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Morphological characterization of two new species of *Dicontophrya* (Ciliophora: Astomatia: Contophryidae) commensal of earthworms (Oligochaeta: Annelida) of Ebebda and Nkolbikogo (Cameroon)

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Abstract

Two new species of astomatous ciliates (*Dicontophrya minus* n. sp. and *Dicontophrya ebebdaensis* n. sp.) endocommensal of Glossoscolecidae earthworms pertaining to the genus *Alma* are described using light-microscopy and a combination of staining techniques (pyridinated ammoniacal siver nitrate and DAPI). Studies reveal the existence of two distinct morphological types as evidence of morphological diversification within the genus *Dicontophrya* de Puytorac and Dragesco, 1969. In the first morphological type represented by *Dicontophrya minus* n. sp., the cell is ovoid (90 x 125 – 35 x 115 µm) with a ribbon like axial macronucleus flanked with a relatively big micronucleus (6.2 µm in diameter). In the second morphological type (*Dicontophrya ebebdaensis* n. sp.), the cell is elongated and wormlike (180 x 215 – 35 x 50 µm) with a nuclear apparatus composed of a long ribbon shape macronucleus (171.1 µm), bearing in its posterior half a globulous micronucleus. In the two cases cells bear in the anterior end a depression in which lodges a skeletal apparatus build in the same plan of organization (a modified V shaped skeletal branch bearing about 7 skeletal fibers) that characterize the genus.

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Introduction

The gut of oligochaetes Annelids can be considered as a succession of many contiguous microhabitats (de Puytorac & Mauret, 1956; Nana *et al.*, 2014; Fokam *et al.*, 2015) in which many microorganisms work cooperatively in the process of soil mineralization (Lavelle, 1997). Diverse ciliated unicellular organisms constitute the major component of this complex microbial community among which mouthless ciliates belonging to the Subclass Astomatia Schewiakoff, 1896, have been only covered by a few studies over the past four decades (de Puytorac and Dragesco, 1968, 1969; de Puytorac, 1969, 1972; Ngassam, 1983; Fokam, 2005; Fokam *et al.,* 2008 ; Fokam *et al.*, 2011, 2012,).

Much work remains to be done for understanding the contribution of astomatous ciliates to earthworms-mediated soil mineralization and for analyzing their diversity as well as host-specificity. Since the Astomes are deprived of an oral region, classification within this enigmatic group of ciliate is based on four major features: (1) the presence and the nature of an attachment apparatus; (2) the presence, arrangement, and number of suture lines in the somatic ciliature; (3) the size and shape of the nuclear apparatus; and (4) the number and arrangement of pulsatile vacuoles.

The aim of the present study was to pursue the inventory of astomatous ciliates living in the gut of earthworms from Cameroon.Two new species are described on the basis of standard cytological methods and fluorescence labeling of nuclear and skeletal components. Morphological traits revealed through this dual approach, especially the topography of the attachment apparatus, clearly indicate the two ciliates as species belonging to the genus *Dicontophrya* de Puytorac and Dragesco, 1969, from *Alma nilotica* collected in the southwestern part of the center region of Cameroon.

Material and methods

Cell isolation procedure

Earthworms were collected between October 2013 and March 2014 on the banks of Sanaga River at Ebebda, 60 km north of Yaoundé (4°00' - 4°30' N; 11°30' - 11°50' E) and Nyong River at Nkolbikogo (3° 65 - 4° N; 11° 30' - 11°70' E) (Fig. 1). Worms were identified as belonging to the family Glossoscolecidae and to the genus *Alma* sp. (Fig. 2), according to the keys described by Sims and Gerard (1999).

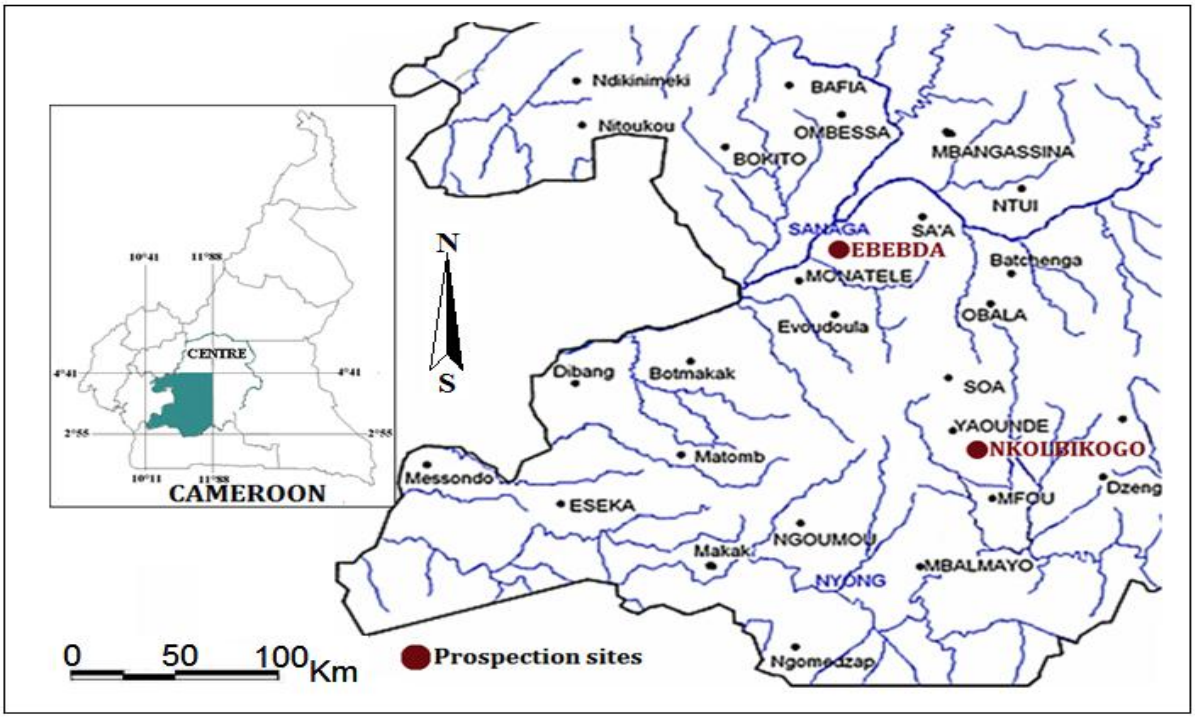

Fig. 1. The study area (National institute of cartography, 2011, modified, 2015).

Fragments of the digestive tract pertaining to each of the tree main portions (fore-, mid-, and hindgut) were opened under a binocular dissecting microscope Wild M5 (Heerbrugg, Germany) at 250 X magnification. This was done in a petri dish containing either a physiological Ringer's solution or a commercial mineral water (Volvic™ in France or Supermont™ in Cameroon). The ciliates were removed from the ingesta using micropipettes, counted, washed further, and then observed in vivo before fixation for cytology.

Cell labeling

For current observations, cells were stained using the ammoniacal pyridinated silver carbonate technique of Fernandez-Galiano (1994). The nuclear apparatus was colored using 4',6-Diamidino-2-Phenyl Indole (DAPI) (Williamson & Fennell, 1975) before observation under a Leica DMR epifluorescence microscope. Aliqotes of DAPI-treated cells were counterstained by immunofluorescence labeling with a FITC–conjugated anti-tubulin antibody as described in a previous study (Diogon *et al.* 2001).

Measurement of cytological parameters

The shape and mobility of ciliates were noted before cell fixation. All of the cell measurements were made with a calibrated ocular micrometer. The following parameters were calculated: arithmetic mean; standard deviation; minimum and maximum values. Morphometric data were gathered by the examination of groups of 30 separate cells of each species. Abundance and frequency of infestation was established from the 30 worms analyzed in each site of study. Drawings of these cells were performed using a camera lucida attached to a Wild M20 microscope. A digital camera was used for light micrographs. Identification and classification was done according to the key of de Puytorac (1994).

Results

The taxonomy based studies require a standard to present the results. Thus to standardize our results, they will be presented in two parts (diagnosis and description) for each species.

Table 1. Morphometric characterisation of *Dicontophrya minus* n. sp.

	Cell length (µm)	cell width (µm)	Ma length (µm)	Ma width (µm)	Mi Diam	Kin. sup.	Kin. inf.	Number of Sk. F	P. v.
Max	125	50	115	6	9	41	40	8	6
Mean	111.6	42.1	92.5	4.9	6.2	31.9	31.8	6.9	3.6
Min	90	35	70	4	4	28	28	5	2
S.D	8.0	4.4	10.9	0.7	1.5	3.1	3.0	0. 9	1.1

Notes: Kin.- Kineties; Max– maximum; Ma– Macronucleus; Mi- Micronucleus; Min– minimum; Mi. Diam. Micronucleus diameter; P. v. - Pulsatile vacuoles; S.D– Standard Deviation; Sk. F- Skeletal Fibers; µm– micrometer; N=30.

1. Dicontophrya minus Fokam, Nana, Ngassam et al. n. sp.

This ciliate cohabits with some species of Hysterocinetians and many others species of astomatous ciliates in the midgut of *Alma Nilotica*

Diagnosis

Commensal of the digestive tract of *Alma nilotica*. Ovoid in shape but slightly elongated: 90 - 125 X 35 - 50 µm. one row of 2 to 6 pulsatile vacuoles. 28 - 41 kineties on each side of the cell. 5 - 8 skeletal fibers. The cytoskeleton includes a V-shape element where converge the anterior borders of skeletal fibers, present only on the lower side of the cell.

This specimens have been collected at Ebebda (frequency: 73, 3 %; mean abundance: 52 per worm) and at Nkolbikobo (frequency: 40 %; mean abundance: 37 per worm).

Type host

earthworm (*Alma nilotica* Sims and Gerard, 1999).

Table 2. Morphometric characterisation of *Dicontophrya ebebdaensis* n. sp.

	Cell length (µm)	cell width (µm)	Ma length (µm)	Ma width (µm)	Mi Diam	Kin. sup.	Kin. inf.	Number of Sk. F	P. v.
Max	215	35	195	5	5	29	29	8	11
Mean	199.6	28. 7	171.1	4	3.4	24.9	24.7	7.1	7.8
Min	180	25	145	3	2	21	21	4	5
S.D	9.7	2.9	14.72	0. 9	0. 7	2.0	1. 9	1.2	1.9

Notes: Kin.- Kineties; Max– maximum; Ma– Macronucleus; Mi- Micronucleus; Min– minimum; Mi. Diam. Micronucleus diameter; P. v.- Pulsatile vacuoles; S.D– Standard Deviation; Sk. F- Skeletal Fibers; µm– micrometer; N=30.

Type locality

Ebebda and Nkolbikobo, Center region, Cameroon.

Habitat

Midgut.

Type specimens

Permanent preparations belonging to this species are kept in the Laboratory of General Biology (Faculty of Sciences, University of Yaounde I Cameroon).

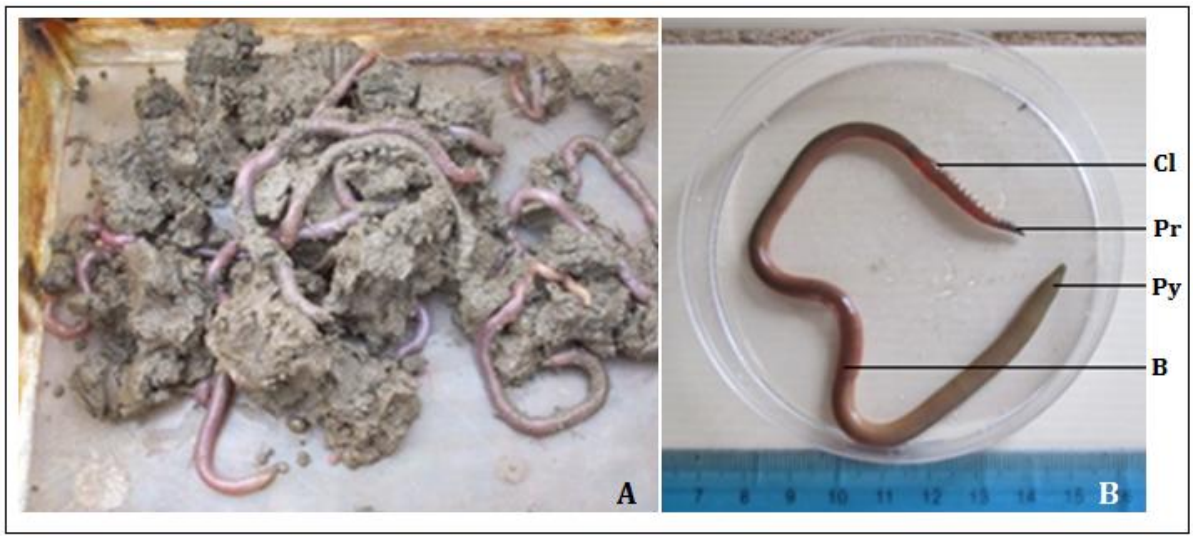

Fig. 2. *Alma nilotica* A) Fresh collection, B) General morphology (scale: cm) Cl: Clitellum, Pr: Prostomium, Py: Pygidium, B: body (Segmented).

Description

General morphology

This cell is ovoid in shape but slightly elongated with the anterior and posterior ends rounded (Figs. 3,4A). It measures 90 - 125 µm long and 35 - 50 µm in its widest part (Table 1). The cell is somewhat flattened on the lower side and show slight convexity on the upper side. The macronucleus is ribbon like, relatively broad (70 – 115 x 4 - 6 µm) and lies axially. The micronucleus (6.2 µm in diameter) flanks equatorially the macronucleus on one the left side (Fig. 4B-C). After silver staining, it is sometimes observed in the cytoplasm of the cell, rod-like endozoic bacteria.

Ciliature

The meridian somatic kineties are made up of about 28-40 equally distributed on each side of the cell, with exception of an anterior glabrous area occupied by the skeletal apparatus of the ciliate (Fig. 3B). In the posterior part of the cell, the kineties of the two sides confront, forming laterally two secant systems (right and left).

Skeletal apparatus

The skeletal apparatus is lodged in an anterior depression of the anterior pole of the cell and formed of a two-part basal piece (a modified V shape skeletal branch uniting all the Radiophryidae) bearing two crochet hooks and on which 4-7 skeletal fibers are fixed, but instead deported on the lower surface (Fig. 3B).

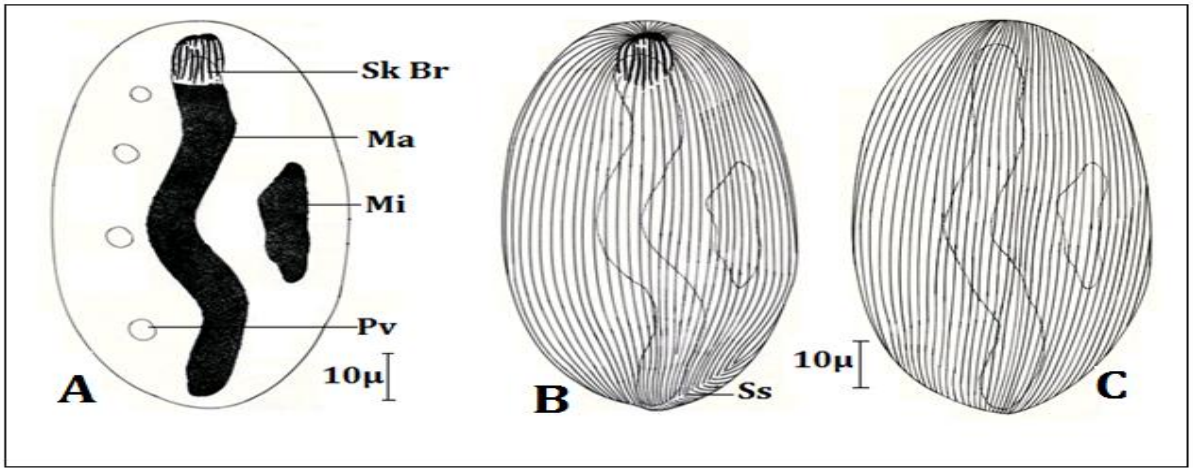

Fig. 3. *Dicontophrya minus* n. sp. (Drawings after silver silver staining). **A**) General morphology, **B**) Ciliature of the lower side, **C**) Ciliature of the upper side. Ma– Macronucleus, Mi– Micronucleus; Pv– Pulsatile vacuoles, SkBr: Skeletal branch, Ss: Secant system.

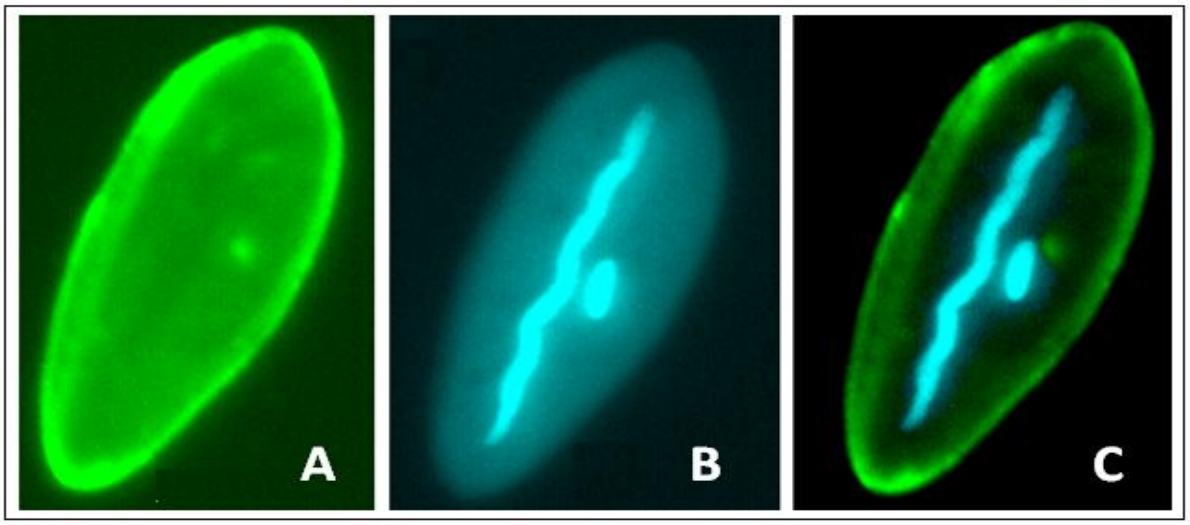

Fig. 4. *Dicontophrya minus* n. sp. (x 400). **A**) General morphology visualized by FITC-conjugated anti-tubulin antibody, **B**) DAPI-stained nuclear apparatus, **C**) Merger of the two previous images.

2. Dicontophrya ebebdaensis Fokam, Nana, Ngassam et al. n. sp.

D. ebebdaensis is found along with some species of Hysterocinetidae and many others species of Astomes ciliates in the midgut and proximal part of the hindgut of *Alma Nilotica* collected in Ebebda.

Diagnosis

The cell have an elongated cylindrical body : 180 - 215 X 25 - 35 µm. 42 – 58 bipolar kineties uniformly distributed on the two faces of the cell. One row of 5-11 pulsatile vacuoles. 4-8 skeletal fibers. (frequency: 73,33 % ; mean abundance: 52 individual per worm).

Type host

Earthworm (*Alma nilotica* Sims and Gerard, 1999).

Type locality

Ebebda, Center region, Cameroon.

Habitat

Midgut and proximal part of the hindgut.

Type specimens

Permanent preparations belonging to this species are kept in the Laboratory of General Biology (Faculty of Sciences, University of Yaounde I Cameroon).

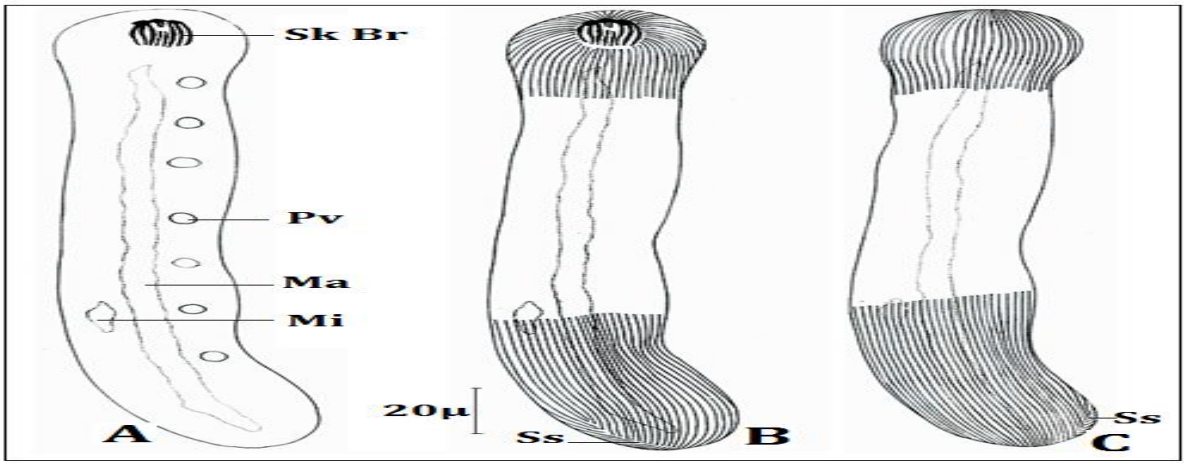

Fig. 5. *Dicontophrya ebebdaensis* n. sp. (Drawings after silver staining). A) General morphology; B) Ciliature of the lower side; C) Ciliature of the upper side. Ma– Macronucleus, Mi– Micronucleus, Pv– Pulsatile vacuoles, SkBr: Skeletal branch; Ss: Secant system.

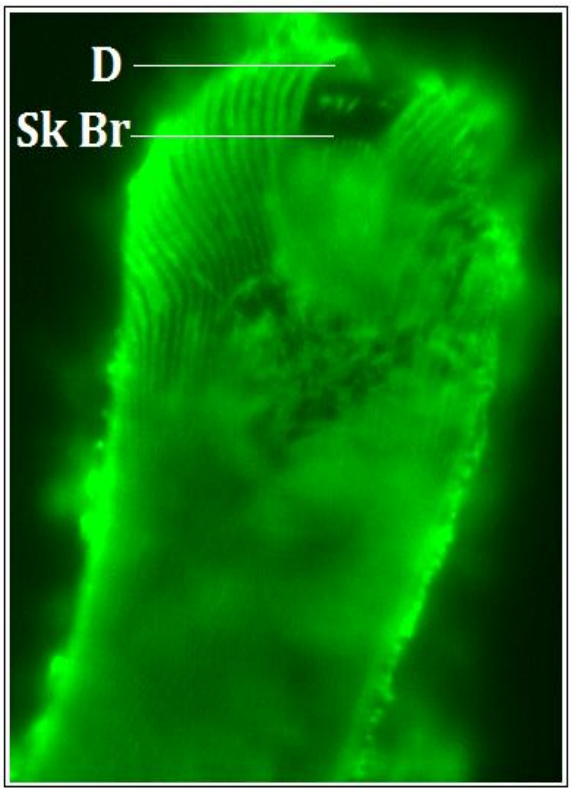

Fig. 6. *Dicontophrya ebebdaensis* n. sp. (x 400) Detail of the anterior pole of a cell labeled with anti-tubulin antibody showing the skeletal apparatus. Note the depression (D) and skeletal branches (Sk Br) of the attachment apparatus.

Description

General morphology

It is a cylindrical and elongated cell (215 µm long over 35 µm width). The macronucleus is axial and ribbon like (171.1 µm long over 4 µm width) (Figs. 5, 7B-C; table 2). It anterior ends are a bit far from the poles. This macronucleus bears in the posterior one-third a lenticular micronucleus (3.4 µm in diameter in its widest part. The vacuolar apparatus is made up of on row of 5-11 pulsatile vacuoles lining the right side of the macronucleus.

Ciliature

The ciliature is composed of 42-56 longitudinal meridian kineties regularly spaced and covering uniformly the ciliate. On in lower face (side) it left glabrous an anterior circular area occupied by the cytoskeleton (Figs. 5B, 6).

Skeletal apparatus

The architecture de skeletal armature is builded in the same plan of organization as the one of the previous species (*Dicontophrya minus*) and is lodged in an

anterior depression (Fig. 6): a « V » shaped element, profoundly modified into a basal piece on which articulate axially, two crochet hooks, and supporting inferiorly skeletal fibers (4-8 in number) (Fig. 5B).

Discussion and affinities

The ciliary pattern and the architecture of the skeletal apparatus of *Dicontophrya ebebdaensis* and *Dicontophrya minus* are quite similar. The description of these features is in accordance with the original description of the genus *Dicontophrya* by de Puytorac and Dragesco (1969) represented by the type species *Dicontophrya grassei* de Puytorac and Dragesco, 1969. The main differences between our two new forms concerns the general morphology, more precisely the wormlike body derived from the considerable elongation of the cell (the ratio length/width: 7.0 and 2.6 respectively is significantly different for the two specimens), and the disposition of the nuclear apparatus (slightly deported in the subequatorial zone in *D. ebebdaensis* while equatorial in *D. minus*). These differences seems to be of specific values, and we consider it is appropriate to distinguish these specimens by distinct specific designations: *Dicontophrya ebebdaensis* for the name of the locality were the ciliate have been collected for the first time, and *Dicontophrya minus* for the ciliate relatively reduced size.

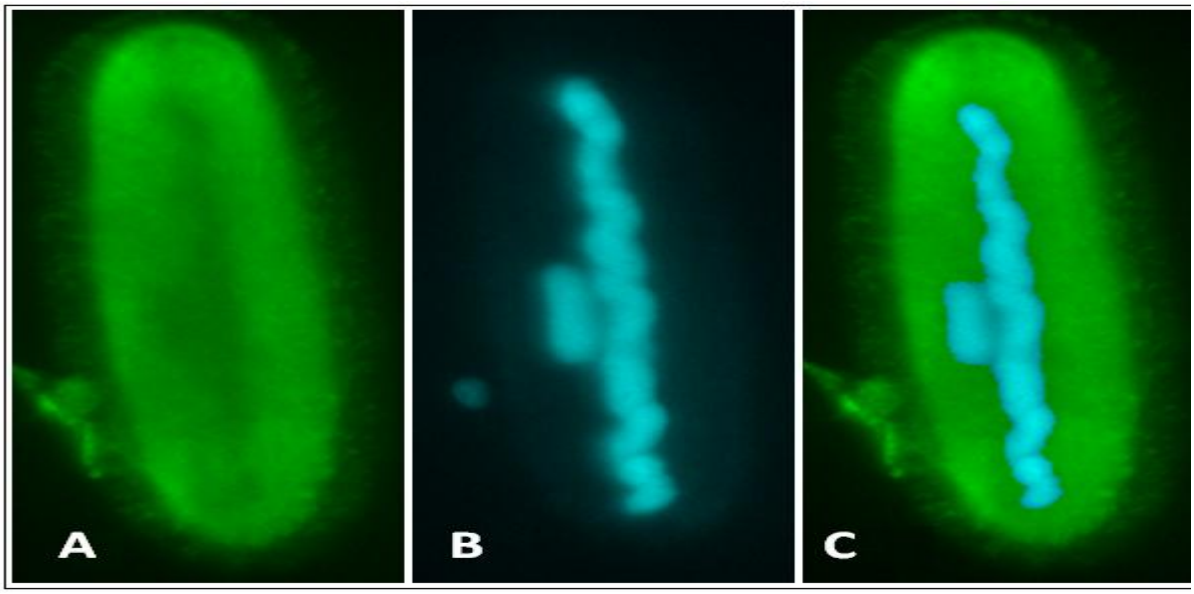

Fig. 7. *Dicontophrya ebebdaensis* n. sp. (x 400). A) General morphology visualized by FITC-conjugated anti-tubulin antibody, B) DAPI-stained nuclear apparatus, C) Merger of the two previous images.

The distinction from the other already known species of this genus resides in the differences in morphometric characteristics: 90 X 50 µm; 56 kineties; about 10 skeletal fibers for *D. grassei* and 111.6 X 42.1 µm; 64 kineties; 7 skeletal fibers for *D. minus*. More over the ciliary topography, marked by the presence of an anterior sagittal secant system in *D. grassei* helps distinguishing them at a specific level.

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