

New details indicated by different stainings during conjugation of ciliated protozoa *Paramecium*

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Abstract: During conjugation of *Paramecium caudatum*, nuclear events occur in a scheduled program. Morphological studies on nuclear behavior during conjugation of *P. caudatum* have been performed since the end of the 19th century. Here we report on new details concerning the conjugation of *P. caudatum* through the staining of conjugating cells with protargol, carbol fuchsin solution, Hoechst 33342 and immunofluorescence labeling with monoclonal antibody of anti- α tubulin. 1) The crescent nucleus is a characteristic of the meiotic prophase of *P. caudatum*, has an unstained area. We stained this area with protargol, which was separated from the chromatin area and was not detected by the other stainings. 2) In regards to the four meiotic products, it has long been considered that only one product enters the paroral cone region (PC) and survives after meiosis. However, our protargol and immunofluorescence labeling results indicated that PC entrance of the meiotic product happened before the completion of meiosis instead of after. 3) In our previous study, protargol staining indicated the presence of a swollen structure around the central part of the “U” and “V” shaped spindles connecting the two types of prospective pronuclei. However, immunofluorescence labeling with anti- α tubulin antibodies gave a different image from protargol. All these observations form the basis for further studies of their molecular mechanisms.

Key words: *Paramecium*; Conjugation; Crescent; PC entrance; Connecting spindles

不同染色方法揭示纤毛原生动物草履虫接合生殖过程的新细节

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摘要: 通过石炭酸品红、Hoechst 33342、蛋白银及免疫荧光标记等染色方法对草履虫接合生殖过程进行了重新观察, 结果发现: 1) 新月核是第一次减数分裂前期小核的主要形态学特征, 在核内有一未被石炭酸品红、Hoechst 33342 着色区域, 蛋白银染色则清楚显示该结构; 2) 4 个单倍体减数分裂产物中的 1 个核进入旁锥完成配前第三次核分裂, 其余 3 核退化。蛋白银染色和抗 α 微管蛋白单克隆抗体进行免疫荧光标记显示, 核进入旁锥的时期在第二次减数分裂末期而非减数分裂结束后; 3) 配前第三次分裂末期, 核间连丝的中间段有一被蛋白银识别的结构, 但免疫荧光标记却无显示, 只表现为纤维状结构与两侧核间连丝相连。观察结果为草履虫接合生殖过程中相关分子生物学机制研究奠定了必要的形态学基础。

关键词: 草履虫; 接合生殖; 新月核; 进入旁锥; 核间连丝

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Morphological studies on nuclear behavior during conjugation of *Paramecium caudatum* have been studied since the end of the 19th century (Maupas, 1889; Calkins & Cull, 1907). In addition, many similar studies on other

Paramecium and ciliates have been performed before and during the 1980s (Nanney, 1980; Wichterman, 1986). Over the last 30 years, much ciliate research has focused on mechanisms to interpret morphological phenomena

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by molecular biological techniques, and some remarkable results have been obtained such as the discoveries of telomere, telomerase and ribozyme (Blackburn & Gall, 1978; Cech & Bass, 1986; Greider & Blackburn, 1985). However, fewer morphological studies have been done. Comparison of images obtained from the same cells by different stainings led to new findings in our previous study (Gao et al, 2010). Therefore, several staining methods were used to re-study the conjugation process of *P. caudatum*, including the protargol technique (Shi, 1987), carbol fuchsin solution staining (Carr & Walker, 1961), Hoechst 33342 (Santos et al, 2000) and immunofluorescence labeling with monoclonal antibody of anti- α tubulin (Yang & Takahashi, 2002). We discovered new details concerning conjugation of *P. caudatum*, which are reported here.

1 Materials and Methods

1.1 Chemicals and preparation of staining solutions

Silver protein from Merck KGaA (Germany), monoclonal antibody of mouse anti- α tubulin, FITC-conjugated goat anti-mouse IgG, propidium iodide (PI) and Hoechst 33342 (HO) purchased from Beyotime Institute of Biotechnology (China) were used. All other chemicals were from Hangzhou Dafang Chemical Reagent Inc (China). Preparations of stock solutions for Hoechst 33342 and carbol fuchsin solution followed previous descriptions (Carr & Walker, 1961; Yang & Takahashi, 1999; Yang et al, 2007).

1.2 Cell culture, conjugation induction and stainings

Two complementary mating types of *P. caudatum* collected from East Lake Campus of Zhejiang A & F University (China) were used. Cell culture, conjugation induction, and collection of synchronized conjugating pairs followed previous studies (Dryl, 1959; Hiwatashi, 1968; Sun et al, 2010; Wei et al, 2008; Yang & Takahashi, 1999). Modified protargol (Shi, 1987) briefly reported before (Shi & Frankel, 1990; Yang & Shi, 2007) was used. Immunofluorescence labeling with anti- α tubulin antibody followed Yang & Takahashi (2002). Temporary preparations were made by means of "volume-fixing" (Yang et al, 2008; Lin et al, 2009). All experiments were performed at room temperature ($\sim 25^{\circ}\text{C}$).

2 Results and Discussion

2.1 A structure in crescent nuclei indicated by protargol technique

The crescent nucleus is a characteristic of the

meiotic prophase of *P. caudatum*, in which there is an unstained area (Fujishima & Hiwatashi, 1981; Harumoto & Miyake, 1996). To determine if protargol provides any new indications about this structure, conjugating pairs during the meiotic prophase were stained (Fig. 1C, C', F, F'). In addition, cells at the same developmental stage were also stained by Hoechst 33342 (Fig. 1A, A', D, D') and carbol fuchsin solution (Fig. 1B, B', E, E'). As in former reports, an unstained structure was observed in the crescent nuclei when cells were stained either by Hoechst 33342 or by carbol fuchsin solution (Fig. 1D, D', E, E'). In contrast to this, a protargol stained structure separated from chromatin was observed (Fig. 1F, F'), which was exactly the area not detected by the other two stainings (compare Fig. 1D, E, F; D', E', F'). Before the crescent stage, micronuclei form a truncated spindle shape when cells were stained by Schiff reagent (Fujishima, 1983). Both Hoechst 33342 and carbol fuchsin solution staining in the current study also showed the micronuclei as truncated spindle shapes (Fig. 1A, B, A', B'). However, protargol staining indicated that the micronucleus was a fully spindle shape consisting of two parts, thread-like chromatin and evenly stained non-chromatin (Fig. 1C, C'). The non-chromatin area was located at one end of the nucleus corresponding to the truncated area indicated by Hoechst 33342 and carbol fuchsin solution. The nature of this structure might be related with microtubule organizing center (MTOC). To date, immunofluorescence labeling with anti-gamma-tubulin antibodies indicated that during conjugation of *Paramecium*, only a transient site of gamma-tubulin and microtubule assembly was observed at the site of nuclear exchange (Klotz et al, 2003). To clarify the relationships among microtubules, gamma-tubulin and MTOCs, further research needs to be done with the help of molecular biological techniques.

2.2 Entrance of meiotic product into paroral cone region

After meiosis, four meiotic products are produced, among which only one enters the paroral cone region (PC) (the area surrounding the disintegrated oral apparatus) and survives to complete the third prezygotic division producing a migratory and stationary pronucleus (Wichterman, 1986; Yanagi, 1987; Yanagi & Hiwatashi, 1985), while the remaining three undergo apoptotic degeneration (Yang et al, 2007; Gao et al, 2010). Recently, immunofluorescence labeling with monoclonal antibodies of anti- α tubulin indicated that at least one

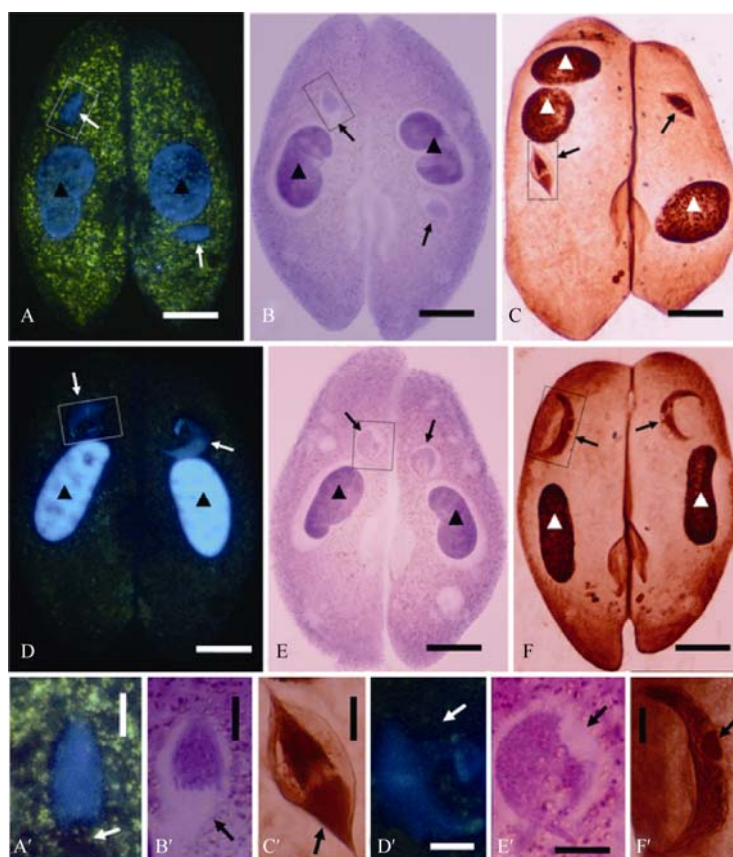


Fig. 1 Micronuclei on meiotic prophase of *Paramecium caudatum*

Framed portions of (A–F) are magnified in (A'–F'), respectively. (A, D) Hoechst 33342 staining; (B, E) Carbol fuchsin solution staining; (C, F) Protargol staining. (A–C) Stage IV of meiotic prophase; (D–F) Stage V of meiotic prophase (crescent). Arrows: in (A–F), denote micronuclei of the first meiotic prophase; in (A'–F'), denote the area in meiotic nuclei unstained by either Hoechst 33342 or carbol fuchsin solution but stained by protargol. Scale bars: 20 μm in (A–F) and 10 μm in (A'–F').

of the meiotic products was located in the PC area at the telophase of the second meiotic division (Gao et al, 2011b). To know the precise timing of PC entrance, many conjugating pairs were stained either by protargol technique or immunofluorescence labeling with anti- α tubulin antibodies. Both stainings indicated two cases of PC entrance (Fig. 2). One was during telophase of the second meiotic division (Fig. 2B, B', G–I), when the meiotic nuclei showed a teardrop form. The other was soon after meiosis (Fig. 2D, D', J), when all meiotic products showed spindle shapes without degenerating symptoms. Protargol staining indicated that PC entrance occurred in 70% of conjugants during the telophase of the second meiotic division, and in 96.3% soon after meiosis. In the case of immunofluorescence labeling, PC entrance was observed in 94.6% of conjugants during telophase of the second meiotic division, and 100% of conjugants after meiosis. However, PC entrance was observed neither at the telophase nor after the completion

of the first meiotic division (Fig. 2E, F). These observations indicated that PC entrance of meiotic products was a specific phenomenon, which mainly happened during telophase of the second meiotic division instead of after meiosis. Based on electron microscopic analysis of transverse sections of jointed *P. caudatum* at the junction zone (André & Vivier, 1962; Vivier & André, 1961; Vivier, 1965), it has been suggested that a paroral cone is not present during the conjugation (Wichterman, 1986). In the current experiment, the paroral cone region was clearly observed (Fig. 2A–D), and might be involved in the protection of meiotic products from degeneration. We are not sure whether this observed difference was from different techniques or different syngens of *P. caudatum*.

2.3 A protargol staining-indicated structure in the spindles connecting prospective migratory and stationary pronuclei

After meiosis, the haploid nucleus located in the PC

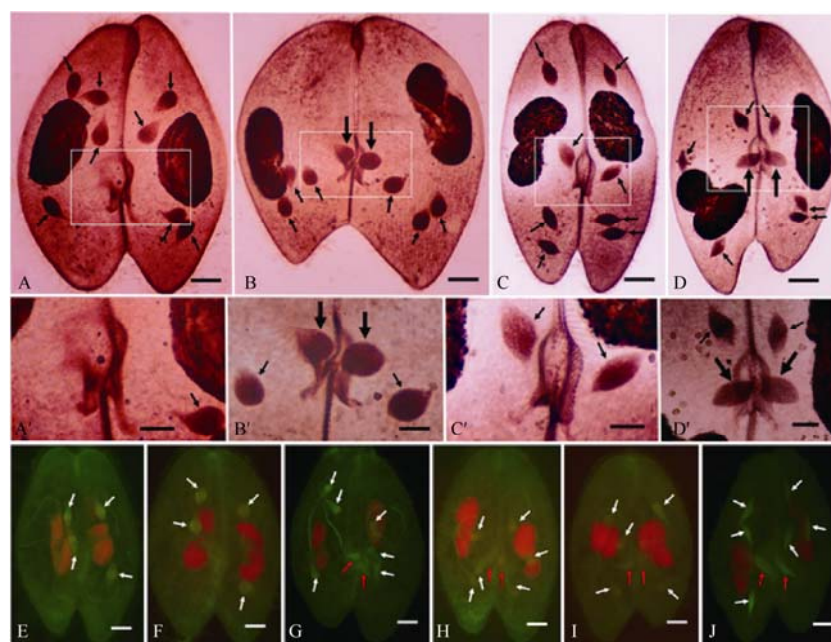


Fig. 2 PC entrance of meiotic products in *Paramecium caudatum*

(A–D) Protargol stained images; (E–J) Immunofluorescence labeling images indicated by anti- α tubulin antibody followed by FITC-labeled goat anti-mouse IgG and propidium iodide (PI). Portioned areas of (A–D) are magnified in (A'–D'), respectively. (A) No meiotic products in the PC at the telophase of the second meiotic division; (B) One meiotic product in the PC at the telophase of the second meiotic division; (C) No meiotic products in the PC soon after meiosis; (D) One meiotic nucleus in the PC soon after meiosis; (E, F) No products of the first meiotic division located in the PC; (E) Telophase of the first meiotic division. (F): Soon after the first meiotic division; (G–I) Three conjugating pairs on the telophase of the second meiotic division. One nucleus was located in the PC. (J) Soon after meiosis. One meiotic nucleus was located in the PC. Thick or red arrows: meiotic products in the PC. Thin or white arrows: meiotic products outside the PC. Scale bars: 20 μ m in (A–J) and 5 μ m in (A'–D').

region divides mitotically (the third prezygotic division) yielding two types of pronuclei (Wichterman, 1986). Recently, it has been indicated by both protargol staining and immunofluorescence labeling with anti- α tubulin antibodies that “U” and “V” shaped spindles connect prospective migratory and stationary pronuclei at the telophase of the third prezygotic division (Gao et al, 2011a, b). For easier comparison, the same experiments were repeated in the current study. The “V”-shaped spindles and side-by-side localization of the two prospective pronuclei were observed (Fig. 3). Protargol staining indicated the presence of a swollen structure at the crossing points of the “V”-shaped spindles (circles in Fig. 3A–C, A'–C'), while immunofluorescence labeling with anti- α tubulin antibodies indicated a slender line structure at the same area. In other words, this protargol-stained structure consisted of microtubules and some other unknown components. It is not clear if this

structure corresponded to the structure in the crescent nuclei indicated in the current study, or functioned as MTOC. Besides these, there were two new points that have never been reported. The first was that the stationary pronuclei were much closer to the migratory pronuclei in Fig. 3C than in Fig. 3A and B. To clarify this, many conjugating pairs covering the third prezygotic division have to be stained and measured and statistically analyzed. The second is that many anti- α tubulin recognized dots were observed surrounding both types of prospective pronuclei. It has been previously reported that both intranuclear and cytoplasmic microtubules play important roles on pronuclear behavior (Nakajima et al, 2001). This current study indicated that both cytoplasmic and intranuclear microtubules appeared much earlier than the stage of pronuclear transfer.

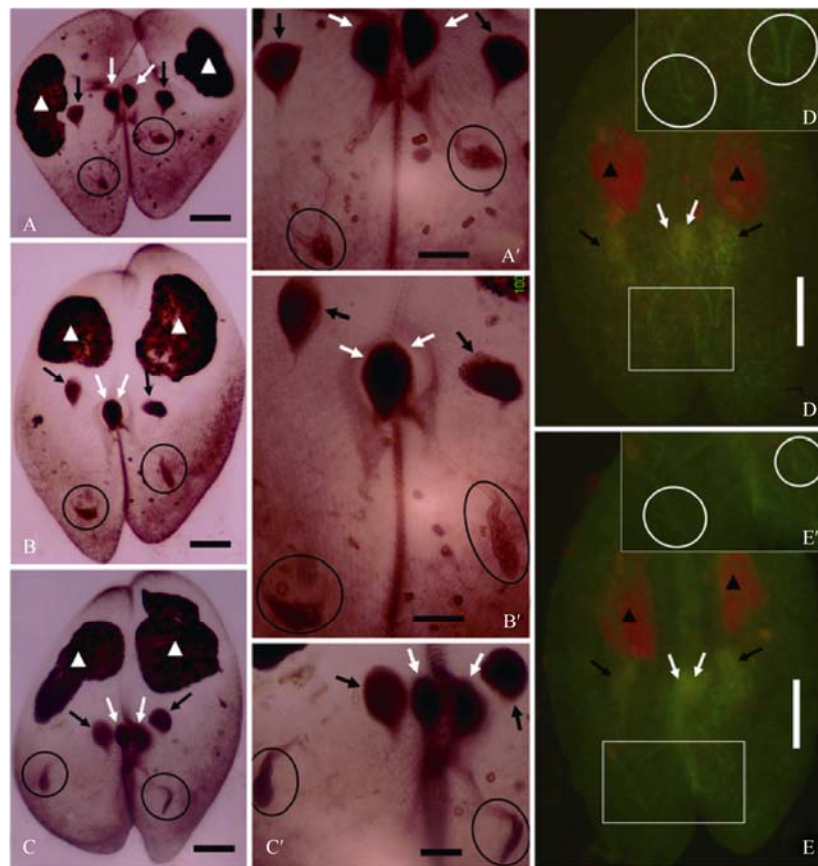


Fig. 3 Telophase of the third prezygotic division of *Paramecium caudatum*

(A–C) Protargol stained images; (D, E) Immunofluorescence labeling images indicated by anti- α tubulin antibody followed by FITC-labeled goat anti-mouse IgG and propidium iodide (PI). Portioned areas of (A–E) are magnified in (A'–E'), respectively. White arrows: prospective migratory pronuclei. Black arrows: prospective stationary pronuclei. Circles: "V"-shaped connecting spindles between two types of pronuclei. Triangles: old macronuclei. Scale bars: 20 μ m in (A–E) and 5 μ m in (A'–C').

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