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Bellec, Laure; Maurer-Alcala, Xyrus X.; and Katz, Laura A., "Characterization of the Life Cycle and Heteromeric Nature of the Macronucleus of the Ciliate *Chilodonella uncinata* Using Fuorescence Microscopy" (2014). Biological Sciences: Faculty Publications, Smith College, Northampton, MA.
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Author Manuscript
J Eukaryot Microbiol. Author manuscript; available in PMC 2015 May 01.

Published in final edited form as:
J Eukaryot Microbiol. 2014 ; 61(3): 313–316. doi:10.1111/jeu.12109.

Characterization of the Life Cycle and Heteromeric Nature of the Macronucleus of the Ciliate *Chilodonella uncinata* Using Fluorescence Microscopy

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Abstract

Only a limited number of studies exist on the life cycles of non-model ciliates such as *Chilodonella uncinata* (Cl: Phyllopharyngea). The handful of papers on this taxon indicates the presence of a heteromeric macronucleus, marked by separate DNA rich and DNA poor regions. Here, we study the life cycle of *C. uncinata* using confocal laser scanning microscopy with DAPI staining, which allows us to differentiate nuclear dynamics of the micronucleus and the macronucleus during life cycle stages. We photo-documented various stages and confirm aspects of the development of the new macronucleus previously characterized by electron microscopy. We further reveal the heteromeric structure of the macronucleus with Z-stacks and 3D reconstructions. We find no evidence for the presence of an endosome at the center of the macronucleus during vegetative growth. In addition to illustrating the life cycle of this ciliate, the approaches developed for this study will enable additional comparative analyses of nuclear dynamics using fluorescence microscopy.

Keywords

nuclear dynamics; macronuclear anlage; ciliate; confocal microscopy; vegetative cell

CILIATES are characterized by the presence of cilia and dimorphic nuclei. Each cell contains two distinct nuclei: a germline micronucleus (MIC) and a somatic macronucleus (MAC). The MAC, which is transcriptionally active during vegetative growth, develops from a zygotic nucleus through chromosomal fragmentation, amplification, and elimination of internal sequences (Juranek and Lipps 2007; Prescott 1994). Within this important group of eukaryotes, with more than 8,000 described species, there is a considerable complexity in life cycles and nuclear structures (Raikov 1982, 1996). The life cycle of ciliates generally includes asexual reproduction (most of the time by binary fission), where the MIC divides by mitosis and the MAC divides by amitosis (i.e. chromatin moves in an unusual manner along microtubules; Raikov 1982), and a separate sexual stage (conjugation or autogamy). With the exception of models like *Euplotes*, *Stylonychia*, *Oxytricha*, *Paramecium*, and

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Tetrahymena species, nuclear dynamics during life cycles are poorly understood for many species.

Chilodonella uncinata (Ehrenberg, 1838) (Phyllopharyngea), the focus of this study, is characterized by the heteromeric nature of its MAC. The heteromeric MAC consists of two zones: 1) the peripheral region or "orthomere" with dense chromatin granules; 2) the central region or "paramere" that shows a diffused stain with an endosome in the center (Pyne 1978). This kind of organization is known as a centric heteromeric nucleus according to the classification of Fauré-Fremiet (1957). The life cycle is still not completely resolved as the development of the new macronucleus was studied only three times earlier through light microscopy (Pyne et al. 1974), electron microscopy (Pyne 1978) and ultrastructural autoradiography (Pyne and Gache 1979). Here we investigate nuclear dynamics during *C. uncinata's* life stages using DAPI staining and confocal laser scanning microscopy. These methods allow us to document a generalized life cycle and confirm the heteromeric structure of the MAC with 3D reconstruction images.

MATERIAL AND METHODS

The POL line of *C. uncinata* was originally isolated in a culture from Stefan Radzikowski and then deposited as ATCC® PRA-256 (Robinson and Katz 2007). Cells were cultured at room temperature in the dark with filtered and autoclaved pond water plus a rice grain to support bacterial growth. Cells were fixed directly to Superfrost microscope slide (Fisher) in 2% paraformaldehyde on ice for 1h and washed twice with PBS (1X). Staining was done at room temperature in the dark for 1 min (1 μg/ml 4′, 6-diamidino-2-phenylindole [DAPI]). Cells were washed twice with PBS (1X) and 1 drop of SlowFade Gold (Invitrogen) was added to minimize loss of fluorescent signal.

Organisms were observed with an upright Olympus BX51 epifluorescent microscope and a confocal laser scanning microscope. Image acquisitions were done with an oil immersion 63/1.4 objective lens (HCX PL APO) on a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Mannheim). Fluorochromes were visualized with either an argon laser with excitation wavelength of 350 nm or a UV laser with excitation wavelength of 405 nm for DAPI. Images were scanned sequentially with a resolution of 1024 × 1024 pixels, an acquisition speed of 100 Hz, and a line average of six to reduce noise. Adobe Photoshop CS5 and Volocity 5.0c (PerkinElmer) were used to analyze images and Z-stacks from the confocal laser scanning microscopy. We also tested another DNA stain; YOYO-1 at 0.05 μM concentration, which yielded the same results as DAPI (data not shown).

RESULTS AND DISCUSSION

We observed more than one hundred cells with fluorescence and confocal microscopes (Table 1). We completed eleven experiments with a DAPI stain, allowing us to visualize both nuclei, differentiate stages, and generate a life cycle (Fig 1). Asexual division happens in less than 15–20 min with a division of the MAC into two equal parts (Fig 1A).

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The sexual cycle of *C. uncinata* appears to begin with meiosis of the MIC (Fig 1C), followed by conjugation, exchange of haploid MIC between cells, and nuclear fusion to form a zygotic nucleus. Based upon our observations (the lack of 4 micronuclei in a single unpaired cell), we propose that, as in most other ciliates, meiosis of the MIC occurs after formation of the conjugating pair (Fig 1 C). Such pairs always have the same morphology with two cells side by side connected near the oral apparatus (Fig 1 D, H). We were able to take images of a conjugating pair with four MICs in each cell (Fig 1 D). Our observations show the separation of the MICs, where the retained haploid MIC (post meiosis II) remains close to the base of the oral cavity, while those destined to degrade remain anterior to the MAC. To facilitate the separation the MAC migrates towards the anterior of the ciliate and acts as an isolating barrier to the MICs. The MIC positioned at the base of the oral apparatus, where the cytoplasmic bridge forms between cells, appears to undergo mitosis in preparation for conjugation. As in other ciliates, we propose that the zygotic nucleus divides by mitosis and one daughter nucleus forms the new macronucleus while the old MAC fragments. With DAPI staining of DNA, we observe the presence of the new MAC anlage, the old MAC and the MIC at different stages (Fig 1 E, F).

Pyne et al. (1974) hypothesized that the development of the macronuclear anlage occurs in three stages, and our DAPI staining supports this hypothesis. Firstly, the anlage typically develops in the posterior region of the cell, and initially appears with a homogenous blue DAPI stain without dense granules of DNA. Most of the time, the MIC is present between the old MAC and the anlage (Fig 1 E, F). During the second stage, the anlage increases in size until it is two or three times the parental macronuclear size (Fig 1E). At the third stage, bright and dense granules of DNA appear inside the anlage, increasing in number and size, as the anlage begins to reduce its size until roughly equal with the old MAC (Fig 1 F). At the end of this stage the old MAC becomes pycnotic and the anlage has the appearance and size of a vegetative MAC (Fig 1 B) (Pyne et al. 1974).

We estimated that between 95 and 98 % of cells are at a vegetative state across all our observations and experiments. During this period, the macronucleus is typically located in the posterior end of the cell (i.e. away from oral apparatus) near the smaller micronucleus (Fig 2A). Macronuclear size in *C. uncinata* varies as 10.34 ± 1.29 μm (7.99–12.80; n=50) and the MIC size as 2.73 ± 0.39 μm (2.06–4.08; n=50) while the MAC to MIC ratio is 3.8 ± 0.46. The ratio is relatively no less variable than MAC size – about 12% CV. With DAPI staining, the micronucleus appears less bright than the larger macronucleus yet stains homogenously, while the macronucleus has a diverse range of intensities (Figs 1B, 2B).

Staining the DNA and using a confocal laser scanning microscope allows us to create three-dimensional images of the heteromeric MAC. With this method, we took sixty images (Z-stack) of the macronucleus and micronucleus of one vegetative cell. We show six images representative of the composition of both nuclei (Fig 2B), where the MIC was observed between only a few layers (Z = 19 and Z = 25). In the MAC, we detected a ring of dense granules of DNA and a light homogenous stained center between layers 25 and 35. At the bottom and the top of the MAC we saw bright granules of chromatin (layers 10, 19, 45, and 51). A 3D reconstruction of this cell reveals the heteromeric nature of the macronucleus; the MAC is made up of dense granules of DNA located at the peripheral region interconnected

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by a diffuse DNA poor region in the center (Fig 2C; http://www.science.smith.edu/departments/Biology/lkatz/movies/Chilodonella_MAC.mov).

The heteromeric nature of the MAC was previously studied in *C. uncinata* with light and electron microscopy (Pyne 1978; Radzikowski and Steinbrück 1990; Radzikowski 1985). Images, Z-stacks, and 3D reconstructions of a vegetative cell stained by DAPI allow us to confirm this remarkable characteristic. However, we cannot validate some electron microscopic observations such as differentiated nucleoli to the dense chromatin body inside the orthomere or, more surprisingly, the presence of a DNA-rich endosome in the center of the paramere. The endosome was reported as a distinct Feulgen-positive body in the middle of the cell during interphase (Fauré-Fremiet 1957; Raikov 1982). We took images of 85 vegetative cells that include 12 Z-stacks and never observed a body in the center of these cells. Our failure to observe an endosome is consistent with the hypothesis that the endosome is a short-lived feature composed of defective DNA that is later eliminated (Radzikowski 1985). In the closely related ciliate *Trithigmostoma steini* (originally published as *Chilodon steini* Blochmann, 1985), the endosome is not visible after the division, but it reappears within two hours after the separation of macronuclei (Radzikowski and Steinbrück 1990). A second hypothesis is that the endosome has a very weak DNA content that is not detectable by DAPI or YOYO-1 staining.

The use of fluorescence microscopy allows us to understand the life cycle and the unusual nature of the heteromeric macronucleus of *C. uncinata* in greater detail. We also provide an important number of images and reconstructions for further morphological study, and demonstrate the power of fluorescence microscopy for characterizing nuclear features in non-model ciliates.

Acknowledgments

We would like to thank Daniel J. G. Lahr, Meaghan S. Hall, and Judith Wopereis for insightful discussions and training on confocal laser scanning microscope. This work was supported by the National Institutes of Health (1R15GM097722).

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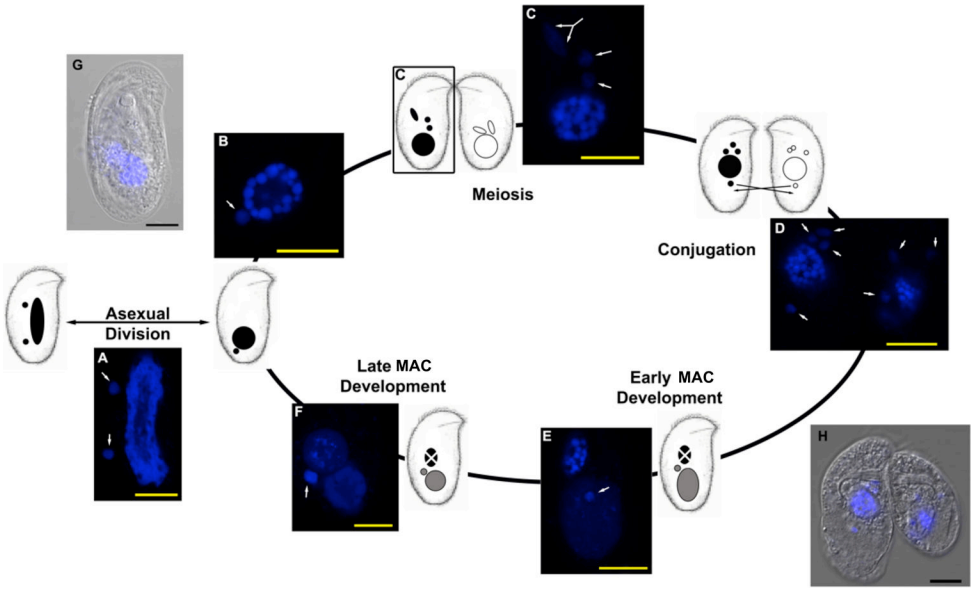

Figure 1.

A generalized life cycle of *Chilodonella uncinata* illustrated by cartoons of cells and fluorescent images. Cartoons show the MIC (smaller black or white circle), the MAC (larger black or white oval/circle) and genetically novel MIC or new MAC (grey oval/circle) that are generated by conjugation. (**A**) Asexual division and MAC amitosis. (**B**) Vegetative Cell. (**C**) MIC meiosis; branched white arrow indicates a MIC in meiosis II. The fluorescence image represents only the left cartoon (inside the rectangle) (**D**) Conjugating pair: haploid MICs are exchanged; one MIC is not visible in current plane in the right cell. (**E**, **F**) MAC development: a new MAC is differentiated and the old MAC begins to fragment. (**G**) DIC/DAPI overlay of the same cell in F; late in macronuclear development. (**H**) DIC/DAPI overlay of a conjugating pair; of note is the rotation and alignment of the oral apparatus. All scale bars are 7.5 μm. White arrows indicate MICs in each cell. DAPI was used for DNA counterstaining to visualize macronucleus and micronucleus (blue) and images were acquired by a confocal laser scanning microscope.

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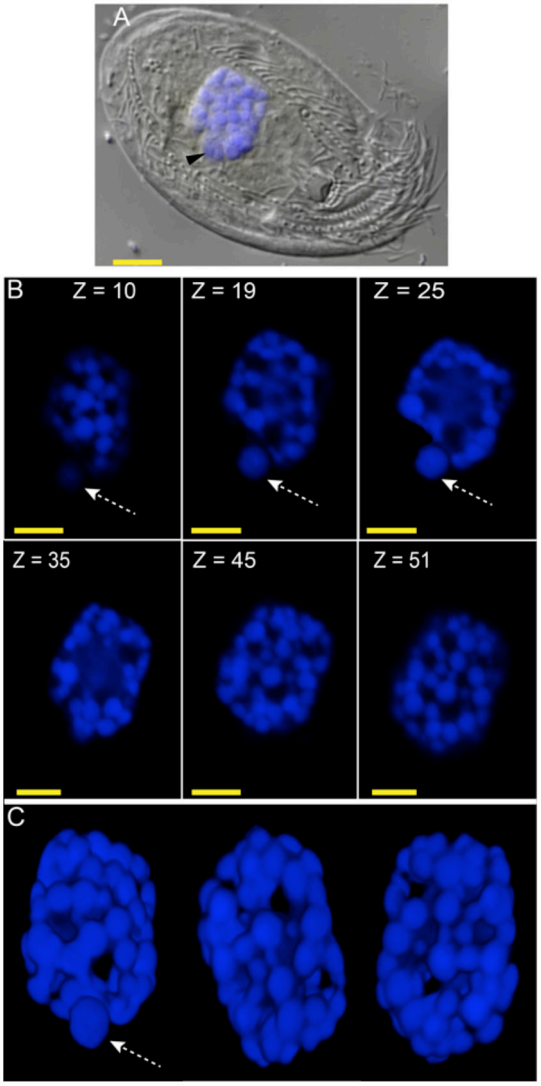

Figure 2.

Both nuclei from a vegetative cell of *Chilodonella uncinata*. Stack of one DAPI-stained vegetative cell was acquired using a confocal laser scanning microscope. (**A**) Overlay of DAPI and DIC stacks (60 images) of a vegetative cell. Black arrowhead shows position of the MIC (**B**) Series of single optical sections from a Z-stack of 60 images (numbers represent the position of the image in the Z-axis). (**C**) Three different views of a 3D reconstruction of the macronucleus in a vegetative cell. All scale bars are 5 μm. DAPI was used for DNA counterstaining to visualize macronucleus and micronucleus (blue).

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Table 1

Number of DAPI-stained images by stage of the life cycle of *C. uncinata* acquired using an inverted fluorescent microscope and a confocal laser scanning microscope (simple image or Z-stack).

State	Fluorescent Microscope	Confocal Microscope		Total of cells
		Images	Z-stack	
Vegetative	55 (5)	18 (4)	12 (4)	85
Conjugation	1 (1)	21 (3)	3 (2)	25
Development	6 (3)	18 (5)	2 (2)	26
Division	2 (2)	4 (2)	0	6
Total of cells	64	61	17	

Notes: numbers in parentheses refer to number of separate experiments performed.