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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

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Abstract The combined use of morphological and molecular studies allowed for the first time the recognition and description of the adult stage of *Clinostomum cutaneum* Paperna, 1964 from the grey heron *Ardea cinerea* L. in Kenya. A redescription of the metacercaria that infect Nile tilapia *Oreochromis niloticus niloticus* (L.) from the same aquatic environment is also presented. *C. cutaneum* differs from all other species of *Clinostomum* Leidy, 1856 in the shape of its uterus. Sequencing the rRNA confirmed the morphological similarity between adults from the grey heron and the metacercarial stage from tilapia, and a level of genetic similarity with the other previously sequenced *Clinostomum* spp. was observed. The need for a reorganisation of *Clinostomum* using both morphological and molecular methods is highlighted.

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Introduction

The Clinostomidae Lühe, 1901 is a family of digeneans the members of which, at the adult stage, live in the oral cavity, pharynx or oesophagus of fish-eating birds, reptiles and occasionally mammals, including man. Among the four subfamilies listed by Kanev et al. (2002) is the Clinostominae Lühe, 1901, which comprises three genera infecting piscivorous birds, such as herons, cormorants and pelicans; *Clinostomum* Leidy, 1856 is the type-genus.

The basic life-cycle of clinostomines, as exemplified by *Clinostomum complanatum* (Rudolphi, 1814), comprises snails as the first intermediate hosts, in which miracidia hatched from eggs laid by adult flukes develop to sporocysts and then rediae that produce brevifurcate cercariae. These brevifurcate cercariae penetrate fish, the second intermediate hosts, developing into metacercariae that are infective for the definitive hosts (Olsen, 1974). *Clinostomum* metacercariae, known as 'yellow grubs' due to the colour of their gut contents, may encyst in different sites (e.g. dermis, muscles, gill-arch) or remain free in the body-cavity. Many fish species have been reported as second intermediate hosts (Bullard & Overstreet, 2008).

Due to the high degree of morphological variability within the same species, in the past, *Clinostomum* has been subjected to several taxonomic revisions.

One of the most important revisions was made by Ukoli (1966), who synonymised 20 previously described species of *Clinostomum* with *C. complanatum*, recognising 13 valid species on the basis of six main morphological characters. Yamaguti (1971) partly accepted the revision made by Ukoli (1966) and listed a total of 26 valid species, among which 16 were described on the basis of the adult stage, seven only as a larval form and three occasionally isolated as adults from the mouths of cats. Subsequently, Feizullaev & Mirzoeva (1983) synonymised all the species of *Clinostomum* with *C. complanatum*, with the exception of *C. sorbens* Braun, 1899, *C. heluans* Braun, 1899, *C. detruncatum* Braun, 1899, *C. ophicephali* (Tubangui & Masiluñgan, 1944), *C. philippinense* Velasquez, 1960 and *C. phalacrocoracis* Dubois, 1931, which were all allocated to different genera. More recently, Matthews & Cribb (1998), in a study of *Clinostomum* species found as adults in Australian fish-eating birds, revalidated *C. australiense* Johnston, 1917 and *C. hornum* Nicoll, 1914 and described a new species, *C. wilsoni* Matthews & Cribb, 1998, in a study which indicated the need for a revision of this genus which includes molecular techniques, as also stressed by Nolan & Cribb (2005). The importance of applying diagnostic molecular approach to clinostomine systematics has recently been confirmed by Dzikowski et al. (2004), who recognised *C. complanatum* and *C. marginatum* (Rudolphi, 1819) as distinct taxa on the basis of 18S rRNA sequences.

The application of a molecular approach in parallel to morphological study may be particularly important for the completion of the life-cycle and identification of those *Clinostomum* species described in the past only on the basis of morphological features of the metacercarial stage without any subsequent description of the relative adult stages from the definitive host. In relation to this, Paperna (1964a, b) described a metacercaria that differed in the shape of the uterus and the position of the gonads from *C. complanatum* and all of the other known clinostomids, naming it first as *Clinostomum* sp. and then as *C. "cutaneum"*. He found these metacercariae in several fish species, including several tilapias [*Tilapia zilli* (Gervais), *T. nilotica* (L.), *T. galilaea* (L.) and *Tristramella simonis* (Günther)] from Israel, without ever describing the adult stage from the definitive host, and Finkelman (1988) described a pre-adult phase of what was apparently the same species from an experimentally infected black-crowned night-heron *Nycticorax nycticorax* (L.). The parallel findings of metacercariae consistent with the *C. "cutaneum"* in Nile tilapia *Oreochromis niloticus niloticus* (L.) farmed in Kenya and adult clinostomids exhibiting strong similarities with these metacercariae in grey herons *Ardea cinerea* L. from the same area led us to carry out morphological and molecular studies aimed at completing the species description.

Materials and methods

Parasites

Mature specimens of clinostomids were recovered from the oesophagus of two grey herons *A. cinerea* found dead and entangled in the fish nets at Sagana Fish Farm, Sagana, Kenya.

Clinostomid larval stages (metacercariae) were isolated from the skin tissue of pond-farmed Nile tilapias *O. niloticus niloticus* at the same fish farm.

Morphological study

Morphological studies were performed on 15 adults (A) and 15 metacercariae (M) fixed in 70% ethanol. Whole-mounts were prepared of 20 parasites (10 A, 10 M), some cleared in Amman's lactophenol and some stained with Mayer's acid carmine or using Malzacher's method (Pritchard & Kruse, 1982). The posterior third was cut from the other 10 specimens (5 A, 5 M) and then processed for molecular analysis after measurement of total length and maximum width. The anterior part of these parasites was cleared in Amman's lactophenol and studied morphologically.

Line drawings were made with the aid of a drawing tube, and measurements are given in micrometres unless otherwise stated. Measurements were taken following Matthews & Cribb (1998).

One adult and one metacercaria were processed for scanning electron microscopy (SEM) and observed using a Jeol 5200 electron microscope.

Molecular analysis

Total DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) following the manufacturer's protocol. The rRNA gene was amplified by single PCR or nested PCR with different sets of primers (Table 1). In all the reactions, *C. complanatum* collected from wild barbel

Table 1 Primers used for the amplification of rRNA

Sequence 5′–3′	References	Gene	
82_f-CAGTAGTCATATGCTTGTCTCAG	Mariaux (1998)	18S rRNA	PCR 1st & 2nd step
81_r-TTCACCTACGGAAACCTTGTTACG			
83_f-GATACCGTCCTAGTTCTGACCA			PCR 2nd step
84_r-TCCTTTAAGTTTCAGCTT GC			
81_f-GTAACAAGGTTTCCGTAGGTGAA	Present study	ITS rRNA	PCR
ITS2.S_r-CCTGGTTAGTTTCTTTTCCTCCGC	Cribb et al. (1998)		
U178_f-GCACCCGCTGAAYTTAAG	Lockyer et al. (2003)	28S rRNA	PCR
L1642_r-CCAGCGCCATCCATTTTCA			
900_f-CCGTCTTGAAACACGGACCAAG			Sequencing
EDC2_r-CCTTGGTCCGTGTTTCAAGACGGG			
1200_r-GCATAGTTCACCATCTTTCGG			

Barbus barbus (L.) from Italian rivers was used as a reference sample. The total PCR volume was 50 µl, which contained 10× PCR buffer (Invitrogen), 200 µM dNTPs (Invitrogen), 0.3 µM of each primer, 1.5 mM $MgCl_2$ and 2.5 U Platinum *Taq* DNA Polymerase (Invitrogen). A Tpersonal (Biometra) thermocycler was used for the amplification, with different annealing temperatures and numbers of cycles depending of the region amplified. Briefly, 18S rRNA: 35 cycles of 30 s at 94°C, 40 s at 56°C and 90 s at 72°C, preceded by a denaturation step at 94°C for 2 min and followed by an extended elongation step at 72°C for 5 min; internal transcribed spacer (ITS) rRNA: annealing temperature 50°C, number of cycles 40; and 28S rRNA: annealing temperature 52°C, number of cycles 40. The PCR products were electrophoresed on a 1% agarose gel (Sigma) stained with SYBR Safe DNA Gel Stain in 0.5× TBE (Molecular Probes-Invitrogen). The PCR products were sequenced (PRIMM, Milan, Italy) in an ABI 3730 DNA Analyser. Sequence assembly was carried out using Vector NTI Advance™ 11 software (Invitrogen) and underwent a database search using BLAST (Altschul et al., 1990). Multiple sequence alignments were constructed using ClustalW (Thompson et al., 1994) and adjusted by eye. The taxa included in the genetic analysis are listed in Table 2.

Clinostomum cutaneum Paperna, 1964

Hosts: Adults (A) from *Ardea cinerea* L. (Ciconiformes: Ardeidae) grey heron; metacercariae (M) from *Oreochromis niloticus niloticus* (L.) (Osteichthyes: Cichlidae) Nile tilapia.

Locality: Sagana Fish Farm, Sagana, Kenya (00°39,755′S, 37°11,777′E; alt. 1,207 m).

Site: Oesophagus (A); skin (M).

Vouchers: Deposited in the Natural History Museum, London (Adults: Reg. No. 2009.10.29.1-3; Metacercariae: Reg. No. 2009.10.29. 4-6).

Previous records: *Clinostomum* sp. and *C.* "*cutaneum*" metacercariae (Paperna 1964a, b) in *Tilapia zilli* (Gervais), *T. nilotica* (L.), *T. galilaea* (L.) and *Tristramella simonis* (Günther) from Israel.

Description of adult (Figs. 1–5; Table 3)

[Based on 15 specimens; measurements in Table 3.] Body stout, widest in region of gonads. Oral sucker small, surrounded by well-developed oral collar. Ventral sucker larger than oral sucker. Pharynx absent; oesophagus very short; intestine bifurcates immediately posterior to level of oral sucker; oesophageal bulb present; intestinal caeca reach close to posterior end of body, distorted and compressed laterally in hindbody by egg-filled uterus, with smooth margins back to level of ventral sucker and several strongly pigmented diverticula between level of gonads and their posterior extremities. Testes entirely in middle third of body; anterior testis broad, irregularly lobed and party obscured by uterus when latter is filled by eggs, with right lobe slightly overlapped and pushed medially by cirrus-sac; posterior testis broad, triangular, lobed, in mid-line, with apex pointing posteriorly. Cirrus-sac anterior to

Table 2 List of the taxa included in the genetic analysis

Taxon	Host species/dev. stage	Locality	GenBank
C. cutaneum	*Ardea cinerea*/A	Kenya	GQ339114*
C. cutaneum	*Oreochromis niloticus*/M	Kenya	FJ609421*
C. phalacrocoracis	*Ardea cinerea*/A	Kenya	FJ609423*
C. phalacrocoracis	*Oreochromis niloticus*/M	Kenya	FJ609422*
C. complanatum	*Barbus barbus*/M	Italy	FJ609420*
C. complanatum	*Egretta garzetta*/A	Israel	AY245701 (Dzikowski et al., 2004)
C. marginatum	*Ardea herodias*/A	USA	AY245760 (Flowers et al., unpubl.)
Clinostomum sp. Australia-PO-2003	*Hypseleotrix galii*/M	Australia	AY222094, AY222175 (Olson et al., 2003)
Clinostomum sp. USA-PO-2003	*Rana catesbeiana*/M	USA	AY222095, AY222176 (Olson et al., 2003)
Clinostomum sp. Australia-MJN-2004	*Hypseleotrix galii*/M	Australia	AY465871 (Nolan & Cribb, 2004)

A, adult; M, metacercaria

*Present study

ovary, at level of anterior testis, contains large seminal vesicles and frequently everted cirrus with several basal papillae. Genital pore medial to cirrus-sac, close to antero-dextral margin of anterior testis. Ovary small, ovoid, located dextrally in intertesticular space. Mehlis' gland between testes, surrounding tubular oötype. Seminal receptacle small, oval, posterior to oötype. Laurer's canal not observed. Vitellarium follicular, extensive, reaching from middle level of ventral sucker to level short of caecal extremities, filling entire body width except for lateral margins. Tubular uterus passes around left margin of anterior testis and opens into uterine sac, occupies all intercaecal space between ventral sucker and anterior testis when filled with eggs, forming distinctive heart-shaped sac immediately posterior to ventral sucker, but has clear Y-shape when not completely filled by eggs; metraterm muscular, connects uterus with genital atrium. Mature eggs in uterine sac, some in proximal uterus. Excretory ducts arranged in complex web; excretory system not clearly visible.

SEM indicate tegument to be covered by papillae between posterior extremity of body and ventral sucker. Cirrus with prominent papillae at its base.

Redescription of metacercaria (Figs. 6–10)

[Based on 15 specimens.] Similar to adult, but slightly smaller; body 5.22–6.70 (6.16 ± 0.58) mm long, 1.77–2.45 (2.14 ± 0.26) mm wide. Oral sucker 220–370 (290 ± 55.8) × 312–492 (394 ± 68.1), not surrounded by distinct oral collar. Ventral sucker larger than oral sucker, 705–1,002 (855 ± 110.1) × 792–1,029 (878 ± 86.7). Glandular structure present in forebody, reaching to ventral sucker. Intestinal caeca run laterally to ventral sucker and genital primordia, with regular outline to level of ventral sucker and then with fewer but larger diverticula in hindbody than in adult, connected at extremities to excretory system by very thin duct. Testes less conspicuous than in adults, with more evident digitations and larger intertesticular space. Cirrus-sac large, rounded, with deep cleft forming 2 lobes; cirrus not evident. Genital pore close to right margin of anterior testis. Ovary irregular in shape, smaller than the cirrus-sac, located dextrally in intertesticular space. Vitellarium not evident. Tubular proximal uterus follows similar pathway to that in adult, opens into Y-shaped uterine sac; muscular metraterm evident. V-shaped excretory vesicle with well defined excretory pore opening to exterior.

Cuticular surface covered by thin papillae with bipartite/tripartite apex visible under light microscope.

Remarks

The main morphological characters of the *Clinostomum* species described at the adult stage and considered valid by Ukoli (1966) and Matthews & Cribb (1998) are

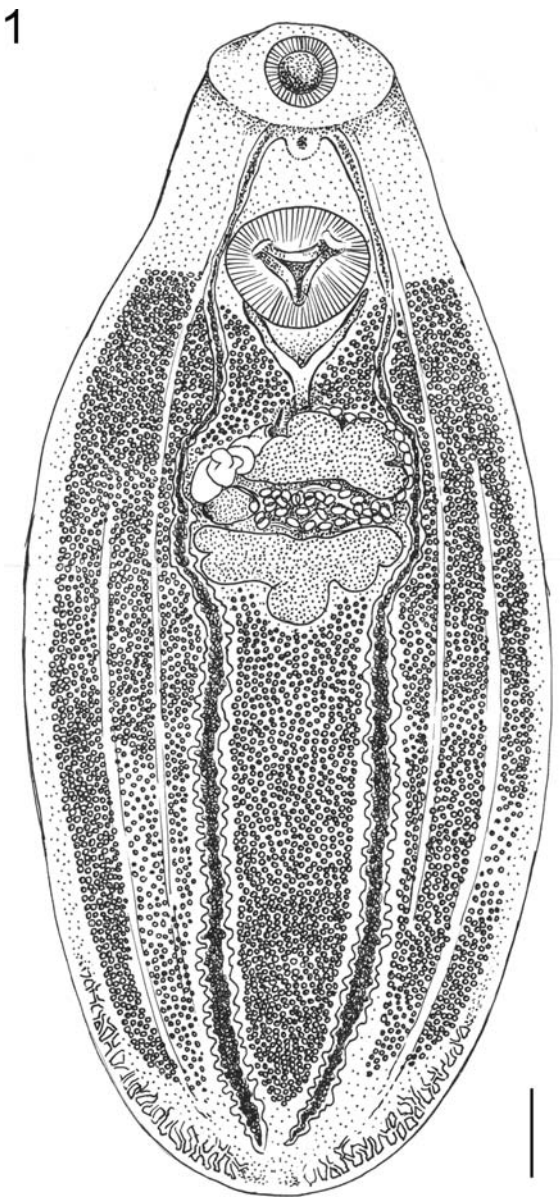


Fig. 1 Adult *Clinostomuum cutaneum. Scale-bar*: 400 μm

reported in Table 4. *C. ophicephali* and *C. sorbens* are not listed here because they were moved to *Clinostomoides* Dollfus, 1950 and *Clinostomatopsis* Dollfus, 1932, respectively (Yamaguti, 1971).

Molecular analysis

The comparisons of the rRNA sequences (entire 18S-ITS1-5.8S-ITS2 and partial 28S) of the adult stage of *C. cutaneum* (GQ339114, present study) with the morphologically similar metacercaria (FJ609421, present study) displayed 100% identity, indicating that they are different developmental stages of the same parasite. The 18S rRNA gene amplified resulted in a 1,913-bp fragment. The consensus sequence analysed by BLAST search gave a 99.8% identity with *C. phalacrocoracis* (FJ609422–FJ609423), 99.1% with *C. complanatum* (AY245701, FJ609420), 99% with *C. marginatum* (AY245760), 99.3% (97% coverage) with *Clinostomum* sp. Australia-PO-2003 (AY222094) and 99% (97% coverage) with *Clinostomum* sp. USA-PO-2003 (AY222095). The alignment of the 18S rRNA of the above-mentioned sequences showed few differences between the species, with distances ranging from 0.1% to 1.7% (Table 5).

In the case of the ITS rRNA region, the total length of the sequence was 1,030 bp: 584 bp belonged to ITS1, 159 bp to 5.8S and 287 bp to ITS2 rRNA. The BLAST analysis gave 98.7% identity with *C. phalacrocoracis* (FJ609422–FJ609423), 97.2% with *C. complanatum* (FJ609420), 97.3% (96% coverage) with *C. complanatum* (AY245701) and, limited to the ITS2 spacer, 95.5% (30% coverage) with *Clinostomum* sp. Australia-MJN-2004 (AY465871).

The alignment of the entire ITS sequences carried out in this research and comprising *C. cutaneum* (FJ609421), *C. phalacrocoracis* (FJ609422–FJ609423) and *C. complanatum* (FJ609420, AY245701) revealed distances ranging from 1.2% to 2.1% over 989 bp (Table 6). Splitting the results, the alignment showed that the 5.8S rRNA gene is identical among the species, whereas the ITS1 (584 bp) of *C. cutaneum* exhibited a 2.5% distance from *C. complanatum* (FJ609420, AY245701) and 1.4% from *C. phalacrocoracis* (FJ609422–FJ609423); and in the ITS2 sequence the divergence was 2.6% and 1.5%, respectively.

The sequence of the 28S rRNA obtained included only the first part of the gene and was 1,671 bp. The BLAST search gave a 99.9% identity with *C. phalacrocoracis* (FJ609422–FJ609423), 99.5% with *C. complanatum* (FJ609420), 99.3% (74% coverage) with *Clinostomum* sp. Australia-PO-2003 (AY222175) and 98.2% (74% coverage) with *Clinostomum* sp. USA-PO-2003 (AY222176). The difference observed between *C. cutaneum* and *C. phalacrocoracis* was only one nucleotide (0.1% divergence), and the greatest difference observed was 1.7% with *Clinostomum* sp. (AY222176) (Table 7).

Table 3 Measurements of adult *Clinostomum cutaneum*

Clinostomum cutaneum	Min–max (mean ± SD)
Oral collar width	933–1,196 (1,039 ± 79.2)
Body length	3,990–5,610 (4,928 ± 440.9)
Body width	2,071–3,014 (2,409 ± 277)
Body length/width	1.68–2.47 (2.06 ± 0.23)
Oral sucker length	209–302 (249 ± 38.46)
Oral sucker width	335–430 (382 ± 36.91)
OS width/body width	0.11–0.19 (0.16 ± 0.03)
Ventral sucker length	554–857 (698 ± 83.9)
Ventral sucker width	612–945 (789 ± 98.2)
VS width/OS width	1.43–2.74 (2.09 ± 0.39)
VS width/body width	0.26–0.37 (0.33 ± 0.03)
Distance between sucker	463–689 (603 ± 86.9)
Anterior testis length	217–532 (393 ± 85.4)
Anterior testis width	732–1,200 (949 ± 121.3)
AT width/length	1.93–4.2 (2.52 ± 0.65)
Posterior testis length	264–595 (390 ± 100)
Posterior testis width	879–1,290 (1,054 ± 141.6)
PT width/length	1.96–3.58 (2.82 ± 0.58)
Distance between testes	220–404 (297 ± 57.3)
Ovary length	86–269 (198 ± 55.2)
Ovary width	188–358 (278 ± 48.2)
Ovary width/length	1.03–2.8 (1.5 ± 0.54)
Cirrus-sac length	152–344 (221.6 ± 55.9)
Cirrus-sac width	173–328 (251.3 ± 45.5)
Cirrus-sac length/body length	0.03–0.07 (0.05 ± 0.01)
Eggs	88–117 (102.8 ± 5.9) × 52–63 (60.4 ± 1.9)

Discussion

Clinostomum cutaneum, redescribed herein, shows a unique morphological feature: a Y-shaped uterus in the metacercarial stage and a heart-shaped uterus, when filled with eggs, in the adult. None of the species presently considered valid (Ukoli, 1966; Yamaguti, 1971; Matthews & Cribb, 1998) display this character.

At the metacercarial stage, all of the features observed are consistent with the *Clinostomum* sp. metacercariae described by Paperna (1964a) from the skin and muscle of *Tilapia zilli*, *T. nilotica*, *T. galilaea* and *Tristramella simonis* in Israel and reported later as *C.* "*cutaneum*" by Paperna (1964b). The adult stage of this species has never been fully described, but a sub-adult specimen from an experimentally-infected black-crowned night-heron *Nycticorax nycticorax* was reported by Finkelman (1988).

A comparison of our description with the original made by Paperna (1964a) indicates a high similarity in terms of both measurements and the morphology of the body. In this study, more detail is provided of the genital complex and the presence of glandular contents in the forebody. Moreover, the intestinal caeca had connections to the excretory system via very thin ducts which were not evident in adults, as previously noted by Yamaguti (1971), with a V-shaped excretory vesicle opening to the exterior as a uroproct via a well-defined excretory pore. The excretory pore was not visible under the SEM, perhaps because of the presence of a cuticular folder, as reported by Dubois (1930) in *C. phalacrocoracis*.

The observations carried out on the adult allowed us to describe the cirrus as a well-developed organ with basal papillae arranged in a regular pattern. This organ, which has not been described in previous morphological studies, may represent a possibly useful character for differentiating *Clinostomum* species. The vitelline follicles of *C. cutaneum* reach to the level of the ventral sucker, as in the majority of the species, but not in *C. kassimovi* Vaidova & Feizullaev, 1958, in which they reach into the forebody. The gonads are located in the middle third of the body, as in *C. kassimovi* and *C. wilsoni* Matthews & Cribb, 1998, but unlike the other valid species. The cirrus-sac is anterior to the ovary, as in species other than *C. tilapiae* Ukoli, 1966, in which it is at the same level, and *C. kassimovi*, in which it is between the testes. Finally, the genital pore opens close to the anterior margin of the anterior testis, as in other *Clinostomum* species, with the exception of *C. attenuatum* Cort, 1913 and *C. phalacrocoracis*, in which it opens at ovarian level and posterior to the anterior testis, respectively.

In order to contribute to the known molecular data on *Clinostomum* species, we sequenced almost all of the rRNA (18S-ITS1-5.8S-ITS2 and partial 28S) of some of the parasites collected during this study: adult and metacercariae *C. cutaneum* (GQ339114, FJ609421), *C. phalacrocoracis* (FJ609423–FJ609422), both adults from the oesophagus of grey herons, and metacercariae from the gill-arches of Nile tilapias in the same fish

Table 4 Major morphological characters of *Clinostomum* species described at the adult stage

Clinostomum species	Uterus	Vitelline extent	Testes position	Cirrus-sac position	Genital pore position
C. cutaneum Paperna, 1964 (present study)	Heart-shaped	To ventral sucker	T1–T2 Middle third	Anterior to ovary at T1 level	Close to T1
C. complanatum (Rudolphi, 1814)	Single	To ventral sucker	T1 middle third T2 posterior third	Anterior to ovary at T1 level	Close to T1
C. attenuatum Cort, 1913	Single	To ventral sucker	T1–T2 posterior third	Anterior to ovary between testes	Close to ovary
C. australiense Johnston, 1917	Single	To ventral sucker	T1 middle third T2 posterior third	Anterior to ovary at T1 level	Close to T1
C. detruncatum Braun, 1899	Single with lateral branches	To ventral sucker	T1–T2 posterior third	Anterior to ovary at T1 level	Close to T1
C. heluans Braun, 1899	Single	To ventral sucker	T1–T2 posterior third	Anterior to ovary at T1 level	Close to T1
C. hornum Nicoll, 1914	Single	To ventral sucker	T1 middle third T2 posterior third	Anterior to ovary at T1 level	Close to T1
C. intermediale Lamont, 1920	Single	To ventral sucker	T1 middle third T2 posterior third	Anterior to ovary between testes	Close to T1
C. kassimovi Vaidova & Feizullaev, 1958	Single	Not reaching ventral sucker	T1–T2 middle third	Between testes	Close to T1
C. marginatum (Rudolphi, 1819)	Single	To ventral sucker	T1 middle third T2 posterior third	Anterior to ovary at T1 level	Close to T1
C. phalacrocoracis Dubois, 1930	Single	To ventral sucker	T1–T2 posterior third	Anterior to ovary between testes	Posterior to T1
C. tilapiae Ukoli, 1966	Single	To ventral sucker	T1 middle third T2 posterior third	Same level as ovary	Close to T1
C. wilsoni Matthews & Cribb, 1998	Single	To ventral sucker	T1–T2 middle third	Anterior to ovary at T1 level	Close to T1

farm, plus metacercariae of *C. complanatum* (FJ609 420) collected in Italy from wild barbel. Comparison of the alignments obtained from sequences of the 18S, 5.8S and 28S rRNA genes and the internal transcribed spacer regions ITS1 and ITS2 of *C. cutaneum* with the *Clinostomum* sequences available in GenBank produced interesting results.

The alignment of the 18S rRNA exhibited few differences between the species, with distances of 0.1–1.7% (Table 5). As described by Hillis & Dixon (1991), this region is highly conserved and is characterised by a slow evolutionary rate, useful for evaluating ancient evolutionary events but useless, in some cases, for discriminating organisms at the species level. In fact, *C. cutaneum* and *C. phalacrocoracis* differ at only three nucleotides, with 99.8% identity over the *c*. 1,913 bp fragment of 18S rRNA, even though the parasitological analysis showed that these parasites are clearly morphologically distinct species.

In the case of the 5.8S rRNA gene, the alignment showed no differences between the species. Several authors (Hillis & Dixon, 1991; Hershkovitz & Lewis, 1996; Coleman, 2003) described this gene as characterised by a high level of conservation similar to that of the 18S rRNA gene, so it is little used in phylogenetic studies (Troitsky & Bobrova, 1986; Troitsky et al., 1991; Suh et al., 1992). In addition, this coding region is too short, *c*. 160 bp in *Clinostomum* species, to produce robust phylogenies across large time scales.

The internal transcribed spacers, ITS1 and ITS2, are relatively conserved regions within species or genera. They have been used as markers in population genetic studies (Hillis & Dixon, 1991) and to explore species boundaries in at least 19 digenean families (Nolan & Cribb, 2005). ITS1 is characterised by the presence of tandem repeat units at the 5′ end that provide the variability in the sequence composition at both the interspecific and intraspecific level.

Table 5 Pairwise distance in the *Clinostomum* spp. sequences available, comparing a *c.* 1,095-bp fragment of 18S rRNA and the identity values obtained by BLAST analysis (in bold)

	1	2	3	4	5	6	7	8	9
1		**100%**	**99.8%**	**99.8%**	**99.1%**	**99.1%**	**99.0%**	**99.3%***	**99.0%***
2	0								
3	0.3	0.3							
4	0.4	0.4	0						
5	1.3	1.3	1.6	1.7					
6	1.3	1.3	1.6	1.7	0				
7	1.3	1.3	1.6	1.7	1.4	1.4			
8	1.1	1.1	1.4	1.5	0.9	0.9	1.5		
9	1.2	1.2	1.5	1.6	1.3	1.3	0.1	1.4	

[1] *C. cutaneum* (GQ339114); [2] *C. cutaneum* (FJ609421); [3] *C. phalacrocoracis* (FJ609423); [4] *C. phalacrocoracis* (FJ609422); [5] *C. complanatum* (AY245701); [6] *C. complanatum* (FJ609420); [7] *C. marginatum* (AY245760); [8] *Clinostomum* sp. Australia-PO-2003 (AY222094); [10] *Clinostomum* sp. USA-PO-2003 (AY222095)

*97% coverage

Table 6 Pairwise distance in the *Clinostomum* spp. sequences available, comparing a *c*. 989-bp fragment of ITS rRNA and the identity values obtained by BLAST analysis (in bold)

	1	2	3	4	5	6
1		**100%**	**98.7%**	**98.7%**	**97.2%**	**97.3%***
2	0					
3	1.2	1.2				
4	1.2	1.2	0			
5	2.1	2.1	2.1	2.1		
6	2.1	2.1	2.1	2.1	0	

[1] *C. cutaneum* (GQ339114); [2] *C. cutaneum* (FJ609421); [3] *C. phalacrocoracis* (FJ609423); [4] *C. phalacrocoracis* (FJ609422); [5] *C. complanatum* (FJ609420); [6] *C. complanatum* (AY245701)

*96% coverage

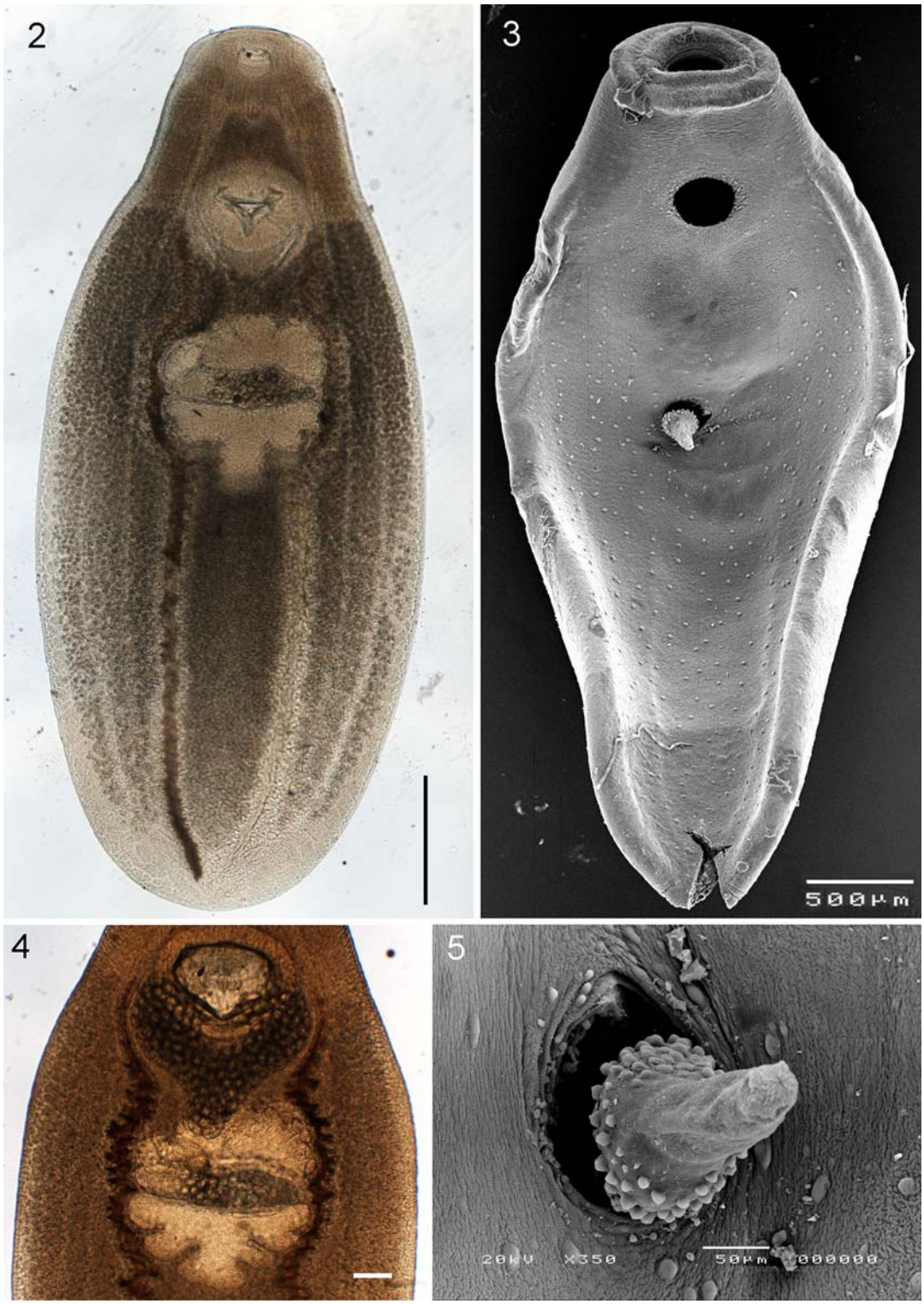
These repeats are known in some digenetic trematode families, such as the Haematoloechidae, Mesometridae, Opecoelidae, Schistosomatidae, Strigeidae and Telorchiidae (see Nolan & Cribb, 2005), but no information is available for the Clinostomidae. The analysis of *C. cutaneum*, *C. phalacrocoracis* and *C. complanatum* indicated no repeat units in these species. No molecular data are available in GenBank for other clinostomid genera. The ITS1 sequences examined were 584 bp long, and no intraspecific variation in the length of the ITS1 was observed, unlike the observations of others authors, such as van Herwerden et al. (1998) for *Schistosoma* spp. and Dvořák et al. (2002) for *Trichobilharzia* spp. The different lengths of ITS1 are due to the presence of

Table 7 Pairwise distance in the *Clinostomum* spp. sequences available, comparing a *c*. 1,258-bp fragment of 28S rRNA and the identity values obtained by BLAST analysis (in bold)

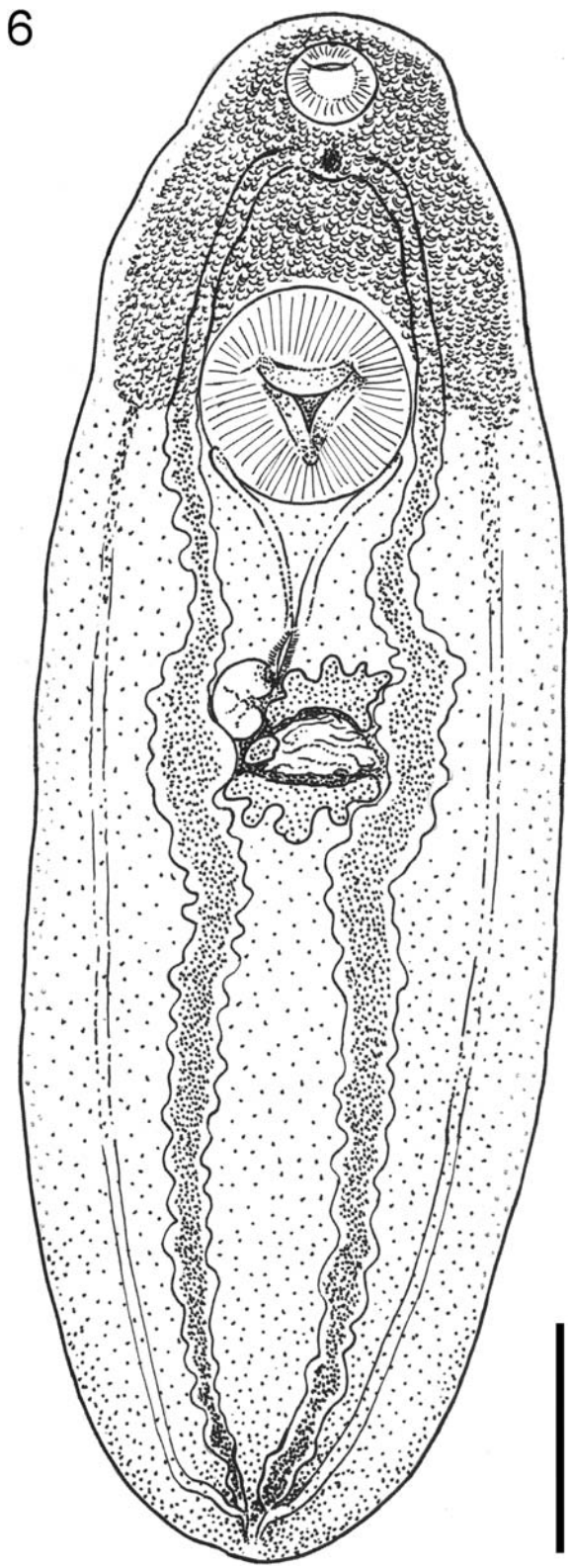
	1	2	3	4	5	6	7
1		**100%**	**99.9%**	**99.9%**	**99.5%**	**99.3%***	**98.2%***
2	0						
3	0.1	0.1					
4	0.1	0.1	0				
5	0.6	0.6	0.5	0.5			
6	1.7	1.7	1.8	1.8	1.6		
7	0.6	0.6	0.6	0.6	0.9	2.0	

[1] *C. cutaneum* (GQ339114); [2] *C. cutaneum* (FJ609421); [3] *C. phalacrocoracis* (FJ609423); [4] *C. phalacrocoracis* (FJ609422); [5] *C. complanatum* (FJ609420); [6] *Clinostomum* sp. Australia-PO-2003 (AY222175); [7] *Clinostomum* sp. USA-PO-2003 (AY222176)

*74% coverage



Figs. 2–5 Adult *Clinostomuum cutaneum*: 2. Light micrograph of entire worm. 3. SEM micrograph of entire worm. 4. Light micrograph of genital complex and the egg-filled uterus. 5. SEM micrograph of everted cirrus. *Scale-bars*: 2, 800 µm; 3, 500 µm; 4, 250 µm; 5, 50 µm



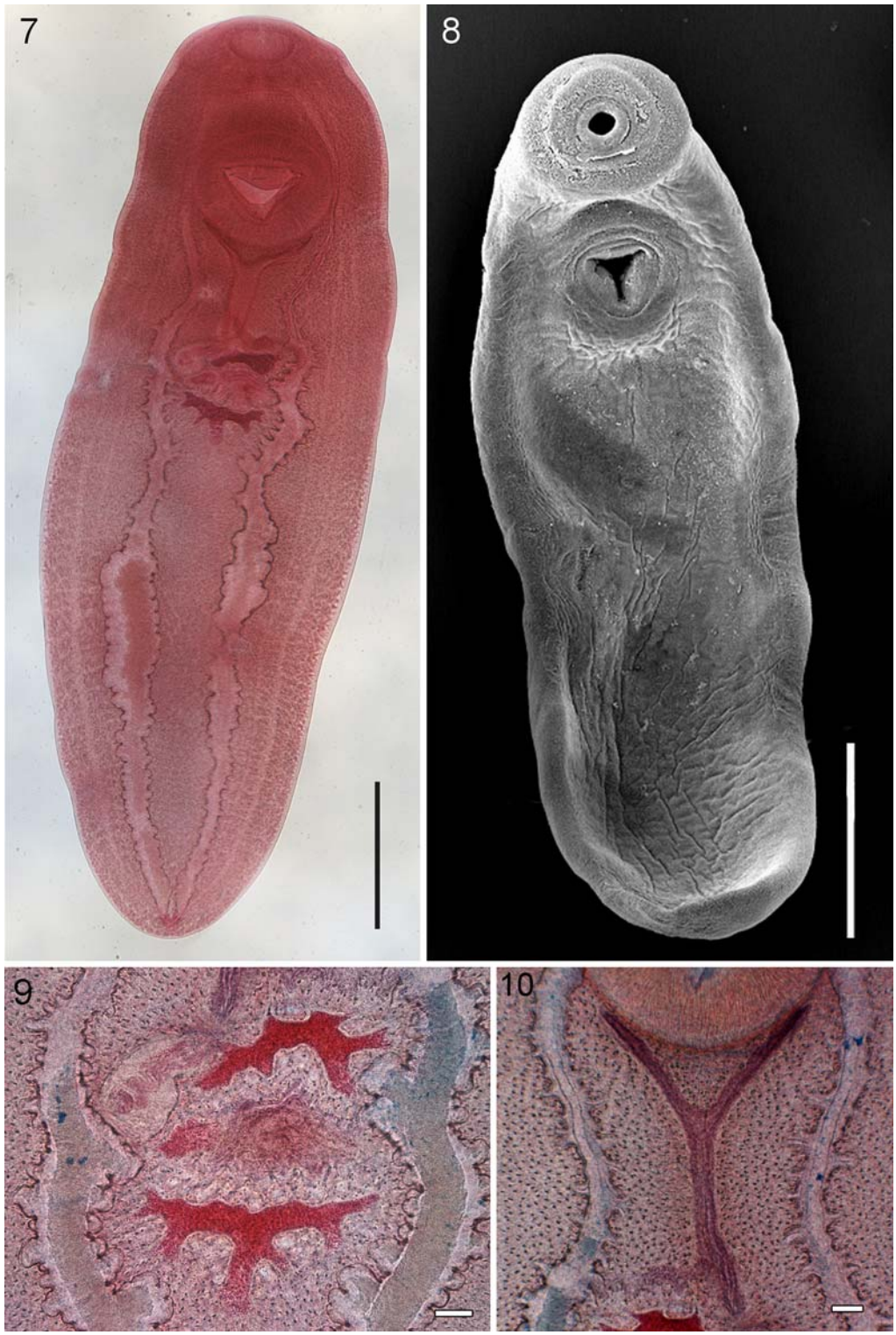
◀**Fig. 6** Metacercaria of *Clinostomuum cutaneum*. *Scale-bar*: 800 μm

the repeat elements (Luton et al., 1992; Kane & Rollinson, 1994; Kane et al., 1996), which in our case are absent, as described above. The ITS2 spacer is characterised by a lower degree of variation with a high degree of conservation at the species level. Usually, it does not contain repeat units, although Morgan & Blair (1995) found repeats in the Echinostomatidae. It is also characterised by differences in length within and between families. In our sequences, we found different lengths: 287 bp in *C. cutaneum* and *C. phalacrocoracis* and 283 bp in *C. complanatum*; moreover, we did not observe intraspecific variations as described by other authors for other digeneans (Luton et al., 1992; Galazzo et al., 2002; Jousson & Bartoli, 2002). The 28S rRNA gene is longer and has more variations in the rate of evolution than the 18S rRNA. This gene has been used in phylogenetic studies (Olson et al., 2003). The alignment of the 28S rRNA sequences obtained showed very low differences between the sequences compared, particularly between *C. cutaneum* and *C. phalacrocoracis* with only one nucleotide difference. Thus, like 18S rRNA, this region has little value for distinguishing species.

Even though sequencing DNA represents the primary approach of modern systematics, the combination with traditional techniques, such as the use of morphological parameters, is essential for describing parasites at the species level. In fact, as suggested by Nolan & Cribb (2005), the best way to approach species identification is to perform morphological descriptions on half the specimens and molecular analysis on the other half, as carried out in this research.

In this study, we described for the first time the adult stage of *C. cutaneum* Paperna, 1964 from grey herons, the definitive host of the parasite, combining a traditional morphological approach with molecular analyses. Besides sequencing the DNA of our parasite, we were also able to link the adult stage to the metacercarial stage found in Nile tilapia from the same environment, confirming the morphological observations.

Following the suggestion of Matthews & Cribb (1998), who suggested the need for a reorganisation



Figs. 7–10 Metacercaria of *Clinostomuum cutaneum*: 7. Light micrograph of entire worm. 8. SEM micrograph of entire worm. 9. Light micrograph of developing genital complex. 10. Light micrograph of Y-shaped uterus. *Scale-bars*: 7, 1 mm; 8, 1.2 mm; 9, 10, 100 μm

of the genus *Clinostomum* using both morphological and molecular approaches, this study hopefully represents the first input towards a systematic revision of this complex group of parasites.

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