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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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Abstract

In the Afrotropic region, the genus *Clinostomum* is represented by four accepted and four unnamed species distinguished using molecular data. Here, we describe one of the four unnamed 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 $I \geq 6.7\%$ divergent from those of other species) and morphological differences from named and unnamed 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.

Keywords Yellow grub · Helminth · Catfish · Halzoun

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 (1966a) 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 15 named species, namely *C. cutaneum* Paperna, 1964 (Gustinelli et al. 2010),

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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 1966a (Caffara et al. 2017), *C. poteae* Rosser et al. 2018, *C. heluans* Braun, 1899 (Briosio-Aguilar et al. 2018), *C. caffarae* Sereno-Uribe et al. 2018, *C. arquus* Sereno-Uribe et al. 2018, *C. cichlidorum* Sereno-Uribe et al. 2018, *C. brieni* (Dollfus, 1950) (Caffara et al. 2019), and *C. sinensis* Locke et al. 2019. In addition to these species, however, a number of lineages await description or identification. Most pertinent here are four unnamed 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-three 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. 2019), *Clinostomum* sp. 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 16 morphometrics normalized to range from − 1 to 1 (total *n* specimens = 102). Analysis of similarities (ANOSIM, with 9999 permutations) was used to test for differences in morphometric variation among species based on Euclidean distances in normalized morphometrics. PCA and ANOSIM were conducted using Primer-E (Auckland, NZ).

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.

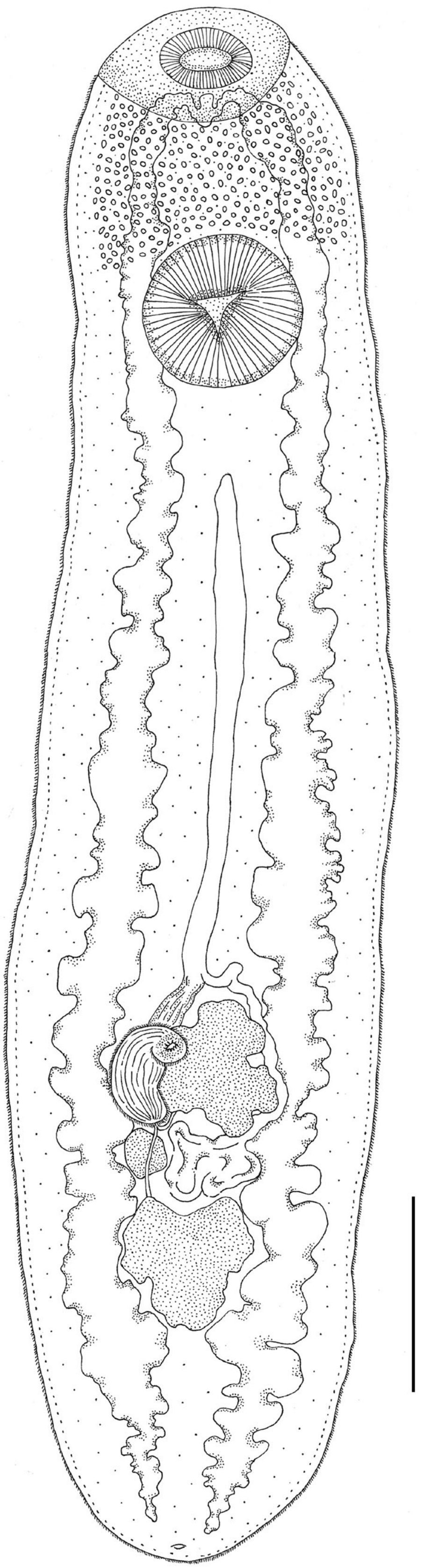

Fig. 1 Line drawing of metacercaria of *Clinostomum ukolii* n. sp. Scale bar = 1000 μm

Table. 1 Morphological data from *Clinostomum ukolii* n. sp. (range (mean ± SD)) in μm. Characters with an asterisk were analyzed in PCA and ANOSIM

Oral collar	723–1254 (1051 ± 144)
Body length*	1818–8801 (6073 ± 1428)
Body width*	1290–3546 (2047 ± 399)
Body length/width	0.87–4.94 (3.02 ± 0.72)
Oral sucker (OS) length*	169–599 (302 ± 88.37)
OS width*	286–796 (494 ± 104)
OS width/body width	0.14–0.36 (0.24 ± 0.05)
Ventral sucker (VS) length*	587–1159 (921 ± 117)
VS width*	669–1146 (936 ± 113)
VS width/OS width	1.16–2.75 (1.95 ± 0.40)
VS width/body width	0.31–0.70 (0.47 ± 0.07)
Distance between OS and VS*	857–2085 (1276 ± 290)
Anterior testis (AT) length*	349–882 (588 ± 144)
AT width*	263–906 (642 ± 114)
AT width/length	0.60–1.68 (1.12 ± 0.21)
Posterior testis (PT) length*	214–857 (554 ± 129)
PT width*	459–1041 (736 ± 125)
PT width/length	0.83–2.68 (1.38 ± 0.32)
Distance between testes*	353–874 (558 ± 120)
Ovary length*	136–410 (242 ± 57)
Ovary width*	150–480 (231 ± 57)
Ovary width/length	0.62–1.97 (1.00 ± 0.33)
Cirrus sac (CS) length*	316–633 (472 ± 83)
CS width*	144–290 (219 ± 32)
CS length/body length	0.04–0.23 (0.08 ± 0.03)

Remarks

Metacercariae of *C. ukolii* n. sp. can be morphologically distinguished from metacercariae of accepted and unnamed species in Africa based on morphological characters such as those of 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) and *C. van der horsti* (Ortlepp 1935)

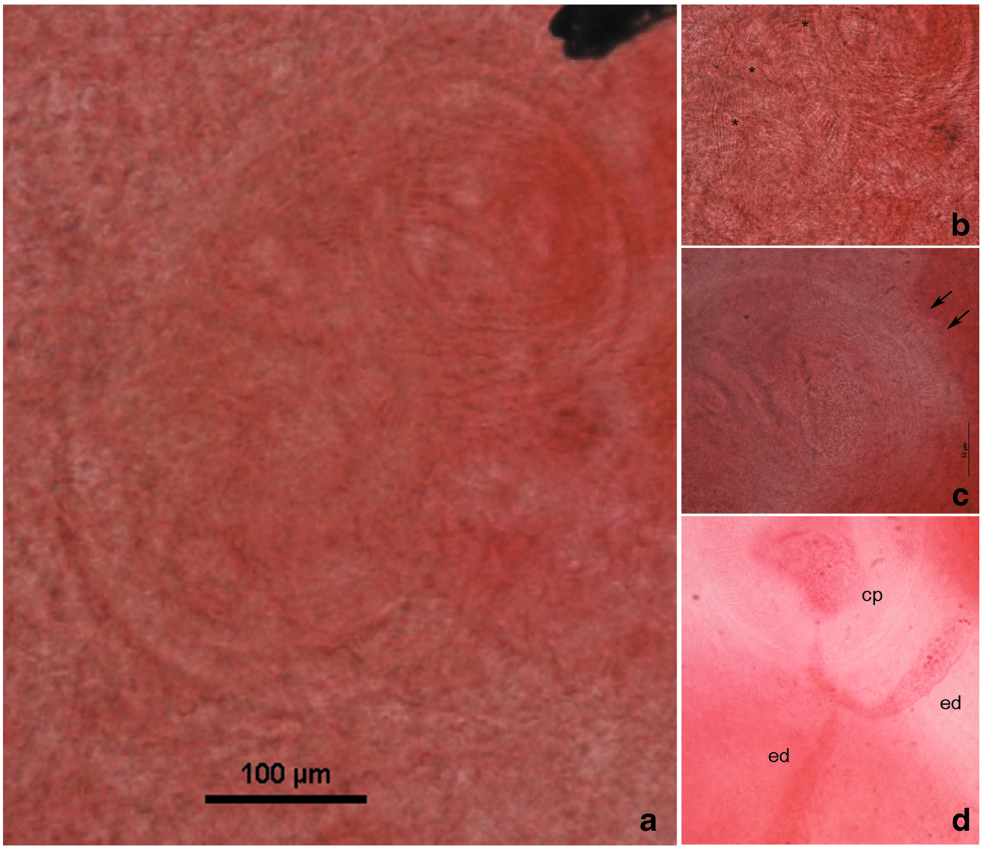

Fig. 2 Cirrus pouch of *Clinostomum ukolii* n. sp. from *Synodontis batensoda*. **a** In toto. **b** Longitudinal muscle fiber (*). **c** Hair-like structures (arrows). **d** Efferent ducts (ef) connected to cirrus pouch (cp)

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 lobes 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 reniform 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; in *C. van der horsti*, it is piriform and smaller. 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. Metacercariae of *C. van der horsti* showed spines posterior to ventral sucker; in *C. brieni*, the spines are from oral sucker and thicker, 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 6762–10,602 µm; data from Caffara et al. 2019) and *C. phalacrocoracis* (mean 12,061, range 9500–15,200 µm; data from Caffara et al. 2014) and larger than *C. van der horsti* (range 5000–6000 µm; Ortlepp 1935).

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

Table 2 Results of analysis of similarity (ANOSIM) among 117 metacercariae of *Clinostomum*, including 54 *C. ukolii* n. sp., based on Euclidean distances among normalized morphometrics. Comparisons show ANOSIM R statistics with *P* value in parenthesis (based on 9999 permutations)

	C. ukolii n. sp.	*C. complanatum*	*C. phalacrocoracis*	*C. tilapiae*	*C. brieni*	*C.* morphotype 2	*C.* morphotype 3
C. ukolii n. sp. (*n* = 54)							
C. complanatum (*n* = 23)	0.728 (0.0001)						
C. phalacrocoracis (*n* = 13)	0.767 (0.0001)	0.965 (0.0001)					
C. tilapiae (*n* = 8)	0.275 (0.0163)	0.644 (0.0001)	0.681 (0.0001)				
C. brieni (*n* = 6)	0.853 (0.0001)	0.778 (0.0001)	0.936 (0.0001)	0.668 (0.0007)			
C. morphotype 2 (*n* = 5)	0.296 (0.0235)	0.961 (0.0001)	0.526 (0.0006)	0.472 (0.0023)	1.000 (0.0022)		
C. morphotype 3 (*n* = 7)	0.338 (0.0074)	0.471 (0.0007)	0.729 (0.0001)	0.089 (0.1279)	0.881 (0.0012)	0.626 (0.0013)	
C. morphotype 4 (*n* = 1)	0.654 (0.0364)	0.215 (0.25)	0.923 (0.0714)	0.402 (0.2222)	0.978 (0.1429)	1.000 (0.1667)	0.578 (0.125)

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.

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.

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 analyses of 54 metacercariae that in combination show *C. ukolii* n. sp. to be distinct from those of all other species in the region.

Matthews and Cribb (1998), Sereno-Uribe et al. (2018), and anonymous reviewers of earlier versions of this communication argued that species of *Clinostomum* or other digeneans should rest on morphological characters of adults, not of metacercariae. We view the practice of morphological description of species based on adult characters as a useful convention but not a strict criterion. One strength of the conventional approach is that, in many digeneans, informative morphological characters are usually more numerous in adults than in metacercariae (Gibson, 1987). However, the terms "metacercaria" and "adult" are misleading if taken to imply a well-defined distinction (Galaktionov and Dobrovolskij 2003). Varying degrees of maturity occur in different digeneans in their second intermediate hosts and consequently the number of informative characters also varies. For example, should the morphology of progenetic metacercariae be considered strictly incomparable to that of "adults" in definitive hosts? Ample species-specific characters have often been noted in the metacercariae of *Clinostomum* by

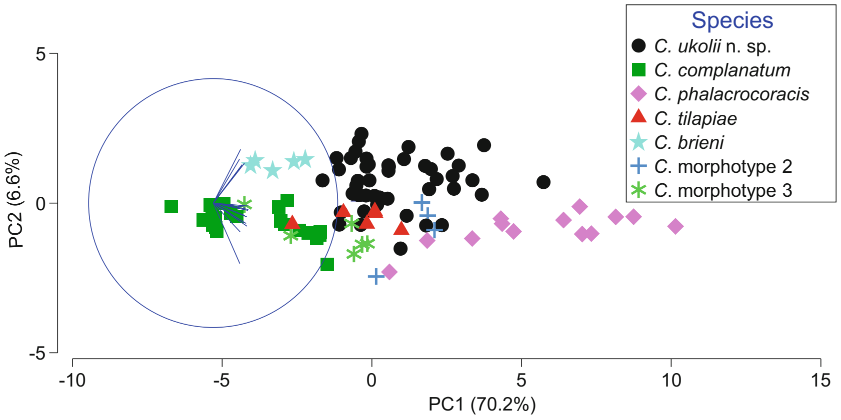

Fig. 3 PCA. Principal components analysis (PCA) of variation in 16 morphometrics (see Table 1) among 102 metacercariae of *Clinostomum* Leidy, 1856. The first two axes of PCA explained 76.8% of morphometric variation among (PC1 70.2%, PC2 6.6%). Vectors show the direction and magnitude of correlations of the morphometric features along both axes, with the circle representing correlation of maximum strength

us and others. For example, Maccagno (1934) observed that *C. complanatum* and *C. marginatum* are more easily distinguished as metacercariae than as adults. Ukoli (1966a, b) reached the same conclusion in detailed studies of *C. tilapiae* and *C. phalacrocoracis*, as well as a review of the entire genus *Clinostomum*. These authors pointed out advantages of the subadult morphology of clinostome metacercaria over the adult form and referred to taxonomically important reproductive structures (testes, cirrus pouch, ovary, and uterus) that are well developed in metacercariae. Comparisons among these and other structures in metacercariae have also been used to discriminate several other species of *Clinostomum* in more recent works (Caffara et al. 2011, 2017; Locke et al. 2019). In contrast, 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). In the clinostomes, therefore, recognizing new species based on metacercariae is not limited by an insufficient number of informative morphological characters.

A second advantage of the conventional focus on adults is that it ensures characters are compared at equivalent stages of development (e.g., in gravid specimens), while development in metacercariae is not as easily gauged. In natural infections, however, it is our experience that clinostome metacercariae display fairly uniform size distributions, which likely reflects an accumulation of specimens with stable sub-adult morphology. Under experimental conditions, clinostome metacercariae show slower growth over a longer period than adults, which are shorter-lived (Liao 1992; Jhansilakshmibai and Madhavi 1997), but this does mean metacercarial morphology is inherently more variable. For example, body lengths of adults are more variable than those of metacercariae in *C. marginatum* and *C. cichlidorum*, and the opposite holds for *C. complanatum*, *C. arquus*, and *C. caffarae* (see morphometrics in Caffara et al. 2011 and Sereno-Uribe et al. 2018). Moreover, some characters vary little in developing metacercariae. Several, such as the position of the cirrus pouch, were recorded by Vianna et al. (2003), and Briosio-Aguilar et al. (2018) observed remarkably stable genital morphology in clinostomid metacercariae with up to threefold variation in total length. In other words, there is no compelling evidence that ontogenetic variation in morphological characters of clinostomid metacercariae is greater than in adults, or that such variation is likely to lead to mistaken taxonomic conclusions in comparisons among metacercariae more often than in comparisons among adults.

We emphasize that the erection of *C. ukolii* n. sp. is tied to a particular taxonomic context. The metacercarial morphology of all regionally known species is well characterized, and *C. ukolii* n. sp. is distinct from them both morphologically (see Remarks) and genetically (Caffara et al. 2017) (with the caveat that DNA sequences have not been obtained from *C. van der horsti*). Species from other regions are unlikely to be present (Locke et al. 2015b). In this special circumstance of a regional *Clinostomum* fauna in which nearly all species have been sequenced and both developmental stages are well-characterized morphologically, we contend that naming a new species based on either form is an appropriate course. In regions where some species of *Clinostomum* are known only as adults, however, or many accepted species have not yet been sequenced, erecting a new name based on metacercariae can be problematic, because of the risk of ascribing a new name to a species already known, but only as an adult. As Ukoli (1966a) argued, the morphology of clinostome metacercariae is informative of that of the adult, but this does not mean metacercariae and adults can be directly compared to adults. Any such comparisons must take into account changes (especially size-related changes) that are likely to vary with overall growth (e.g., Caffara et al. 2019).

Authors such as Ferris (1928) have cautioned against undue haste in naming new species, but a stable, accepted name provides great value in the context of modern molecular surveys. Because the distinct status of *C. ukolii* n. sp. is well supported, we believe that naming it is preferable to adding to provisional names accumulating from molecular prospecting studies while awaiting data from adults. 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, and confusing provisional names will become more numerous. For example, most sequence records of a species called *Clinostomum* lineage 5 (Pérez-Ponce de León et al. 2016) or *Clinostomum* L5 (Briosio-Aguilar et al. 2018) are labeled *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*). Data from adults of *C. ukolii* n. sp. would clearly be of interest, but even if adults of *C. ukolii* n. sp. proved to be morphologically indistinguishable from another African species, *C. ukolii* n. sp. would still stand based on molecular data and differences in metacercarial morphology.

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; Olivier et al. 2009; Ejere et al. 2014; Echi et al. 2012; Aboel Hadid and Lotfy 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 specimens figured in these studies differ from those of *C. complanatum* and resemble *C. phalacrocoracis*, although the total lengths of the worms in both papers are 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 African clinostomes with both DNA and morphology in both larvae and adults.

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Compliance with ethical standards

Applicable international, national, and institutional guidelines for the care and use of animals were followed.

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

Disclaimer Any opinion, finding, and conclusion or recommendation expressed in this material is that of the authors and the NRF does not accept any liability in this regard.

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