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**Original**

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**Detection of haploid nuclear death in living *Paramecium caudatum***Xianyu YANG<sup>1\*</sup>, Xin GAO<sup>1</sup> and Xinlu SHI<sup>2</sup>

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**Key Words:** *Paramecium*; conjugation; apoptosis; acridine orange; Hoechst 33342**SUMMARY**

During conjugation of *Paramecium caudatum*, four haploid nuclei are formed after meiosis, only one of those that enters the paroral region survives and divides once more to form two gametic nuclei, and the remaining three degenerate. Here, we applied the method of co-staining the living cells with acridine orange and Hoechst 33342, which had been found effective to identify the apoptotic nuclei in *Tetrahymena*, to clarify whether the meiotic nuclear degeneration in *P. caudatum* is an apoptotic process. We found that three haploid nuclei outside the paroral region were stained green-yellow, while the survivor in the paroral region was stained blue. When the conjugating pairs separated and the synkaryon divided twice, three degenerating haploid nuclei were still observed and stained yellow. The

old fragmented macronuclei, which are known to be still alive in exconjugants, were stained blue, in sharp contrast with the case in *Tetrahymena*. These results strongly suggest that the haploid nuclei degenerate in an apoptotic way in *P. caudatum*.

**INTRODUCTION**

Ciliates are a group of unicellular organisms having two kinds of nuclei, small diploid germinal micronuclei and large polygenic somatic macronuclei (Nanney, 1980), and nuclear events follow a programmed schedule during their sexual process, i.e., conjugation. *Paramecium caudatum* is one of the well-known ciliates with one micronucleus and one macronucleus, and during conjugation nuclear events include meiosis, gametic nuclear formation and exchange, formation of synkaryon (fertilized nucleus), three successive postzygotic nuclear divisions (synkaryon divisions), new macronuclear differentiation and development, and old macronu-

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clear fragmentation and degeneration, meanwhile old oral apparatus is disintegrated and replaced by a new one (Wichterman, 1986). In fact, during conjugation of *P. caudatum*, there are three stages in which the micronuclei encounter fate determination. 1). After meiosis, only one meiotic product survives, while the other three degenerate (Calkins and Cull, 1907). 2). Soon after the 3<sup>rd</sup> synkaryon division, four posterior nuclei are differentiated into macronuclear anlagen; four anterior nuclei remain as presumptive micronuclei (Mikami, 1980). 3). In exconjugants, only one presumptive micronucleus is selected as a germinal nucleus for the next generation, and the remaining three degenerate (Calkins and Cull, 1907). In the case of old macronuclei, they break into approximately fifty pieces, and finally, the old macronuclear fragments degenerate and are replaced by the new macronuclei. Therefore, nuclear degeneration happens three times during the sexual process of *P. caudatum*: degeneration of haploid meiotic nuclei, extra-presumptive micronuclei elimination, and old macronuclear degeneration. However, the old macronuclear fragments in exconjugants remain functional and keep their ability to undergo DNA synthesis and regenerate into the macronucleus (Mikami, 1988). Recently, it was reported that the old macronuclear fragments finally undergo an apoptotic process in *P. caudatum* (Kimura *et al.*, 2004). Concerning the degradation of meiotic haploid nuclei and the extra-presumptive micronuclear elimination, it is still not clear whether they undergo an apoptotic process.

Apoptosis is characterized by oligonucleosome-sized DNA fragmentation, which can be detected by agarose gel electrophoresis (Compton, 1992), and it also can be detected by a TUNEL technique (Gavrieli *et al.*, 1992). In *Drosophila*, acridine orange (AO), a vital fluorescent dye, has been used to identify apoptotic cells (Abrams *et al.* 1993; White *et al.*, 1994). In *Tetrahymena*, AO was combined with another vital fluorescent dye, Hoechst

33342 (HO), and, by use of these two fluorescent dyes, the normal nuclei were stained blue, and the degenerating meiotic nuclei and old macronuclei were stained yellow-orange or yellow-green (Mpoke and Wolfe, 1997; Santos *et al.*, 2000). The various advantages of AO/HO staining over the TUNEL technique to detect apoptosis were discussed, and AO/HO double staining was proposed as a useful method for specifically identifying apoptotic cells (Mpoke and Wolfe, 1997). Therefore, in this experiment, we focused on the study of meiotic haploid nuclear degradation in *P. caudatum* to clarify whether this process is apoptotic by AO/HO staining. Here, we will report these results and discuss some related experiment results.

## MATERIALS and METHODS

### Chemicals and Stock Solutions

AO was purchased from Shanghai Chemical Reagent Company (China), and Hoechst 33342 from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Other chemicals were from Hangzhou Dafang Chemical Reagent Inc (China). Stock solutions (50x) of both AO (1 µg/ml) and Hoechst 33342 (10 µg/ml) were prepared with autoclaved water and kept in 4°C refrigerator.

### Cell Culture and Conjugation Induction

Two complementary mating types of *P. caudatum* were cultivated in 2.5% fresh lettuce juice diluted with modified Dryl's solution (Dryl, 1959), prepared by substituting KH<sub>2</sub>PO<sub>4</sub> for NaH<sub>2</sub>PO<sub>4</sub> (K-DS). *Klebsiella pneumoniae* was inoculated one day before use (Hiwatashi, 1968). The conjugation was induced by mixing highly reactive cells of complementary mating types (Hiwatashi, 1968). To obtain more concentrated conjugating pairs, we used iron-dextran particles (Vosskühler and Tiedtke, 1993) to separate conjugating pairs of *P. cau-*

*datum* (Yang and Takahashi, 1999). All the experiments were performed at room temperature ( $\sim 23^{\circ}\text{C}$ ).

### Staining, Observation, and Photographing

Ten minutes or more before the observation, 10  $\mu\text{l}$  each of AO and Hoechst 33342 stock solutions was added to a 480  $\mu\text{l}$  cell suspension (final concentration: 0.02  $\mu\text{g/ml}$  of AO; 0.2  $\mu\text{g/ml}$  of Hoechst 33342). To make the preparations, the cells were transferred to a slide glass with as little solution as possible, and the movement of cells was slowed down by placing a cover glass on four tiny drops of soft flour paste around the cells and pressing the four corners of the cover glass, as reported by Shi *et al.* (1985). When the cells became flattened and started to move very slowly,

they were observed and photographed under a Nikon 50i fluorescence microscope.

### RESULTS

#### Micronuclear Behavior at Vegetative Stage and during Meiosis

After cells of two complementary mating types are mixed to induce conjugation, the conjugating pairs are formed; the micronuclei then migrate out of the concavity of the macronucleus (Fig. 1a) and undergo meiosis through a long prophase including a crescent stage (Fig. 1b) to form 4 haploid nuclei (Fig. 1c, 1d). Figure 2 shows a vegetative cell in which the micronucleus (arrow) is located in the concavity of macronucleus

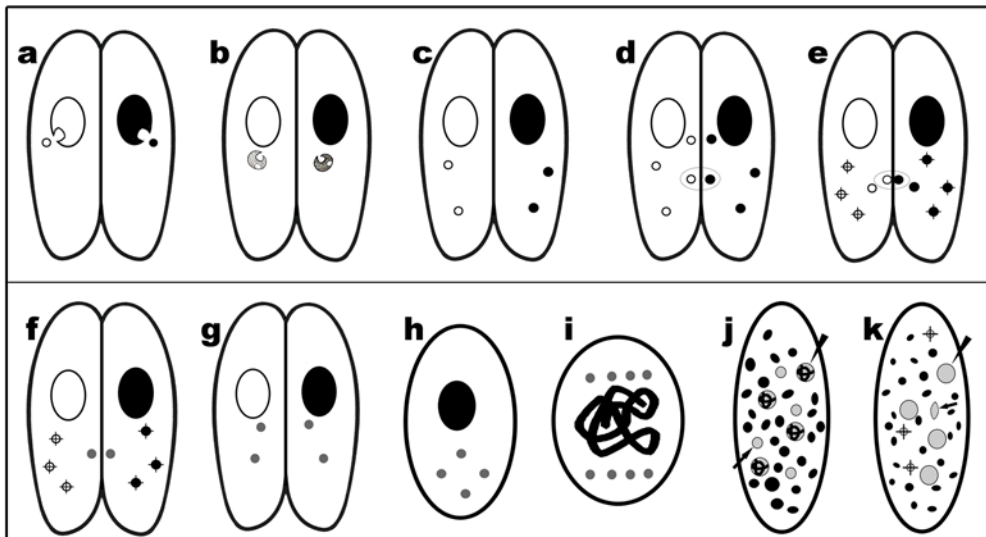


Fig. 1. Schematic representation of major nuclear stages during the conjugation of *Paramecium caudatum*. **a**. Soon after the formation of a conjugating pair. Micronuclei have left the concavity of the macronuclei. **b**. Crescent stage of 1<sup>st</sup> meiotic prophase. **c**. 1<sup>st</sup> meiotic division. **d**. 2<sup>nd</sup> meiotic division. One meiotic product enters the paroral region (dot-lined areas). **e**. 3<sup>rd</sup> prezygotic nuclear division. The nucleus enters the paroral region and divides mitotically to form gametic nuclei, and the remaining three degenerate (crosses). **f**. Synkaryon formation. **g**. 1<sup>st</sup> synkaryon division. **h**. 2<sup>nd</sup> synkaryon division. **i**. 3<sup>rd</sup> synkaryon division. At this stage, cells become rounder and shorter. Four nuclei locate in the posterior region, and the other four locate in the anterior region of the cell. The old macronuclei show a thick skein form. **j**. Nuclear differentiation. Arrowhead: macronuclear anlagen having aggregated chromatin; arrow: spherical presumptive micronuclei. There are many old macronuclear fragments. **k**. Development of macronuclear anlagen and germinal nuclear selection. Arrowhead: more developed macronuclear anlagen; arrow: one selected micronucleus; crosses: presumptive micronuclei destined to be degenerated.

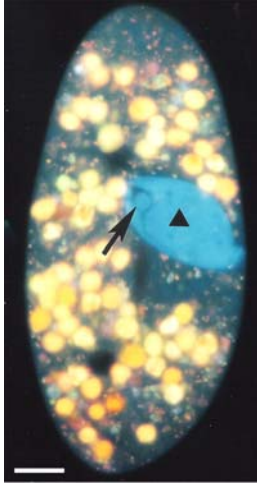


Fig. 2. An AO/HO co-stained vegetative *Paramecium caudatum*. Arrow: a micronucleus in the concavity of the macronucleus. Triangle: a macronucleus. Many yellow-orange or yellow food vacuoles were observed.

(triangle); both of them are stained blue by co-staining of AO and Hoechst 33342, while the food vacuoles are stained yellow or yellow-orange. Figure 3A shows a conjugating pair soon after pair formation approximately 2 h after conjugation, and the micronuclei migrated out of the concavity of the macronucleus. Figure 3B shows a conjugating pair soon after 1<sup>st</sup> meiotic division, and Fig 3C shows one that entered 2<sup>nd</sup> meiotic division approximately 12 h after the induction of conjugation. Figure 3D shows a conjugating pair after 2<sup>nd</sup> meiotic division. Except in Fig. 3C, both micronuclei (arrows) and macronuclei (triangle) are stained blue (Fig. 3A, 3B, 3D), while both are stained greenish-blue in Fig. 3C. Many trapped AO particles show brilliant orange-red color in Fig. 3C.

### The Process of Meiotic Nuclear Degeneration

Soon after meiosis, one of the haploid nuclei that enters the paroral region survives (Fig. 1d) and completes 3<sup>rd</sup> prezygotic nuclear division to form two gametic nuclei: a migratory pronucleus and a stationary pronucleus (Fig. 1e). In contrast, the

remaining three nuclei outside the paroral regions degenerate (Fig. 1e, 1f). Figure 3E shows a conjugating pair at the stage of 3<sup>rd</sup> prezygotic division, and Fig. 3F shows one soon after 3<sup>rd</sup> prezygotic division, approximately 14 h after conjugation; here, both the surviving meiotic nuclei or the gametic nuclei (solid arrows) and macronuclei (triangles) are stained blue, while two or three green-yellow degenerating nuclei (hollow arrows) are observed in each conjugant. In this experiment, 52 cells around the third prezygotic division were observed; and two green-yellow degenerating nuclei were observed in 24 cells, and three were observed in 28 cells.

### A Long Process of Haploid Degeneration

To see how long the degenerating haploid nuclei remain in the cells, the cells at later stages were also observed. After reciprocal exchange of migratory pronuclei, synkaryons are formed and divide three times successively to produce eight nuclei (Fig. 1g-1i). Usually conjugating pairs separate after 1<sup>st</sup> synkaryon division and become exconjugant cells (exconjugants) (Wichterman, 1986). Approximately 16 h after conjugation, most exconjugants finished 2<sup>nd</sup> synkaryon division and entered 3<sup>rd</sup> division. Here, 13 such cells were observed, and, in each cell 4 synkaryon division products and 1 macronucleus were stained blue, while 3 round structures were stained yellow, as shown in Fig. 4A, which were considered to be the degenerating meiotic nuclei. Around 18 h after the induction of conjugation, most cells were at the anaphase or early telophase of 3<sup>rd</sup> synkaryon division, and one or two yellow-stained structures were still observed in 3 out of 11 cells (Fig. 4B), while no such structures were observed in the remaining cells.

### Old Macronuclear Behavior in Exconjugants

Soon after 3<sup>rd</sup> synkaryon division, four of the eight division products are differentiated into

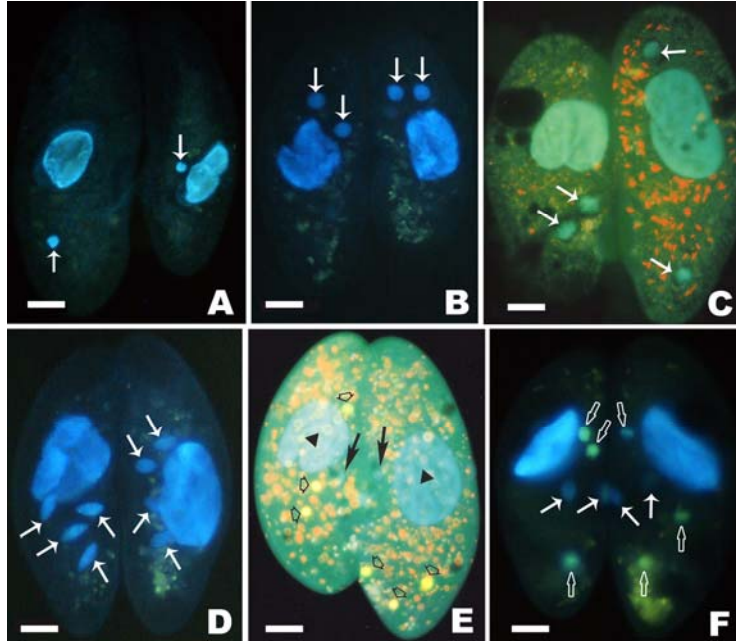


Fig. 3. AO/HO co-stained conjugating pairs of *P. caudatum*. Triangles: macronuclei; solid arrows: micronuclei or meiotic products; hollow arrows: degenerating haploid meiotic nuclei. **A – D.** Both micronuclei or meiotic products and macronuclei were stained blue or greenish-blue. **E and F.** Both surviving meiotic products and gametic nuclei were stained blue, while the degenerating haploid nuclei out of the paroral cone were stained green-yellow. **A.** A conjugating pair soon after the pair formation approximately 2 h after conjugation and the micronuclei migrated out of the concavity of macronucleus. Both micronuclei and macronuclei were stained blue. **B.** A conjugating pair soon after 1<sup>st</sup> meiotic division. There were 2 round 1<sup>st</sup> meiotic division products. **C.** A conjugating pair soon after 1<sup>st</sup> meiotic division approximately 12 h after the induction of conjugation. Many chromosomes were observed in each division product. Many trapped AO particles showed bright orange-red. **D.** A conjugating pair soon after 2<sup>nd</sup> meiotic division approximately 13 h after conjugation. **E.** A conjugating pair at 3<sup>rd</sup> prezygotic division approximately 14 h after conjugation. Many trapped AO particles showed bright orange-red. **F.** A conjugating pair soon after 3<sup>rd</sup> prezygotic division approximately 14 h after conjugation. Scale bars: 20  $\mu$ m.

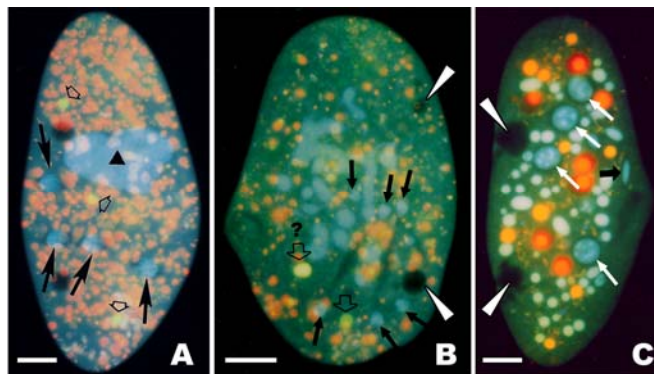


Fig. 4. AO/HO co-stained exconjugants of *Paramecium caudatum*. White arrowheads: two contractile vacuoles. **A.** An exconjugant after 2<sup>nd</sup> synkaryon division approximately 16 h after conjugation. Arrows: 4 synkaryon division products; hollow arrows: degenerating haploid meiotic nuclei. **B.** An exconjugant at the earlier telophase of 3<sup>rd</sup> synkaryon division approximately 18 h after conjugation. Arrows: telophase nuclei of 3<sup>rd</sup> synkaryon division; hollow arrows: degenerating haploid nuclei; question mark: a haploid nucleus delayed to degenerate. The old macronucleus started to break. **C.** An exconjugant 72 h after the induction of conjugation of *P. caudatum*. White arrows: macronuclear anlagen; black arrow: a presumptive micronucleus. Scale bars: 20  $\mu$ m.

macronuclear anlagen, and the remaining four are presumptive micronuclei (Fig. 1j). Through a process of complicated DNA rearrangement, the macronuclear anlagen become more developed, and one of the presumptive micronuclei is selected, showing a spindle-like shape, while the other three eventually degenerate (Fig. 1k) (Yang and Takahashi, 2000). In all 17 exconjugants, 72 h after conjugation, 4 macronuclear anlagen and 1 to 4 spindle-shaped presumptive micronuclei were stained blue, and there were about 50 extra blue-stained structures and some orange-red or orange structures (Fig. 4C).

## DISCUSSION

During conjugation of *Paramecium caudatum*, 4 haploid nuclei are formed after meiosis, and only one of the nuclei that enters the paroral region survives and finishes 3<sup>rd</sup> prezygotic division to form gametic nuclei, while the remaining three degenerate (Fig. 1e) (Calkins and Cull, 1907; Yanagi, 1987). In *Tetrahymena thermophila*, 3 of 4 haploid meiotic products also degenerate, and both the meiotic nuclear degeneration and the old macronuclear degeneration have been demonstrated to be an apoptotic process (Davis *et al.*, 1992; Mpoke and Wolfe, 1996, 1997; Santos *et al.*, 2000). Therefore, it is reasonable to predict that the meiotic products of *P. caudatum* degenerate in a similar way, even though the issue has not been thoroughly examined yet. To clarify this issue, the degeneration of meiotic products of *P. caudatum* was here studied by AO/HO co-staining method. The results obtained show that the meiotic products outside the paroral region were stained yellow-orange or green-yellow at around 3<sup>rd</sup> prezygotic nuclear division (hollow arrows in Fig. 3E and 3F), while the nuclei located in the paroral region were stained blue. In contrast to this, the micronuclei or the meiotic products at the earlier

stages were always stained blue (Fig. 3B – 3D). All these results suggested that haploid nuclei degenerate in an apoptotic way in *P. caudatum* as they do in *Tetrahymena* (Santos *et al.*, 2000).

Concerning when the meiotic products completely disappear from the conjugating cells, there have been no clear descriptions. The current study showed that degenerating meiotic nuclei existed until 3<sup>rd</sup> synkaryon division (Fig. 4A and 4B), and, in *Tetrahymena thermophila*, the degenerating haploid nuclei were observed in the conjugating pairs after the end of 1<sup>st</sup> synkaryon division (synkaryon divides twice in *Tetrahymena* instead of three times in *P. caudatum*) (Santos *et al.*, 2000) indicating a longer degenerating process of meiotic nuclei in *Paramecium* than in *Tetrahymena*.

Regarding the old macronuclei of *P. caudatum*, their fragments have been demonstrated to be functional in exconjugants that keep their ability for DNA synthesis, and, when the new macronuclear anlagen fail to develop into functional macronuclei they are able to regenerate into the macronuclei (MR) (Mikami, 1988). In this report, there were about fifty blue-stained structures and 16 orange-red or orange structures in the exconjugants of *P. caudatum* 72 h after mixing of the complementary mating types (Fig. 4C). The blue structures were demonstrated to be the macronuclear fragments by staining the same cells with carbol fuchsin solution (Carr and Walker, 1961) (data not shown), and this observation indicated that the old macronuclear fragments were functional and their degeneration was not initiated in exconjugants of *P. caudatum* (Mikami, 1988). In two other species of *Paramecium*, the macronuclear fragments (in *P. tetraurelia*) or condensed old macronuclei (in *P. bursaria*) were also stained blue in the exconjugants 48 hours or more after conjugation by AO/HO co-staining (data not shown). Recently, it was reported that the old macronuclear degeneration was an apoptotic process and that it was initiated around the fifth cell cycle after the

conjugation, indicating the delay of old macronuclear degeneration in *P. caudatum* (Kimura *et al.*, 2004), which might be the reason that degenerating macronuclear fragments were not observed in this study. In *Tetrahymena*, the degeneration of old macronuclei has also been demonstrated to be an apoptotic process, which is initiated soon after nuclear differentiation (Mpoke and Wolfe, 1997) and much earlier than that in *P. caudatum*.

Concerning the three extra presumptive micronuclei in exconjugants, their degradation was confirmed morphologically by carbol fuchsin staining (Yang and Takahashi, 2000). In contrast to this, Taka *et al.* (2006) demonstrated the division potency of the extra-presumptive micronuclei even after the germinal micronuclear selection by micronuclear elimination. In exconjugants as shown in Fig. 4C, there are about 50 old macronuclear fragments (stained blue) in different sizes and 16 orange-red or orange colored structures, which might be food vacuoles or the degenerating presumptive micronuclei themselves, as was mentioned above. Therefore, it is difficult to distinguish the degenerating presumptive micronuclei from the other structures. To clarify whether the extra-presumptive micronuclei degenerate in an apoptotic way, special efforts might be needed to eliminate the interference of other structures such as food vacuoles.

Finally, we have to explain the different colors of Figs. 2 – 4. Based on the colors, the pictures could be divided into a “blue group” including Figs. 2, 3A, 3B, 3D, and 3F and a “green” group including Figs. 3C, 3E, and 4A - 4C. In the course of the observation of the cells co-stained by AO and HO, both micronuclei and macronuclei were stained blue at the beginning, except for the degenerating meiotic nuclei (Figs. 3E and 3F) or food vacuoles (Figs. 2 and 4C), and the cytoplasm was stained so faintly that it was hard to recognize the outline of the cells. However, when the observation time of cells became longer, both the nuclei

and the cytoplasm changed gradually to greenish. Therefore, depending on the timing of photography, the functional surviving nuclei were sometimes blue and sometimes greenish blue.

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