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A new species of *Clinostomum* Leidy, 1856 based on molecular and morphological analysis of metacercariae from African siluriform fishes

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Short title: New species of *Clinostomum* from African siluriform fishes

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Abstract

In the Afrotropic region, the genus *Clinostomum* is represented by four valid and four putative species distinguished using molecular data. Here we describe one of the putative species as *Clinostomum ukolii* n. sp. based on metacercariae from siluriform fishes (*Synodontis batensoda*, *Schilbe intermedius*) collected in Nigeria and South Africa. The new species is distinguished by molecular data (39 new sequences of partial cytochrome *c* oxidase I ≥6.7% divergent from those of other species) and morphological differences from valid and putative species in the same region. Metacercariae of *C. ukolii* n. sp. can be distinguished based on size, tegumental spines, and various aspects of the genital complex, including its position, lobation of the anterior testis, and the disposition and shape of the cirrus pouch. Although descriptions of new species of digeneans are typically based on the morphology of adults, we argue that in cases where data are available from metacercariae from regionally known species, new species can be described based on metacercariae, particularly when supported by molecular data, as here. Moreover, sub-adult reproductive structures can be clearly visualized in metacercaria of *Clinostomum*. Considering metacercariae as potential types for new species could advance clinostome systematics more rapidly, because metacercariae are encountered much more often than adults in avian definitive hosts.

Introduction

The genus *Clinostomum* Leidy, 1856 (Digenea: Clinostomidae) was first reported in Africa in 1930 when Dubois described *C. phalacrocoracis* from Angola. In Nigeria, Ukoli (1966) provided the first revision of the genus together with the description of the species *C. tilapiae* from Ghana, which was recently redescribed by Caffara et al. (2017). The latter morphological redescription was supported with molecular comparisons to one named and four unnamed, genetically distinguished species from Africa, along with a review of African reports (see table 1 in Caffara et al. 2017) highlighting incomplete or absent morphological regional descriptions of *Clinostomum*.

Following a pioneering study by Matthews and Cribb (1998), since 2010, morphological data coupled to molecular analyses have been used to characterize species of *Clinostomum* and other clinostomids in Africa and elsewhere. This approach has now been applied to 14 named species, namely *C. cutaneum* Paperna, 1964 (Gustinelli et al. 2010), *C. complanatum* (Rudolphi, 1814) and *C. marginatum* (Rudolphi, 1819) (Caffara et al. 2011), *C. tataxumui* Sereno-Uribe et al. 2013, *C. phalacrocoracis* Dubois, 1930 (Caffara *et al.* 2014), *C. detruncatum* Braun, 1899 (Acosta *et al.* 2016), *C. album* Rosser et al. 2017, *C. tilapiae* Ukoli, 1966 (Caffara *et al.* 2017), *C. poteae* Rosser et al. 2018, *C. heluans* Braun, 1899 (Briosio-Aguilar *et al.* 2018), and *C. caffarae* Sereno-Uribe et al. 2018, *C. arquus* Sereno-Uribe et al. 2018, *C. cichlidorum* Sereno-Uribe et al. 2018, and the recent combination *C. brieni* (Dollfus, 1950) (Caffara *et al.* 2019). In addition to these species, however, a number of lineages await description or identification. Most pertinent here are four putative species distinguished by Caffara et al. (2017) based on genetic diversity and a brief account of morphological differences in the genital complex.

In the present study, we provide a complete morphological description of the metacercariae previously identified as *Clinostomum* morphotype 1 (Caffara et al. 2017) collected from *Synodontis batensoda* and *Schilbe intermedius* from Nigeria and South Africa respectively, which we erect as a new species, namely *Clinostomum ukolii* n. sp.

Materials and methods

Sixty-two metacercariae of *Clinostomum* sp. morphotype 1, were removed from fresh skin tissue of *Synodontis batensoda* (Siluriformes: Mochokidae) collected in the Anambra River Basin, Nigeria, and 6 from the abdominal cavity or gill chambers of *Schilbe intermedius* (Siluriformes: Schilbeidae) sampled in different areas of Limpopo province, South Africa. Of these 24 (18 from Nigeria and 6 from South Africa) were morphologically and molecularly described as *Clinostomum* sp. morphotype 1 in our previous work (Caffara et al. 2017). The new specimens were excysted, washed in saline and preserved in 70% ethanol for morphological analysis, all at room temperature. The posterior end was removed for molecular analysis (Caffara et al. 2017). Thirty-eight new ITS rDNA and 39 CO1mtDNA sequences were generated and published in GenBank (COI: MN044350-MN044388, ITS: MN059670-MN059707). 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). Specimens of *C. ukolii* n. sp. were morphologically compared with those of *C. complanatum* (data from Caffara et al. 2011; Locke et al. accepted), *Clinostomum* morphotypes 2-4, *C. tilapiae* (Caffara et al. 2017), *C. brieni* (Caffara et al. 2019) and *C. phalacrocoracis* (Caffara et al. 2014) using principal components analysis (PCA) of morphometrics normalized to range from -1 to 1. Analysis of similarities (ANOSIM) was used to test for differences in morphometric variation among species based on Euclidean distances in normalized morphometrics.

Results

Morphological description

Clinostomum ukolii n. sp. (Fig. 1, Table 1)

Type host: *Synodontis batensoda*

Type locality: Anambra River Basin, Nigeria

Other host and locality: *Schilbe intermedius*, Limpopo Province, South Africa

GenBank Accession numbers: ITS - KY865609-26, KY865656-60 and MN059670-MN059707; CO1 - KY865626-43, KY865676-81 and MN044350-MN044388

Type specimens deposited in the Museum of the Southwestern Biology, Division of Parasites, University of New Mexico (Accessions MSB: Para: 29098- 29101)

Etymology: *Clinostomum ukolii* n. sp. is named after Professor F.M.A. Ukoli who provided the first important revision of the genus *Clinostomum*.

Morphological features of the metacercariae (n=54) from *S. batensoda* and *Sch*. *intermedius* from Nigeria and South Africa. Body stout, widest in gonadic region. Oral sucker small, surrounded by oral collar (not always visible). Pharynx small, opening into pharyngeal bulb (visible only in some specimens). Ventral sucker larger than oral sucker. Intestinal caeca with small lateral pouches from ventral sucker to posterior end of body. Testes digitated. Anterior testis across middle and posterior third of body, irregularly lobed, slightly displaced to left. Posterior testis in anterior part of posterior third of body, symmetrical, triangular, with sublobes more or less evident. Efferent ducts from lateral right margin of testes to left margin of cirrus pouch (Fig. 2d). Cirrus pouch bean-shaped with tapering anterior margin, embracing right margin of anterior testis, overlapping it, with well evident longitudinal and radial muscle fibers and hair-like structures (Fig. 2a-c). Genital pore medial to cirrus sac, close to right anterior margin of anterior testis. Ovary small, irregular, sometimes slightly lobed, not median, in intertesticular space dextrally alongside cirrus pouch. Uterus running straight from ventral sucker to anterior testis. Uteroduct passing around left margin of anterior testis, forming knee-like bend before opening into uterine sac above anterior testis very close to metraterm. Metraterm muscular, sometimes cup-like, connecting uterus to genital atrium. Tegument completely covered with minute spines. Dome-like structures on tegument surface, between suckers, in some specimens.

Remarks

In an alignment of partial sequences of CO1 overlapping by at least 554 bp, 39 newly generated sequences averaged 99.58 (range 98.14-100%) similarity to the 23 sequences of *Clinostomum* morphotype sp. 1 published by Caffara *et al*. (2017) (KY865627-43, KY865676-81), with 20 of the newly generated sequences identical to two or more of those of Caffara *et al*. (2017). The CO1 sequences of *C. ukolii* n. sp. differ by at least 6.74% from those of other species, the most similar being from *Clinostomum* sp. morphotype 2 (KY865662-6).

Twenty-five of 38 newly generated ITS rDNA sequences were identical to over half the 23 sequences of *Clinostomum* morphotype sp. 1 published by Caffara *et al*. (2017) (KY865609-26, KY865656-60). The new ITS sequences differed by average 0.12% (range 0-0.5%) from those previously published from *Clinostomum* morphotype sp. 1. The most similar ITS sequences were those of *Clinostomum* sp. morphotype 2 (KY865645-7), which differed by 0.1-0.4% from those of *C. ukolii* n. sp.

Among 117 metacercariae, including 54 of *C. ukolii* n. sp., substantial morphometric variation was attributable to species (global ANOSIM R=0.636, p=0.0001). Metacercariae of *C. ukolii* n. sp. differed morphometrically from all other species (Table 2). The pairwise ANOSIM results, which are based on ranks of Euclidean distances, correspond well to the metric ordination of Euclidean distances in PCA (Fig. 3), in which *C. ukolii* n. sp. was well separated from *C. brieni*, *C. phalacrocoracis*, and *C. complanatum* (pairwise ANOSIM R values 0.728-0.853, Table 2), but less so from *Clinostomum* sp. morphotypes 2 and 3 and *C. tilapiae* (pairwise ANOSIM R values 0.275-0.338). Along PC1, all characters scored between 0.211 and 0.283, indicating that no single measurement is particularly discriminating along this dimension, which explained 70.2% of morphometric variation.

Discussion

In this study we describe *C. ukolii* n. sp., which was provisionally identified as morphotype 1 based mainly on molecular data (Caffara et al. 2017). The new species is based on phylogenetic analysis of 62 CO1 and 61 ITS sequences (39 CO1 and 38 ITS sequences newly generated here, see also Caffara et al. 2017), and morphological and morphometric analysis of 54 metacercariae that in combination show *C. ukolii* n. sp. to be distinct from those of other valid and putative species in the region.

Matthews and Cribb (1998), Sereno-Uribe et al. (2018) and two anonymous reviewers of an earlier version of this communication argued that species of *Clinostomum* (or other digeneans) should rest on the morphology of adults, not metacercariae. We agree that the morphology of metacercariae is often not comparable to that of adults (e.g., Caffara et al. 2019). Consequently, in regions where some species are known only as adults, erecting a new name based on metacercariae can be problematic. However, in the present case, the metacercarial morphology of all regionally known species is well characterized, and *C. ukolii* n. sp. is distinct from them both morphologically (see below, Fig. 3) and genetically (Caffara et al. 2017). In this special circumstance of a regional fauna in which both developmental stages are well characterized, naming a new species is an appropriate course. The new name is accompanied by molecular and morphological data from a substantial and representative sample of isolates collected in different hosts and localities. These data will allow identification of other developmental stages of *C. ukolii* n. sp. and comparisons with other regional species, known and new, thus increasing biological knowledge. Ferris (1928) cautioned against undue haste in naming new species, but the value of the stability that a valid name provides should not be underestimated in the context of modern molecular surveys. We believe that erection of *C. ukolii* n. sp. will reduce the accumulation of confusing, conflicting provisional names that arise in molecular prospecting studies of poorly known taxa. For example, most sequence records of a putative species called *Clinostomum* lineage 5 (Pérez-Ponce de Léon et al. 2016) or *Clinostomum* L5 (Briosio-Aguilar et al. 2018) are labelled *Clinostomum* sp. lineage 2 on GenBank, and this is a different species from what Locke et al. (2015b) had earlier called *Clinostomum* spp. 2 or 5 (see Locke et al. 2015a, for other examples from *Diplostomum*). While data from adults of *C. ukolii* n. sp. would be valuable, such data are unnecessary for the establishment of *C. ukolii* n. sp., and it is unclear when such information will be obtained. Collecting definitive hosts of *Clinostomum* is logistically challenging and highly regulated due to conservation concerns (e.g., seven ardeid species are critically endangered, endangered, or vulnerable, IUCN, 2019). Consequently, metacercariae from fish and amphibians will continue to be encountered and studied with greater frequency than adults. Like Ukoli (1966), we believe that metacercariae merit full consideration from a taxonomic perspective, and that this practice could allow clinostome systematics to advance reliably and more rapidly than limitation to adult morphology in all circumstances. Moreover, the sub-adult morphology of clinostome metacercaria presents some advantages over the adult form. Nearly all taxonomically important reproductive structures (testes, cirrus poach, ovary and uterus) are well developed and more clearly visible when the parasite is not sexually mature. Comparisons among these and other structures in metacercariae have been used to discriminate several species of *Clinostomum* (Caffara et al. 2011, 2017). The principal morphological structures unique to adults, eggs and vitellaria, do not play a critical role in distinctions of most species (Matthews and Cribb, 1998; Gustinelli et al. 2010; Caffara et al. 2011; Sereno-Uribe et al. 2018), although they do provide a clear demarcation of maturity. Under experimental conditions, clinostome metacercariae show slower growth over a longer period than adults, which are shorter-lived (Jhansilakshmibai and Madhavi, 1997; Liao, 1992). In natural infections, however, it is our experience that the majority of metacercariae display fairly uniform size distributions, which likely reflects an accumulation of specimens with stable sub-adult morphology. For example, adults body lengths are more variable than metacercariae in *C. marginatum* and *C. cichlidorum*, but the opposite holds for *C. complanatum*, *C. arquus*, and *C. caffarae* (see morphometrics in Caffara et al. 2011 and Sereno-Uribe et al. 2018). In other words, there is no compelling empirical basis for rejecting morphometric analysis of metacercariae because of allegedly greater variability ascribable to unknown specimen age. Most importantly, as discussed below, the metacercariae of *C. ukolii* n. sp. can be morphologically (and genetically) distinguished from all others in the Afrotropic region, which leaves no doubt that it represents a new species.

Metacercariae of *C. ukolii* n. sp. can be morphologically distinguished from valid and putative species in Africa and Europe based on the genital complex. We view detailed morphological comparisons with species in other regions as unnecessary because there is little evidence of transcontinental distributions in *Clinostomum* (Locke et al. 2015b) and the molecular distinction of *C. ukolii* n. sp. from such species is clear from prior phylogenetic analysis (Caffara et al. 2017). In *C. ukolii* n. sp., the genital complex lies between middle and posterior third of the body, while in *C. tilapiae* (Caffara et al. 2017) it occupies 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; the genital complex is entirely in the middle third in *C. cutaneum* (Gustinelli et al. 2010) and *Clinostomum* sp. morphotype 3 (Caffara et al. 2017), and entirely in the posterior third of body in *C. brieni* (Caffara et al. 2019). The genital complex in *C. ukolii* n. sp. is similar in position to that of *C. phalacrocoracis* (Caffara et al. 2014), *C. complanatum* (Caffara et al. 2011) and *Clinostomum* sp. morphotypes 2 and 4 (Caffara et al. 2017), but different in structure. The irregular lobation of the anterior testis of *C. ukolii* n. sp. is unlike the fan shape of the anterior testis of *C. phalacrocoracis* or the triangular, digitated anterior testis of *Clinostomum* sp. morphotypes 2 and 4 and *C. cutaneum*. In *C. ukolii* n. sp., the anterior testis is also less lobed than the posterior, while in *C. tilapiae*, the anterior is more lobed, with two main lateral and one posterior lobe on the posterior testis; in *C. complanatum*, the anterior testis is strongly left-dislocated by the cirrus pouch. In *C. brieni*, the anterior testis is bow-tie shaped while the posterior varies from Y to crescent shaped. Only in *Clinostomum* sp. morphotype 3 does the structure of the testes resemble that of *C. ukolii* n. sp.

The cirrus pouch of *C. ukolii* n. sp. overlaps the anterior testis, as in *Clinostomum* sp. morphotypes 2 and 3, while in *Clinostomum* sp. morphotype 4, the cirrus pouch is in the intertesticular space close to the right posterior margin of anterior testis. In *C. tilapiae*, the cirrus pouch is oval and lies between the testes, almost in contact with the right cecum, while in *C.*

phalacrocoracis it is bean-shaped 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 *C. brieni* it is comma-shaped, intertesticular and in close contact to both testes. Interestingly, in *C. ukolii* n. sp. we were able to see the longitudinal muscular fibers of the cirrus pouch described previously only by Maccagno (1934) in *C. complanatum*.

The tegument of metacercariae of *C. ukolii* n. sp. is completely covered with minute spines over the whole body, as in *C. tilapiae* and *Clinostomum* sp. morphotypes 3 and 4. In *C. brieni*, the spines are thicker and present from oral sucker to posterior end of body, while other African species are devoid of spines. Metacercariae of *C. ukolii* n. sp. are smaller (mean total length 6169, range 3726-8804 µm) than those of *C. brieni* (mean 8683, range 6762-10602 µm, data from Caffara et al. 2019) and *C. phalacrocoracis* (mean 12061, range 9500–15200 µm, data from Caffara et al. 2014).

Clinostomum ukolii n. sp. is now added to the list of species of *Clinostomum* in the Afrotropic ecozone that have been validated with a combined molecular and morphological approach, namely *C. cutaneum, C. phalacrocoracis, C. tilapiae*, and the recent combination *C. brieni* (Gustinelli et al. 2010, Caffara et al. 2014, 2017, 2019). We do not include *C. complanatum* in this fauna because its presence in the Afrotropic region has not been confirmed with molecular data, although it has often been reported in Africa (Batra, 1984; Barson et al. 2008; Oliver et al. 2009; Ejere et al. 2014; Echi et al. 2012; Aboel Hadid and Lofty, 2007). Many of these morphology-based records are open to other interpretation. For example, El-Shahawy et al. (2017) and El-Dakhly et al. (2018) recently reported *C. complanatum* in Egypt, but the distinctive, fan-shaped testes of the specimens figured in these studies clearly differ from those of *C. complanatum* and resemble *C. phalacrocoracis,* although the total lengths of the worms in both papers is far smaller or bigger than any record of either *C. complanatum* or *C. phalacrocoracis* of which we are aware. This situation illustrates the ongoing need to reassess the diversity of Afrotropic clinostomes with both DNA and morphology in both larvae and adults.

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Fig. 1 Line drawing of metacercaria of *Clinostomum ukolii* n. sp. Scale bar = 1000 µm.

Fig. 2 Cirrus pouch of *Clinostomum ukolii* n. sp. from *Synodontis batensoda*: (A) *in toto*; (B) longitudinal muscle fiber (*), (C) hair-like structures (arrows) (D) efferent ducts (ef) connected to cirrus pouch (cp).

Fig. 3 PCA. Principal Components Analysis (PCA) of variation in 16 morphometrics among 102 metacercariae of *Clinostomum* Leidy, 1856. The first two axes of PCA explained 76.8% of morphometric variation among (PC1 70.2%, PC2 6.6%). Vectors show the direction and magnitude of correlations of the morphometric features along both axes, with the circle representing correlation of maximum strength.