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Gametic nuclear exchange during the conjugation of *Paramecium polycaryum*

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SUMMARY

The migratory gametic nuclear exchange is an important event during the conjugation of ciliates. So far, it is unclear whether this process occurs in *Paramecium polycaryum*, and there are also some other contradictory descriptions of this species. To clarify this issue, we studied the conjugation process in the Chinese strain of *P. polycaryum* by means of a modified protargol technique. The reciprocal gametic nuclear exchange and the formation of fertilized nuclei were observed in detail. The results indicated the following: 1) *P. polycaryum* has cross-fertilization during its conjugation. 2) The transfer of migratory gametic nuclei into the partner cell seems to be an active process. 3) The process of fertilized nuclear formation includes the attachment of a migratory gametic nucleus to a stationary one, membrane fusion of two kinds of gametic nuclei with separate nucleoplasm, and the fusion of the nucleoplasm of two gametic nuclei to form a typical fertilized nucleus.

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INTRODUCTION

Paramecium polycaryum is a unicellular eukaryotic organism belonging to the *Paramecium* genus that was originally reported by Woodruff and Spencer (1923). The cells of this species are 70 to 110 μm in length and dorsal-ventrally flattened, with a slightly obliquely truncated anterior end and a rounded posterior end. Each cell has a somatic nucleus (macronucleus), one to eight germinal nuclei (micronuclei), and two canal-fed contractile vacuoles, and it spirals to the left when swimming. There is a centrally located mouth that leads into the buccal cavity (oral apparatus, cytopharynx), which consists of several ciliary structures (Wichterman, 1986).

Concerning the sexual process (conjugation) of *Paramecium polycaryum*, two reports have been published so far, the first one by Diller on an In-

dian strain (1958), and the second one by Hayashi and Takayanagi on a Japanese strain (1962). This conjugation is induced by specific cell-cell interaction by mixing two complementary mating types followed by formation of a conjugating pair (conjugant). The whole process of conjugation includes a series of nuclear events, such as three prezygotic nuclear divisions, formation of zygotic nuclei (fertilized nuclei), three postzygotic nuclear divisions, new macronuclear differentiation and development, and old macronuclear fragmentation and degeneration (Wichterman, 1986). The disintegration and regeneration of the ciliary structures on the cytopharynx also happen during the conjugation. The first two prezygotic divisions are meiotic, and all micronuclei take part in this process, while only one of the meiotic products enters the paroral region (the area surrounding the degenerated oral apparatus) and survives. The surviving nucleus then undergoes the third prezygotic division to form two gametic nuclei, one migratory gametic nucleus (MiN) and one stationary gametic nucleus (StN) corresponding to the male and female gametic cells of higher organisms, respectively. Following the fusion of the MiN and StN, a fertilized nucleus forms and undergoes three successive postzygotic nuclear divisions. Four out of the eight postzygotic division products differentiate into new macronuclei (macronuclear anlagen), and the other four become micronuclei (Diller, 1958; Hayashi and Takayanagi, 1962). However, it is still unclear whether true cross-fertilization (reciprocal migratory gametic nuclear exchange) occurs in *P. polycaryum*. Diller's observation suggested self-fertilization rather than cross-fertilization (1958), while Hayashi and Takayanagi (1962) observed gametic nuclei lying against a conjugant boundary and mentioned cross-fertilization but did not provide any evidence of a reciprocal gametic nuclear exchange.

Concerning the inheritance of the mating types, Diller (1958) described caryonidal heredity, while Hayashi and Takayanagi (1962) described clonal heredity. In *Paramecium*, there are three kinds of mating type inheritance, i.e., "synclonal," "caryonidal," and "clonal" (Wichterman, 1986). A synclone is a culture of the progeny of two cells from one conjugating pair (exconjugant); when all the cells in a synclone show the same mating type, it is called synclonal heredity. When an exconjugant divides, micronuclei divide mitotically, while macronuclear anlagen (usually two or four depending on the number of postzygotic nuclear divisions in different *Paramecium* species) segregate into each of the daughter cells (Wichterman, 1986). A cell line from one such daughter cell is designated as a caryonide; when different mating types appear among the caryonides from the same exconjugant, it is called caryonidal heredity. In clonal heredity, all the caryonides from one exconjugant show the same mating types, and the caryonides from a partner exconjugant show another mating type, but these two mating types are complementary. In Diller's experiment, exconjugants from the conjugating pairs were separately cultured, but conjugating pairs were observed in each culture of the exconjugant; therefore, caryonidal inheritance was concluded (1958). In contrast to Diller's result, Hayashi and Takayanagi did not observe any conjugating pairs in the exconjugant culture except when mixing the two exconjugant cultures from one conjugating pair; they, therefore, concluded that the outcome was clonal inheritance (1962).

To clarify this issue, we studied the entire conjugation process of *P. polycaryum* using a strain collected from Harbin, a city located in northeast China, by means of the protargol method and obtained more detailed information on gametic nuclear exchange and fertilized nuclear formation. Here, we report the outcome and discuss the possible mechanisms of gametic nuclear exchange and the process of fertilized nuclear formation in *Paramecium*.

MATERIALS AND METHODS

Cell culture

Cells of *P. polycaryum* were isolated from water samples collected from Majia Ditch, Harbin, China. Collections were obtained from the ditch under the bridge that was part of Hexing Road and cultivated according to the method described by Sonneborn (1950) except that rice straw was used instead of lettuce powder. Conjugation was induced by mixing highly reactive cells of complementary mating types at room temperature (23-25 °C). Conjugating pairs remained on the bottom of the Petri-dish and were concentrated by micropipettes.

Staining and observation

After the induction of conjugation, cells were fixed with saturated $HgCl_2$ periodically and stained using a modified protargol method. This modification was performed by Shi and reported at the 4th Symposium of Chinese Protozoological Society in 1987. The protocol used for staining consisted of two parts, one for staining infraciliature, published by Shi and Frankel (1990), and the other for staining nuclei (Symposium of the Chinese Protozoological Society, 1987). The second part of this protocol is as follows: 1) Fix cells on a slide when their nuclei have been scattered by the pressure from the cover slip. 2) Perform step 7 to step 16 of Shi and Frankel (1990), including secondary fixing in a 1:1 (v/v) mixture of 95% ethyl alcohol and formalin (10 min); seal cells with 0.5% celloidin in absolute ethyl alcohol, wash slides with distilled water, and bleach with 0.5% $KMnO_4$ (1-3 min) followed by 5% oxalic acid treatment (1-3 min). 3) After bleaching and washing (step 16), add a step of treatment with a 1% NaCl aqueous solution at room temperature for one hour. 4) Continue from step 17 up to the completion of the procedure outlined by Shi and Frankel (1990), including immersion of slides in 1-2% protargol dissolved in distilled water at 50°C for 30 min, development of the cells in 0.1% hydroquinone in a 5% Na_2SO_3 aqueous solution, fixing in 5% $Na_2S_2O_3 \cdot 5H_2O$, dehydration in ethanol series, clearance in xylene, and mounting of the cells. This method can be used to stain spindles, fibril systems, nuclear membranes, nucleoli, chromatin, and chromosomes. Preparations were observed and photographed under a Nikon microscope.

Measurement of the immature period and mating-type inheritance

To investigate the mating-type inheritance in this experiment, exconjugants from the same conjugating pairs were separately grown, and the caryonides of some exconjugants were also separately grown. After ciliate conjugation, caryonides go through a period during which they have no mating ability (activity), known as the immature period, which is measured by the number of cell divisions. Here, the daily isolation method (Takagi and Yoshida, 1980) was used to count the cell divisions; according to this method, one cell is kept in a fresh culture medium; then, the number of cell divisions is calculated the following day. Again, only one cell is transferred to a fresh culture medium, while the other cells are gathered and supplied with enough medium to keep them growing (mass culture), as in the case of the daily isolated cells. This operation is repeated daily until the appearance of mating activity, which was tested by mixing the caryonide mass culture with the original cell cultures that had been used to induce the conjugation.

RESULTS

Mating-type inheritance in P. polycaryum

Forty-three clones of *P. polycaryum* were established from the water samples of Majia Ditch, and grouped into two complementary mating types. To

know the inheritance of mating types, the exconjugants of ten and caryonides of five conjugating pairs were separately grown, and their mating activity was periodically checked. All caryonides were mature by 45 fissions. No conjugating pairs appeared in the exconjugant cultures. Four caryonides from one exconjugant showed the same mating type, while two caryonide groups from two exconjugants of one conjugating pair showed different but complementary mating types in all cases. These results indicated that the inheritance of mating types in the Chinese strain of *P. polycaryum* was clonal rather than caryonidal, a finding which is in agreement with the conclusion of Hayashi and Takayanagi (1962).

Gametic nuclear exchange in P. polycaryum

In *Paramecium*, the micronuclear division is mitotic (the macronuclear division is amitotic), as in other ciliates, but the process is strikingly different from that in metazoan cells in two respects (Wichterman, 1986). One is that the nuclear envelope remains intact during all the stages of division, and the other is that centrioles are absent in ciliates. The modified protargol method developed by Shi is an excellent staining technique that yields high resolution for both fibrillar systems (Shi and Frankel, 1990) and nuclei-related structures, such as spindles, nuclear membranes, nucleoli, chromatin, and chromosomes of ciliates (Shi *et al*., 1997; Watanabe *et al*., 1996). To discern the contradictory reports on the issue of the reciprocal migratory gametic nuclear exchange in *P. polycaryum* (Diller, 1958; Hayashi and Takayanagi, 1962), we studied the entire conjugation process using a cell strain collected from Harbin by means of the protargol method. A typical pattern of three prezygotic nuclear divisions and three postzygotic nuclear divisions was observed in our study, in agreement with previous reports (Diller, 1958; Hayashi and Takayanagi, 1962).

The strain of *P. polycaryum* used in this experi-

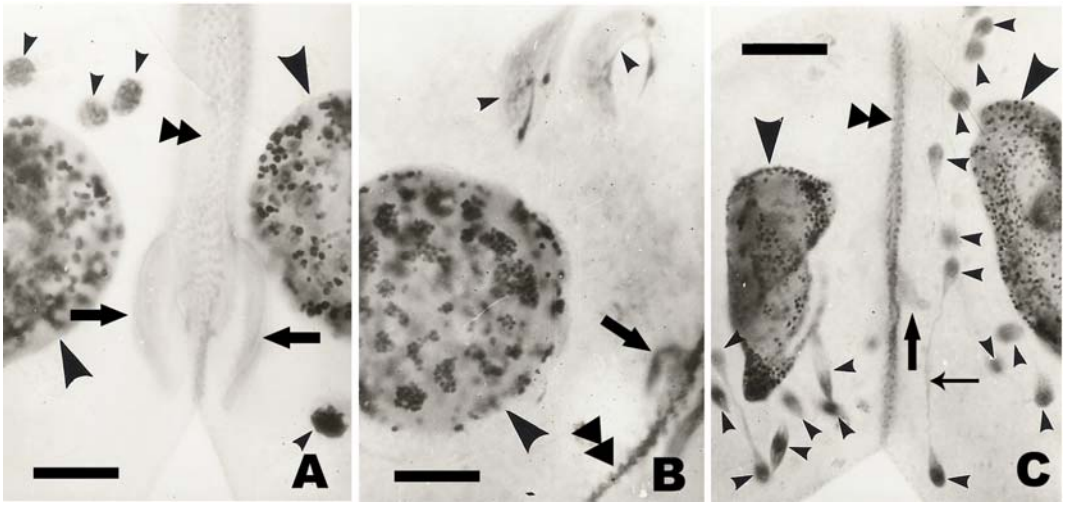

Fig. 1. Meiotic divisions of micronuclei during the conjugation of *P. polycaryum*. Double arrowheads indicate conjugant boundaries of each conjugating pair in all panels. A. A conjugating pair at the early stage. No obvious morphological changes in either micronuclei (small arrowheads) or macronuclei (large arrowheads). The oral apparatus began disintegrating (solid arrows). B. Late meiotic prophase - crescent stage. Small arrowheads: two crescent micronuclei; solid thick arrow: disintegrated oral apparatus. C. Second prezygotic nuclear division. Small arrowheads: nuclei at different stages (metaphase, anaphase, and telophase); solid thick arrow: faint conical structure of the former oral apparatus; thin small arrow: spindle of the telophase nucleus. Scale bars: 20 μm.

ment had 1-6 micronuclei, all of which entered meiosis through a long prophase; on the other hand, the ciliary structures on the cytopharynx disintegrated gradually and disappeared mostly before the first meiotic division (Fig. 1A, B). Finally, a conical structure remained as the trace of the former oral apparatus (thick arrow in Fig. 1C); its surrounding area is the so-called paroral region. After meiosis, only one of the meiotic products that entered the paroral region survived and underwent the third prezygotic division, while the remaining ones degenerated (Fig. 2A, A'). During this division, the nucleus destined to be the migratory gametic nucleus (MiN) kept its location in the paroral regions (right cell in Fig. 2B, B'), but the nucleus destined to be the stationary gametic nucleus (StN) was far from the paroral region (black arrow in Fig. 2C). After this division, one MiN and one StN formed in each mate of the conjugating pair (left cells in Fig. 2B, B', C, C'). MiN was still in the paroral region and seemed to have attached to the conjugant boundary (left cell in Fig. 2C, C'),

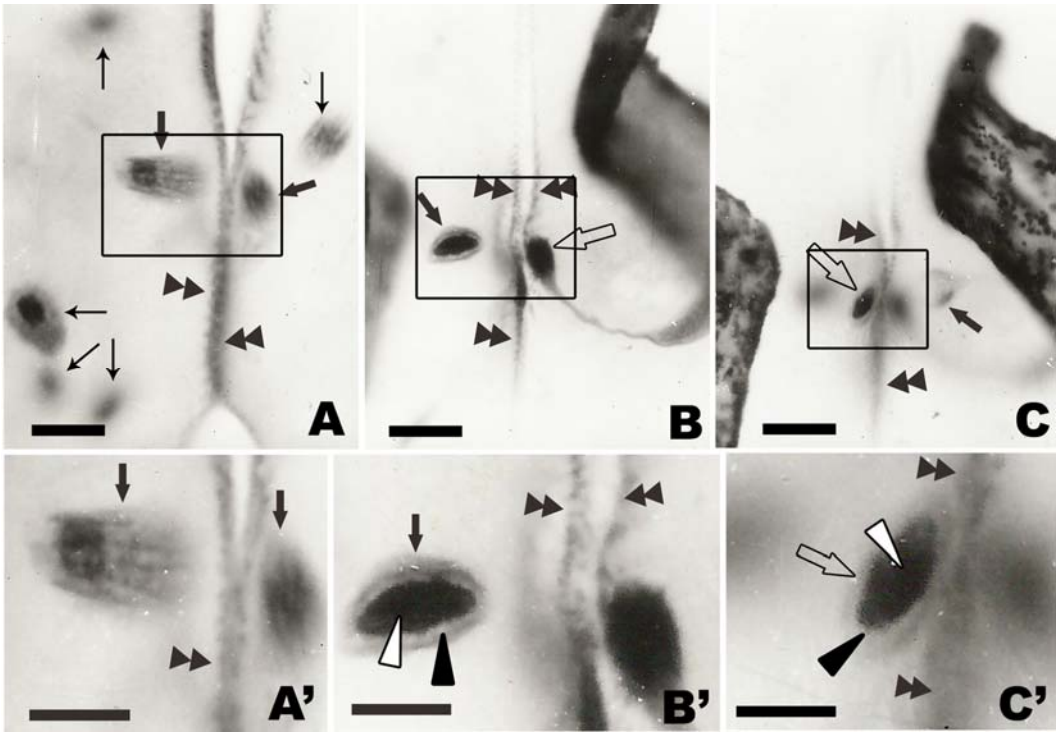

Fig. 2. One of the meiotic products or the migratory gametic nuclei (MiN) located in the paroral regions during the conjugation of *P. polycaryum*. The framed portions in A, B, and C are magnified in A', B', and C', respectively. Double arrowheads: conjugant boundaries in all panels. A. A conjugant after meiosis. Thick arrow: one meiotic product entered the paroral regions; thin arrows: meiotic products outside the paroral region. A'. One meiotic nucleus entered the paroral regions (arrows). B. A conjugating pair around the stage of the third prezygotic nuclear division. Right cell: telophase of the third prezygotic division. Hollow arrow: nucleus destined to be the MiN localizing in the paroral region; thin arrow: spindle. Left cell: after the third prezygotic nuclear division. Black arrow: a round-end spindle-shaped stationary gametic nucleus (StN). B'. Right cell: MiN lying against the conjugant boundary. Left cell: two parts of the StN (arrow). White arrowhead: chromatin; black arrowhead: clear area surrounding the chromatin. C. The same conjugating pair as in B but with a different focus. Right cell: a StN out of focus (solid arrow). Left cell: a round-end spindle-shaped MiN (hollow arrow). C'. A migratory gametic nucleus (hollow arrow). White arrowhead: chromatin part; black arrowhead: clear area surrounding the chromatin. Scale bars: 20μm in A, B, and C and 10μm in A', B', and C'.

while StN remained slightly far from the boundary (right cell in Fig. 2B, B'). Although both MiN and StN located differently in the cells, both of them showed similar morphological changes: a round-end spindle-shaped nucleoplasm (chromatin) surrounded by a thin layer of a clear area (left cells in Fig. 2B, B', C, C'). When the MiN was crossing the conjugant boundary, the chromatin part became thinner and longer (Fig. 3A, B), while the clear area at the anterior part of the MiN became larger and sharper, containing some faint filaments (thin arrows in Fig. 3B).

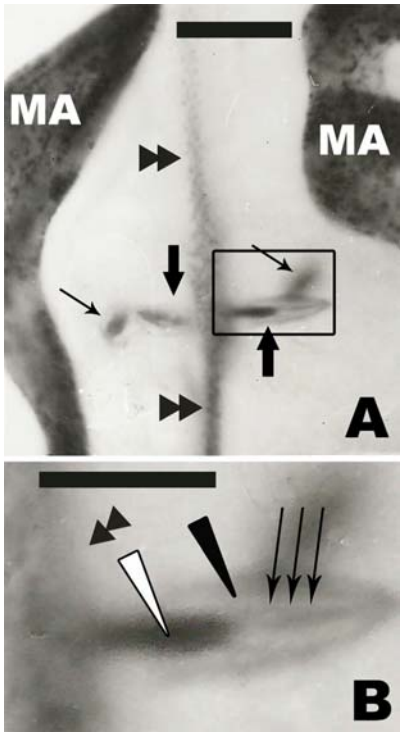

Fig. 3. Reciprocal gametic nuclear exchange in *P. polycaryum*. Framed portion of A magnified in B. A. A conjugating pair at the stage of MiN exchange. Thick arrow: a crossing MiN; thin arrow: a stationary gametic nucleus. An elongated macronucleus is observed in each conjugant. Double arrowhead: conjugant boundary; MA: old macronuclei. B. A crossing migratory gametic nucleus. White arrowhead: thinner and longer chromatin part; black arrowhead: enlarged and sharp clear area at the anterior part of the MiN containing some faint filaments (thin arrows). Scale bars: 20μm in A and 10μm in B.

Zygotic nuclear formation in P. polycaryum

Following the reciprocal MiN exchange, the process of nuclear fusion between MiN and StN (zygotic nuclear formation) initiated. At the beginning, the MiN attached to the StN when both of them extended, showing a bipolar-spindle shape, and were arranged side by side along their longitudinal axes (Fig. 4A, A'); at that time, the membrane border between two gametic nuclei was observed (small black arrow in Fig. 4A'). The membrane border then disappeared, while two parts of the nucleoplasm still remained separated, showing a heterogenous appearance (Fig. 4B, B'). Finally, two parts of the nucleoplasm fused completely, and a typical zygotic nucleus formed (Fig. 4C, C').

DISCUSSION

In this report, we found a "clonal" mating-type determination in the Chinese strain of *P. polycaryum*, which is in agreement with the results reported by Hayashi and Takayanagi on a Japanese strain (1962) and contradictory to Diller's report (1958). All caryonides obtained in this study showed an immature period of mating activity (mating activity appeared by 45 fissions after conjugation), while in *P. tetraurelia*, one of the well-studied species and known to have a "clonal" mating type inheritance, its progenies only have autogamy immature period rather than immature period of mating activity (Wichtermen, 1986). These results, in conjunction with our observation of cross-fertilization might suggest that *P. polycaryum* of Chinese strain represents a different mating type determination style of *Paramecium* genus. Our results might also suggest that certain differences exist between the Chinese and Japanese strains and the Indian strain and that these different strains belong to different sibling groups,

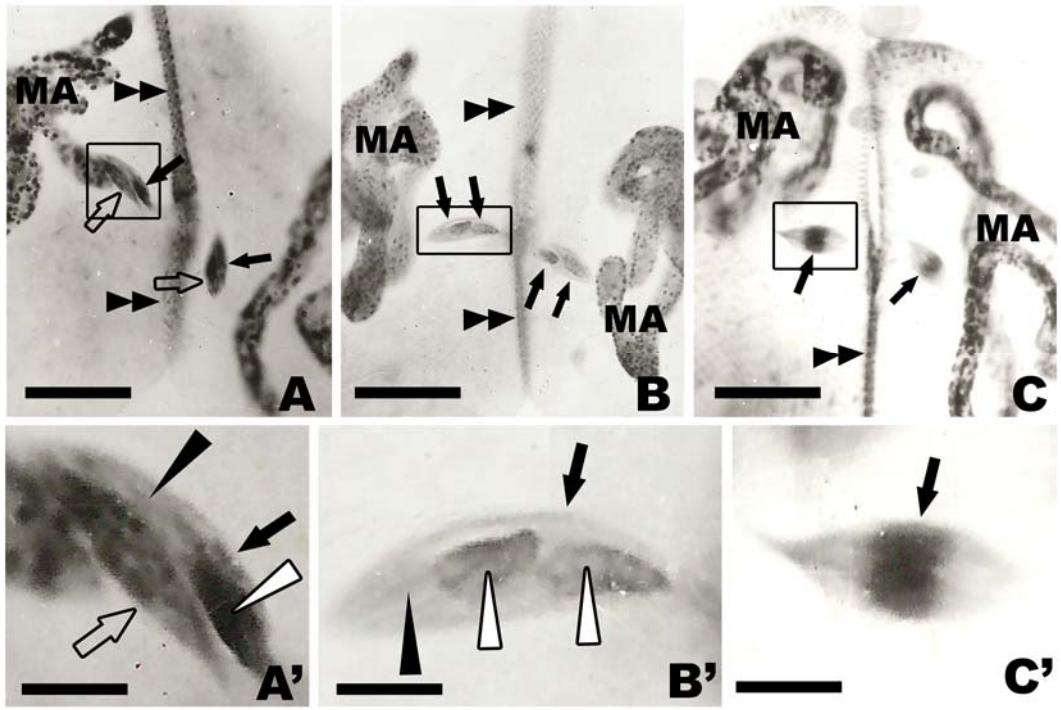

Fig. 4. Process of fertilized nuclear formation in *P. polycaryum*. A-C. Conjugants at different stages of fertilized nuclear formation. Framed portions in A, B, and C are magnified in A', B', and C', respectively. MA: old macronuclei assumed a twisted rope-like shape. Double arrowheads: conjugant boundaries. A. The MiN (solid arrows) attached to the StN (hollow arrows). A'. Bipolar spindle-shaped MiN (solid arrow) and StN (hollow arrow). White arrowhead: chromatin part of the MiN; black arrowhead: clear area at the anterior part of the MiN; thin small arrow: boundary between two gametic nuclei. B. Two parts of the nucleoplasm from two MiN and StN. B'. Fused nuclear membrane of the MiN and StN (arrow) and their separate nucleoplasm (white arrowheads). Black arrowhead: clear area at the anterior part of the MiN. C and C'. Typical fertilized nuclei (arrows). Scale bars: 20 μm in A, B, and C and 4 μm in A', B', and C'.

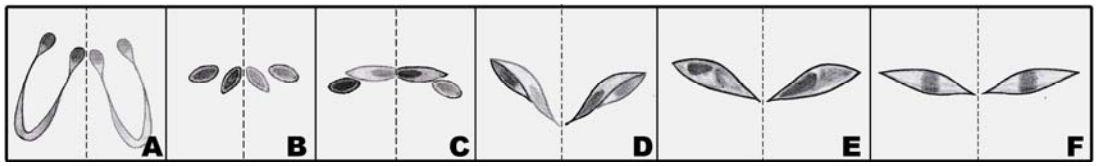

Fig. 5. Schematic representation of gametic nuclear exchange and fertilized nuclear formation of *P. polycaryum* based on the protargol staining images. Only gametic nuclei or fertilized nuclei are shown; the dotted line indicates the conjugating boundary. A. Telophase of the third prezygotic division. B. Soon after the third prezygotic division, the MiN locates in the paroral regions lying against the conjugating boundary, and the StN locates farther from the boundary. Both pronuclei show a round-end spindle shape and are covered by a thin clear area. C. Stage of gametic nuclear exchange showing enlarged and sharp clear areas at the anterior parts of the MiN. D. The MiN attaches to the side of the StN, and both of them show a bipolar-end spindle morphology. The membrane border between the MiN and the StN is observed. E. The membrane borders between MiN and StN disappear; two parts of nucleoplasm show heterogenous appearance. F. Formation of typical fertilized nuclei.

such as different syngens in *P. caudatum* and *P. bursaria* (Wichterman, 1986).

The gametic nuclear exchange was here studied using the modified protargol method. As reported above, this modified protargol method developed by Shi for staining the nucleus can stain not only chromatin but also the nuclear membrane, and the image obtained by this method gives higher resolution than the traditional nuclear staining methods of Feulgen or DAPI. Therefore, this method is extremely useful when the nuclear behavior of ciliates, especially, nuclear divisions, is studied. Our observations indicated the existence of reciprocal gametic nuclear exchange and true cross-fertilization in the Chinese strain of this species (Fig. 3, Fig. 4). More detailed nuclear behavior during the processes of nuclear exchange and fertilized nuclear formation is summarized in Fig. 5.

After the third prezygotic division, the shape of both the MiN and the StN changed into round-end spindles, and each of them consisted of a chromatin part and the clear area surrounding the chromatin (Fig. 2, B', C, C', Fig. 5B). These round-end spindle-shaped gametic nuclei probably correspond to the description of biconvex lens-shaped migratory gametic nuclei, and the clear area surrounding the chromatin corresponds to the structure of intranuclear microtubules lining the gametic nuclear membrane in *P. caudatum* (Nakajima *et al.*, 2001). During the MiN transfer, we observed that the clear area at the anterior part of the MiN became enlarged and sharper (Fig. 3A, A', Fig. 5C), containing some faintly stained filaments (thin arrows in Fig. 3B'); this structure might correspond to the tip of the crossing MiN of *P. caudatum*, in which many microtubules are aligned in the direction of its movement (Nakajima *et al.*, 2001). Other morphological studies on *P. caudatum* (Shi, 1998) and *P. dubosqui* (Watanabe *et al.*, 1996) using the protargol method also indicated the sharp and pointy anterior part of the crossing migratory gametic nuclei. All these observations might suggest that gametic nuclear transfer does not take place through amoeboid movement but, rather, as an active style.

Concerning the gametic nuclear exchange in ciliates, there are mainly two opinions. One focuses on the amoeboid movement of the MiN (André and Vivier, 1962; Inaba *et al.*, 1966), and the other emphasizes that extranuclear microtubule structures, either the microtubule meshwork on the back of MiN in *Tetrahymena* (Orias *et al.*, 1983) or the extranuclear bundles of microtubules in *P. aurelia* (Jurand, 1976), play an essential role in gametic nuclear transfer. Recent studies by Nakajima *et al.* (2001) indicated that both cytoplasmic (extranuclear) microtubules and intranuclear microtubules are necessary for gametic nuclear transfer in *P. caudatum*, and they suggested an active process of MiN migration. The pointy and sharp anterior part of the crossing MiN shown by protargol staining (Shi, 1998; Watanabe *et al.*, 1996; current study) provides support for the idea of an active process of MiN transfer.

After the third prezygotic nuclear division, both the MiN and the StN assumed a round-end shape, and the chromatin part was surrounded by a clear layer (Fig. 2B, B', C, C', Fig. 5B) consisting of microtubules (intranuclear microtubules) (Nakajima *et al.*, 2001). These intranuclear microtubules of the MiN may function in the active migration of the MiN itself (Nakajima *et al.*, 2001), while the function of the similar structure in the SP is not yet understood. After reciprocal gametic nuclear exchange, the MiN first attached to the side of the StN when both assumed the bipolar-spindle shape (Fig. 4A, A', Fig. 5D); then, the nuclear membranes of two gametic nuclei fused while their respective nucleoplasm remained separated (Fig. 4B, B', Fig 5E). Finally, the nucleoplasm from two gametic nuclei fused completely, and a typical fertilized nucleus formed as a result (Fig. 4C, C', Fig. 5F). The question of how the MiN recognizes the StN and fuses with it then has to be answered.

Both morphological and structural changes occur in both kinds of gametic nuclei before and after gametic nuclear transfer. All these changes suggest some certainly active preparatory processes for gametic nuclear transfer and/or MiN - StN recognition.

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