus carpio* L. (Cyprinidae) from Mozambique. *Aquaculture* **284**, 59–61.

**Borchsenius, F.** (2009). *FastGap 1·2*. Department of Biosciences, Aarhus University, Denmark. Published online at http://www.aubot.dk/FastGap_home.htm.

**Britz, J., Van As, J. G. and Saayman, J. E.** (1984). Notes on the morphology of the metacercaria and adult of *Clinostomum tilapiae* Ukoli, 1966 (Trematoda: Clinostomatidae). *South African Wildlife Research* **14**, 69–72.

**Britz, J., Van As, J. G. and Saayman, J. E.** (1985). Occurrence and distribution of *Clinostomum tilapiae* Ukoli, 1966 and *Euclinostomum heterostomum* (Rudolphi, 1809) metacercarial infections of freshwater fish in Venda and Lebowa, Southern Africa. *Journal of Fish Biology* **26**, 21–28.

**Caffara, M., Bruni, G., Paoletti, C., Gustinelli, A. and Fioravanti, M. L.** (2014*a*). Metacercariae of *Clinostomum complanatum* (Trematoda:

Digenea) in European newts *Triturus carnifex* and *Lissotriton vulgaris* (Caudata: Salamandridae). *Journal of Helminthology* **88**, 278–285.

**Caffara, M., Davidovich, N., Falk, R., Smirnov, M., Ofek, T., Cummings, D., Gustinelli, A. and Fioravanti, M. L.** (2014*b*). Redescription of *Clinostomum phalacrocoracis* metacercariae (Digenea: Clinostomidae) in cichlids from Lake Kinneret, Israel. *Parasite* **21**, 32.

**Caffara, M., Locke, S. A., Cristanini, C., Davidovich, N., Markovich, M. P. and Fioravanti, M. L.** (2016). A combined morphometric and molecular approach to identifying metacercariae of *Euclinostomum heterostomum* (Digenea: Clinostomidae). *Journal of Parasitology* **102**, 239–248.

**Caffara, M., Locke, S. A., Gustinelli, A., Marcogliese, D. J. and Fioravanti, M. L.** (2011). Morphological and molecular differentiation of *Clinostomum complanatum* and *Clinostomum marginatum* (Digenea: Clinostomidae) metacercariae and adults. *Journal of Parasitology* **97**, 884–891.

**Coulibaly, N. D., Salembere, S. and Bessin, R.** (1995). Larval *Clinostomum* infection of cichlid fish in the lake of Kompienga in Burkina Faso: a menace to haleutic exploitation and public health. *Cahiers Santé* **5**, 189–193.

**Dollfus, R. P.** (1950). Trematodes récoltés au Congo Belge, par le Professeur Paul Brien. *Annales Du Musée Du Congo Belge* (*Zoologie*) **1**, 1–136.

**Dougnon, J., Montchowui, E., Daga, F. D., Houessionon, J., Laleye, P. and Sakiti, N.** (2012). Cutaneous and gastrointestinal helminth parasites of the fish *Synodontis schall* and *Synodontis nigrita* (Siluriformes: Mochokidae) from the Lower Ouémé Valley in South Benin. *Research Journal of Biological Sciences* **7**, 320–326.

**Dubois, G.** (1930). Matériaux de la mission scientifique Suisse en Angola. *Bulletin de la Société Neuchâteloise des Sciences Naturelles* **55**, 73–88.

**Echi, P. C., Eyo, J. E., Okafor, F. C., Onyishi, G. C. and Ivoke, N.** (2012). First record of co-infection of three Clinostomatid parasites in Cichlids (Osteichthyes: Cichlidae) in a tropical freshwater lake. *Iranian Journal Public Health* **41**, 86–90.

**Finkelman, S.** (1988). *Infections of Clinostomatidea in the Sea of Galilee fish*. MSc thesis. Faculty of Agriculture, Hebrew University of Jerusalem, Jerusalem.

**Fischthal, J. H. and Thomas, J. D.** (1970). Some metacercariae of digenetic trematodes in fishes from Nungua Lake, Ghana. *Anales del Instituto de Biologia Universidad Nacional Autonoma de Mexico Serie Zoologia* **1**, 73–80.

**Gustinelli, A., Caffara, M., Florio, D., Otachi, E. O., Wathuta, E. M. and Fioravanti, M. L.** (2010). First description of the adult stage of *Clinostomum cutaneum* Paperna, 1964 (Digenea: Clinostomidae) from grey herons *Ardea cinerea* L. and a redescription of the metacercaria from the Nile tilapia *Oreochromis niloticus niloticus* (L.) in Kenya. *Systematic Parasitology* **76**, 39–51.

**Jansen van Rensburg, C., van As, J. G. and King, P. H.** (2003). *Clinostomum* species infecting the sharptooth catfish, *Clarias gariepinus* in the Okavango Delta, Botswana. *Parasitological Society of Southern Africa* **74**, 87–101.

**Kabunda, M. Y. and Sommerville, C.** (1984). Parasitic worms causing the rejection of tilapia (*Oreochromis* species) in Zaire. *The British Veterinary Journal* **140**, 263–268.

**Katoh, K., Misawa, K., Kuma, K. I. and Miyata, T.** (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**, 3059–3066.

**Khalil, L. F. and Thurston, J. P.** (1973). Studies on the helminth parasites of freshwater fishes of Uganda including the descriptions of two new species of digeneans. *Revue de Zoologie et de Botanique Africaines* **87**, 209–248.

**Locke, S. A., Al-Nasiri, F. S., Caffara, M., Drago, F., Kalbe, M., Lapierre, A. R., McLaughlin, J. D., Nie, P., Overstreet, R. M., Souza, G. T. and Takemoto, R. M.** (2015*a*). Diversity, specificity and speciation in larval Diplostomidae (Platyhelminthes: Digenea) in the eyes of freshwater fish, as revealed by DNA barcodes. *International Journal for Parasitology* **45**, 841–855.

**Locke, S. A., Caffara, M., Marcogliese, D. J. and Fioravanti, M. L.** (2015*b*). A large-scale molecular survey of *Clinostomum* (Digenea: Clinostomidae). *Zoologica Scripta* **44**, 203–217.

**Lombard, G. L.** (1968). A survey of fish diseases and parasites encountered in Transvaal. *Limnological Society of Southern Africa Newsletter* **11**, 23–29.

**Luus-Powell, W. J., Molele, R. A., Tavakol, S., Matla, M. M., Halajian, A. and Fouché, P. S. O.** (2015). The parasite diversity of *Amphilius uranoscopus* and *Chiloglanis pretoriae* in the Vhembe biosphere reserve, Limpopo province, South Africa. In *9th International Symposium on Fish Parasites, Valencia, Spain, 31 August–4 September 2015*, p. 164.

**Madanire-Moyo, G. N., Luus-Powell, W. J. and Olivier, P. A.** (2012). Diversity of metazoan parasites of the Mozambique tilapia, *Oreochromis mossambicus* (Peters, 1852), as indicators of pollution in the Limpopo and Olifants River systems. *Onderstepoort Journal of Veterinary Research* **79**, 1–9.

**Manter, H. W. and Pritchard, M. H.** (1969). Some digenetic trematodes of Central Africa chiefly from fishes. *Revue de Zoologie et de Botanique Africaines* **80**, 51–61.

**Marwan, A. and Mohammed, T. A.** (2003). Scanning electron microscopy of *Clinostomum* metacercaria from *Oreochromis niloticus* from Assiut, Egypt. *Egyptian Journal of Biology* **5**, 70–77.

**Matthews, D. and Cribb, T. H.** (1998). Digenetic trematodes of the genus *Clinostomum* Leidy, 1856 (Digenea: Clinostomidae) from birds of Queensland, Australia, including *C. wilsoni* from *Egretta intermedia*. *Systematic Parasitology* **39**, 199–208.

**Moszczynska, A., Locke, S. A., McLaughlin, J. D., Marcogliese, D. J. and Crease, T. J.** (2009). Development of primers for the mitochondrial cytochrome *c* oxidase I gene in digenetic trematodes illustrates the challenge of barcoding parasitic helminths. *Molecular Ecology Resources* **9**, 75–82.

**Nolan, M. J. and Cribb, T. H.** (2005). The use and implications of ribosomal DNA sequencing for the discrimination of digenean species. *Advances in Parasitology* **60**, 101–163.

**Ochieng, V. O., Matolla, G. K. and Khyria, S. K.** (2012). A study of *Clinostomum* affecting *Oreochromis niloticus* in small water bodies in Eldoret-Kenya. *International Journal of Scientific & Engineering Research* **3**, 1–6.

**Okaka, C. E. and Akhigbe, J. E.** (1999). Helminth parasites of some tropical freshwater fish from Osse River in Benin, southern Nigeria. *Tropical Freshwater Biology* **8**, 41–48.

**Okoye, I. C., Abu, S. J., Obiezue, N. N. R. and Ofoezie, I. E.** (2014). Prevalence and seasonality of parasites of fish in Agulu Lake, Southeast, Nigeria. *African Journal of Biotechnology* **13**, 502–508.

**Olurin, K., Okafor, J., Alade, A., Asiru, R., Ademiluwa, R., Owonifari, K. and Oronay, O.** (2012). Helminth parasites of *Sarotherodon galilaeus* and *Tilapia zilli* (Pisces: Cichlidae) from River Oshun, Southwest Nigeria. *International Journal of Aquatic Science* **3**, 49–55.

**Olurin, K. B. and Somorin, C. A.** (2006). Intestinal helminths of the fishes of Owa Stream, South-west Nigeria. *Research Journal of Fisheries and Hydrobiology* **1**, 6–9.

**Onyedineke, N. E., Obi, U., Ofoegbu, P. U. and Ukogo, I.** (2010). Helminth parasites of some freshwater fish from River Niger at Illushi, Edo state, Nigeria. *Journal of American Science* **6**, 16–21.

**Ortlepp, R. J.** (1935). On the metacercaria and adult of *Clinostomum van der horsti* sp. n. a trematode parasite of fish and herons. *Onderstepoort Journal of Veterinary Science and Animal Industry* **5**, 51–58.

**Paperna, I.** (1980). *Parasites, Infections and Diseases of Fish in Africa*, Vol. **7**. CIFA Technical Papers, FAO, Roma.

**Pérez-Ponce de León, G., García-Varela, M., Pinacho-Pinacho, C. D., Sereno-Uribe, A. L. and Poulin, R.** (2016). Species delimitation in trematodes using DNA sequences: middle-American *Clinostomum* as a case study. *Parasitology* **143**, 1773–1789.

**Pinto, H. A., Caffara, M., Fioravanti, M. L. and Melo, A. L.** (2015). Experimental and molecular study of cercariae of *Clinostomum* sp. (Trematoda: Clinostomidae) from *Biomphalaria* spp. (Mollusca: Planorbidae) in Brazil. *Journal of Parasitology* **101**, 108–113.

**Pritchard, M. H. and Kruse, G.** (1982). *The Collection and Preservation of Animal Parasites*. University of Nebraska Press, Lincoln, Nebraska.

**Ramollo, P. P., Luus-Powell, W. J. and Jooste, A.** (2006). Impact of water quality on abundance of parasites from *Oreochromis mossambicus* at the Phalaborwa Industrial Complex (PIC) and Barrage: preliminary results. *Parasitological Society of Southern Africa* **77**, 92–103.

**Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. and Huelsenbeck, J. P.** (2012). MrBayes 3·2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**, 539–542.

**Rosser, T. G., Alberson, N. R., Woodyard, E. T., Cunningham, F. L., Pote, L. M. and Griffin, M. J.** (2017). *Clinostomum album* n. sp. and *Clinostomum marginatum* (Rudolphi, 1819), parasites of the great egret *Ardea alba* L. from Mississippi, USA. *Systematic Parasitology* **94**, 35–49.

**Senapin, S., Phiwsaiya, K., Laosinchai, P., Kowasupat, C., Ruenwongsa, P. and Panijpan, B.** (2014). Phylogenetic analysis of parasitic trematodes of the genus *Euclinostomum* found in Trichopsis and Betta fish. *Journal of Parasitology* **100**, 368–371.

**Sereno-Uribe, A. L., Pinacho-Pinacho, C. D., García-Varela, M. and Pérez-Ponce de León, G.** (2013). Using mitochondrial and ribosomal DNA sequences to test the taxonomic validity of *Clinostomum complanatum* Rudolphi, 1814 in fish-eating birds and freshwater fishes in Mexico, with the description of a new species. *Parasitology Research* **112**, 2855–2870.

**Smit, W. J. and Luus-Powell, W. J.** (2012). The occurrence of metazoan endoparasites of *Schilbe intermedius* Rüppell, 1832 from the Nwanedi-Luphephe Dams in the Limpopo River System, South Africa. *African Zoology* **47**, 35–41.

**Taher, G. A.** (2009). Some studies on metacercarial infection in *Oreochromis niloticus* in Assiut governorate and their role in transmission of some trematodes to dogs. *Assiut University Bulletin for Environmental Researches* **12**, 63–79.

**Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S.** (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–2729.

**Ukoli, F. M.** (1966). On *Clinostomum tilapiae* n. sp., and *C. phalacrocoracis* Dubois, 1931 from Ghana, and a discussion of the systematics of the genus *Clinostomum* Leidy, 1856. *Journal of Helminthology* **40**, 187–214.

**Williams, M. O. and Chaytor, D. E. B.** (1966). Some helminth parasites of fresh water fishes of the Freetown peninsula, Sierra Leone. *Bulletin de l'Institut Fondamental d'Afrique Noire. Série A: Sciences Naturelles* **28**, 563–575.

**Yimer, E.** (2000). Preliminary survey of parasites and bacterial pathogens of fish at Lake Ziway. *Ethiopian Journal of Science* **23**, 25–33.

**Yimer, E. and Enyew, M.** (2003). Parasites of fish at Lake Tana, Ethiopia. *Ethiopian Journal of Science* **26**, 31–36.