**SEM evidence for existence of an apical disc on the scolex of *Clestobothrium crassiceps* (Rudolphi, 1819): comparative results of various fixation techniques**

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**Abstract**

The scolex of the bothriocephalidean cestode *Clestobothrium crassiceps* was studied by means of scanning electron microscopy (SEM). The comparative results of various fixation procedures are presented. The scolex of *C. crassiceps* is oval to globular and exhibits two deep bothria which appear in the form of two lobes separated by a longitudinal groove. At the apex of the scolex, resembling a beret, an apical disc is present (oval, flattened and with a sinuous edge). Our results are compared with those previously reported in other species of *Clestobothrium*. This study representes the first report which highlights the presence of an apical disc in the scolex of *C. crassiceps*. It describes the effects of different procedures applied to our material during preparation and a comparative analysis results obtained using these various methods.

**Keywords:** *Clestobothrium crassiceps*, Bothriocephalidea, Cestoda, apical disc, scolex, SEM

**Running head:** Apical disc on *Clestobothrium crassiceps* scolex

## Introduction

Species belonging to the order Bothriocephalidea are predominantly intestinal parasites of teleost fish. This recently erected order contains 46 genera distributed into four families (Bothriocephalidae, Echinophallidae, Philobythiidae and Triaenophoridae) and was formerly included in the suppressed order "Pseudophyllidea" (Kuchta *et al.* 2008a,b). According to Kuchta *et al.* (2008a), the study of the bothriocephalidean cestodes presents numerous difficulties because only a few characters can be used for the diagnosis and differentiation of the members of this order, and the species are morphologically quite homogeneous. Species included in this order are typified mainly by the possession of two bothria on the scolex (Yamaguti 1959, Schmidt 1986, Bray *et al.* 1994). These bothria, located ventrally and dorsally, present different shapes and depths depending on the individual taxa.

The genus *Clestobothrium* belongs to the family Bothriocephalidae along with 13 other valid genera (see Kuchta *et al.* 2008a). The genera belonging to this family are characterized by the median position of the genital pore. Among other characters, the absence of an apical disc in the scolex traditionally characterized species of *Clestobothrium* (Bray *et al.* 1994, Kuchta *et al.* 2008a).

The present contribution demonstrates for the first time the presence of a well-developed apical disc in the scolex of *Clestobothrium crassiceps* (Rudolphi, 1819).

## Materials and methods

Live adult specimens of *C. crassiceps* were collected from the intestine of the European hake *Merluccius merluccius* (Linnaeus, 1758) (Gadiformes: Merlucciidae) caught by artisanal fisheries in the littoral region off Girona (NE of Spain).

Live cestodes were fixed using different fixative agents for the morphoanatomical study, but also for transmission and scanning electron microscope analysis. Thus, some specimens were fixed in hot 4% formaldehyde and in hot 70% ethanol, and later stored in 70% ethanol. Other specimens were fixed in cold 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.4. For the present SEM study, cestodes fixed according to these three methods were treated with 1% osmium tetroxide, dehydrated through an ethanol series, and critical point dried with carbon dioxide in a Polaron CPD 7501. Finally, specimens were mounted on stubs with an adhesive tape and colloidal silver, sputter-coated with gold in a Fisons Instrument SC 510, and examined using a Zeiss DSM 940A scanning electron microscope at 15 kV.

**Results**

Electronmicrographs show that the scolex of *C. crassiceps* is oval to globular and divided by longitudinal grooves, highlighting two dorsoventral hemispheres, each presenting a deep bothrium (Figs 1A,B, 2A, 3A,B). A well-developed apical disc is present (Figs 1A-D, 2A,C,D, 3A,B). This is an oval structure, with undulating margins and situated at the anterior extremity of both hemispheres, resembling a beret (Fig. 1A,C,D). However, the aspect of this apical disc is variable depending on the above-mentioned fixation methodologies. Surprisingly, the apical disc exhibits an extended aspect when the fixation used was cold glutaraldehyde (Fig. 1A-D), whereas hot formaldehyde produced specimens with an apical disc which was more contracted and invaginated (Fig. 2A-D). The latter are the specimens that exhibit a lip-like appearance of the apical disc. On the other hand, cestodes fixed in hot ethanol exhibit an intermediate morphology of the apical disc (Fig. 3A,B).

**Discussion**

Morphologically, species of *Clestobothrium* differ from other bothriocephalids because they possess a sphincter surrounding the aperture of the bothria (Schmidt 1986, Bray *et al.* 1994). To date, this genus comprises only five valid species: (1) *C. crassiceps*, (2) *C. gibsoni*, (3) *C. neglectum* (see Kuchta *et al.* 2008b), and two recently erected new species (4) *C. cristinae* and (5) *C. splendidum* (see Gil de Pertierra *et al.* 2011).

Species of *Clestobothrium* are prevalent mainly among the Gadiformes and the type-species of the genus, *C. crassiceps* (Rudolphi, 1819), is usually encountered parasitizing the European hake *M. merluccius* but has been reported from other fishes (Gil de Pertierra *et al.* 2011). It has a wide distribution, including the Mediterranean Sea, the North Sea, and both Atlantic and Pacific Oceans (Schmidt 1986, Bray *et al.* 1994, Kuchta *et al.* 2008a, Gil de Pertierra *et al.* 2011).

Numerous authors (Rees 1958, Bray *et al.* 1994, Kuchta *et al.* 2008a) failed to mention the presence of an apical disc in the scolex of *C. crassiceps*. Therefore, for many years, *C. crassiceps*, as well as *C. gibsoni* and *C. neglectum*, have been considered as lacking this stucture (Lühe 1899; Dronen and Blend 2003, 2005). However, the presence of an apical disc was reported in both of the recently described species belonging to *Clestobothrium* (*C. cristinae* and *C. splendidum*) by Gil de Pertierra *et al.* (2011). The latter work also pointed out the existence of this apical structure in *C. crassiceps* where, based on the observation of Figure 1K in the revision Kuchta *et al.* (2008a), it was described as a *"weekly developed apical disk"* [sic]. Unfortunately, the orientation of the scanning electron micrograph presented in Kuchta *et al.* (2008a) does not permit the recognition of an apical disc as demonstrated in both of the other species by Gil de Pertierra *et al.* (2011). Moreover, Kuchta *et al.* (2008a), in the diagnosis of *Clestobothrium*, remarked on the absence of an apical disc. Thus, the

present report clearly demonstrates the presence of an apical disc in a third species of *Clestobothrium* and shows for the first time the presence of this structure in *C. crassiceps*. Thus, in our opinion, the presence of this apical disc in the scolex is probably a typical character of the genus, but its presence in *C. gibsoni* and *C. neglectum* needs be verified.

*Clestobothrium crassiceps*, as is the case for other species of the genus, is a medium-sized cestode, flattened dorsoventrally and presenting a more or less globular scolex. According to Dronen and Blend (2003), the identification of species of the genus is largely based on the morphology of the scolex, which is robust and generally oval to spherical. Unlike the other species of *Clestobothrium*, the scolex of *C. crassiceps* is much less robust and the bothria are deeper and larger.

The apical disc in *C. cristinae* and *C. splendidum* forms two lip-like structures (see Gil de Pertierra *et al.* 2011). This is the same appearance as observed in the present study of *C. crassiceps* when specimens were fixed in hot formaldehyde (see Fig. 2A,C,D). However, in *C. crassiceps* the apical disc is larger than in *C. cristinae* and *C. splendidum*. Specimens of *C. crassiceps* fixed in cold glutaraldehyde exhibit a stretched apical disc covering the entire apical scolex surface, lacking a central invagination but bearing a sinuous edge (see Fig. 1A-D).

After necropsy of the host, observation of live specimens under a stereomicroscope during dissection of intestinal tract showed the scolex to be very motile with a clearly visible apical disc resembling a beret. The similar appearance of an apical disc in both live specimens and in specimens fixed in cold glutaraldehyde may be explained by similar conditions of temperature in the intestinal habitat of this marine cestode. In relation to this, there are many studies concerning the different techniques of fixation and preservation for helminths. One of the problems during fixation is the

contraction of worms in different ways and to different extents which is not only influenced by the fixative used but also by the general condition of the specimens at fixation. Moreover, in order to choose the best fixative, the importance and influence of other aspects, such as the size of cestode, the possible presence of caducous rostellar hooks or the fragility of specimens, have been emphasised by several authors (see Gibson 1984, Andersen and Gibson 1989).

Our observations confirm the morphological features described by other authors for species of *Clestobothrium,* especially the presence of an apical disc in the type-species, and indciate possible inter-specific variation in the shape of both the scolex and the apical disc.

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**References**

Andersen K.I., Gibson D.I. 1989. A key to three species of larval *Diphyllobothrium* Cobbold, 1858 (Cestoda: Pseudophyllidea) occurring in European and North American freshwater fishes. *Systematic Parasitology*, 13: 3–9.

Bray R.A., Jones A., Andersen K.I. 1994. Order Pseudophyllidea Carus, 1863. In: (Eds. L.F. Khalil, A. Jones and R.A. Bray) *Keys to the cestodes parasite of vertebrates*. CAB International, Wallingford, UK, 205–247.

Dronen N.O., Blend C.K. 2003. *Clestobothrium neglectum* (Lönnberg, 1893) n. comb. (Cestoda: Bothriocephalidae) from the tadpole fish *Raniceps raninus* (L.) (Gadidae) from Sweden. *Systematic Parasitology*, 56, 189–194. DOI: 10.1023/B:SYPA.0000003805.07946.cd

Dronen N.O., Blend C.K. 2005. *Clestobothrium gibsoni* n. sp. (Cestoda: Bothriocephalidae) from the bullseye grenadier *Bathygadus macrops* Goode & Bean (Macrouridae) in the Gulf of Mexico. *Systematic Parasitology*, 60, 59–63. DOI: 10.1007/s11230-004-1376-7

Gibson D.I. 1984. Technology as applied to museum collections: the collection, fixation and conservation of helminths. *Systematic Parasitology*, 6, 241–255.

Gil de Pertierra A.A., Incorvaia I.S., Arredondo N.J. 2011. Two new species of *Clestobothrium* (Cestoda: Bothriocephalidea), parasites of *Merluccius australis* and *M. hubbsi* (Gadiformes: Merlucciidae) from the Patagonian shelf of Argentina, with comments on *Clestobothrium crassiceps* (Rudolphi, 1819). *Folia Parasitologica*, 58, 121–134.

Kuchta R., Scholz T., Brabec J., Bray R.A. 2008b. Suppression of the tapeworm order Pseudophyllidea (Platyhelminthes: Eucestoda) and the proposal of two new orders, Bothriocephalidea and Diphyllobothriidea. *International Journal for Parasitology*, 38, 49–55. DOI: 10.1016/j.ijpara.2007.08.005

Kuchta R., Scholz T., Bray R.A. 2008a. Revision of the order Bothriocephalidea Kuchta, Scholz, Brabec & Bray, 2008 (Eucestoda) with amended generic

diagnoses and keys to families and genera. *Systematic Parasitology*, 71, 81–136. DOI: 10.1007/s11230-008-9153-7

Lühe M. 1899. Zur Anatomie und Systematik der Bothriocephaliden. *Verhandlungen der Deutschen Zoologischen Gesellschaft*, 9, 30–55.

Rees G. 1958. A comparison of the structure of the scolex of *Bothriocephalus scorpii* (Müller 1776) and *Clestobothrium crassiceps* (Rud. 1819) and the mode of attachment of the scolex to the intestine of the host. *Parasitology*, 48, 468–492. DOI:10.1017/S0031182000021399

Schmidt G.D. 1986. *CRC handbook of tapeworm identification*. Boca Raton: CRC Press, 675 pp.

Yamaguti S. 1959. Systema Helminthum. Vol. II . *The Cestodes of Vertebrates*. Interscience Publishers Inc., New York, USA, 860 pp.

**Figure captions**

**Fig. 1.** SEM micrographs of the scolex of *Clestobothrium crassiceps* fixed in cold 2.5% glutaraldehyde. **A.** Ventrolateral view of the scolex. **B.** Ventral view of the scolex. **C.** Ventrolateral view of the apical disc. **D.** Apical view of the apical disc.

**Fig. 2.** SEM micrographs of the scolex of *Clestobothrium crassiceps* fixed in hot 4% formaldehyde. **A.** Ventrolateral view of the scolex. **B.** Detail of the apical disc-bothrium junction. **C.** Apical view of the apical disc. **D.** Ventrolateral view of the apical disc.

**Fig. 3.** SEM micrographs of the scolex of *Clestobothrium crassiceps* fixed in hot 70% ethanol. **A.** Lateral view. **B.** Apical view.

**Abbreviations to all figures:** AD – apical disc, B – bothrium, G – longitudinal groove.