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Early Embryogenesis and Anterior-Posterior Axis Formation in the White-Tip Nematode *Aphelenchoides besseyi* (Nematoda: Aphelenchoididae)

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Abstract: We followed the early embryogenesis of *Aphelenchoides besseyi* from fertilization to the 4-cell stage under Nomarski optics and examined the chromosome number and structure by DAPI staining. After an oocyte is fertilized by a sperm, the eggshell forms and the male and female pronuclei are reconstructed. The male pronucleus moves toward the female pronucleus, which is located at the center of the egg. They meet, rotate 90°, and fuse. The embryo then divides unequally into a larger anterior AB cell and a smaller posterior P_1 cell. The site of sperm entry into the oocyte seems to become the future anterior pole of the embryo, and thus the formation of an anterior-posterior axis formation is the same as that for *Bursaphelenchus xylophilus*, but opposite to that for *Caenorhabditis elegans*. From immunostaining, the fertilizing sperm appears to bring the centrosome into the oocyte. The chromosome structure during the pronuclear meeting as observed by DAPI staining suggests that a haploid sperm (N = 3) fertilizes a haploid oocyte (N = 3) to form a diploid embryo (2N = 6) and that all chromosomes appear to be of a similar size. Unlike *C. elegans* does, the P_1 cell first divides anterior-posteriorly followed by the AB anterior-posteriorly. These divisions produced the 4-cell stage embryo with 4 cells arranged in a linear fashion, again in contrast to that for *C. elegans* or *B. xylophilus* configured in a rhomboid shape.

Key words: reproduction, amphimictic, chromosome number, centrosome, sex ratio, cell biology.

The nematode has classically been a popular organism for studying animal development for more than 100 years because of its handy size, availability, simple and transparent body, and reproducible development. Since a free-living soil nematode, *Caenorhabditis elegans*, was introduced as a model organism to study animal development and behavior (Brenner, 1974), various novel biological phenomena have been discovered and an enormous amount of sophisticated knowledge has been accumulated through research on this nematode. Completion of the embryonic and postembryonic cell lineages of *C. elegans* has laid a strong foundation for the molecular understanding of animal development (Sulston and Horvitz, 1977; Sulston et al., 1983). The connection and wiring diagram of all neuronal cells (White et al., 1986) has made it possible to analyze animal responses to environmental stimuli in cellular and molecular terms. The *C. elegans* genome was completely sequenced with about 19,000 predicted gene-coding regions in 100 million pairs of nucleotides (The *C. elegans* sequencing consortium, 1998). From the WormBase homepage, all published *C. elegans* data are retrievable by personal computer (WormBase, http://elegans.swmed.edu/). Its fundamental characteristics, features, and properties can now be compared with those of other free-living and parasitic nematodes to grasp evolutionary divergence among members of the phylum Nematoda (Fitch, 2005).

The white-tip nematode *Aphelenchoides besseyi* is the causal agent of white tip disease of rice, a disease whose occurrence and nematode distribution have been reported in almost every rice-growing region of the world (Fortuner and Williams, 1975; Duncan and Moens, 2006). This nematode is a facultative ecto- and endoparasite; it feeds on the host's leaves and young tissues, causing whitening of the tops of the leaves, which then die off and shred (Togashi and Hoshino, 2001; Duncan and Moens, 2006). In spite of its parasitic nature, *A. besseyi* is easily cultured in the laboratory on any of several fungi, such as *Alternaria alternatus*, *Curvularia lunata*, or *Fusarium verticillioides*, as food (Jamali et al., 2008) and is suitable for laboratory experimentation.

Although we have observed that this nematode has two sexes, male and female, it has been reported to have a strongly biased sex ratio or even to reproduce parthenogenetically (Fortuner and Williams, 1975; Gokte-Narkhedkar et al., 2001). During early embryogenesis of the nematodes, we could easily observe their chromosome structure and behavior and could confirm their reproductive strategy as being parthenogenetic or amphimictic (Hasegawa et al., 2006). In addition to clarifying the anterior-posterior axis formation and the orientation, the timing of cell cleavage during early embryogenesis is also important for understanding their basic biology (Goldstein et al., 1998; Goldstein, 2001; Schierenberg, 2006; Hasegawa and Miwa, 2008). Here we report the early embryogenesis of *A. besseyi* from fertilization to the 4-cell stage under Nomarski optics and confocal laser microscopy, as well as the chromosome number by DAPI staining. We also report the anterior-posterior axis formation by antibody staining.

Materials and Methods

Nematode strain and culturing: The nematode *Aphelenchoides besseyi* used in this experiment was isolated in Hiroshima prefecture Japan, a gift from Dr. Togashi, K., the University of Tokyo. Nematodes were cultured on a sparse fungal mat of *Botrytis cinerea* grown on 1/10 potato dextrose agar (PDA) or on a dense *B. cinerea* mat grown on sterilized barley (Hasegawa et al., 2006).

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Mass collection of early embryos: Like the pine wood nematode *Bursaphelenchus xylophilus* (Hasegawa et al., 2004), a gravid adult *A. besseyi* female usually has only one fertilized egg in the uterus. Unlike *B. xylophilus*, however, it rarely lays eggs in the absence of food. To collect many *A. besseyi* early embryos, we established the following method: *A. besseyi* was cultured on a fungal mat of *B. cinerea* grown on PDA for a few days at 30°C (Fig. 1A); while the nematodes were reproducing, some of them migrated from the surface of the PDA to the lid of the Petri dish (Fig. 1B). Nematodes in web-like aggregations were collected from the lid with sterile water, transferred to new fungal mats of *B. cinerea* grown on 1/10 PDA, and incubated for 24 to 48 hours at 30°C (Fig. 1C). After the incubation, the nematodes laid many eggs on the PDA plate. Freshly-laid eggs (prior to the first mitotic cell division) were collected by mouth pipette and either transferred to an agar pad on a microscope slide (Sulston and Horvitz, 1977) for viewing of living embryos or washed and collected by sterile water for DAPI staining or immunostaining (Fig. 1D). Living embryos were observed with Nomarski differential interference contrast optics (Nikon E 600). All living embryos observed for pronuclear meeting and cell division were followed until hatching.

After the nematodes were washed away from the lid, those remaining on the PDA surface continued to reproduce and gathered again on the surface of the lid (Fig. 1A, B). The plates were reusable for collecting early embryos until the fungi were exhausted.

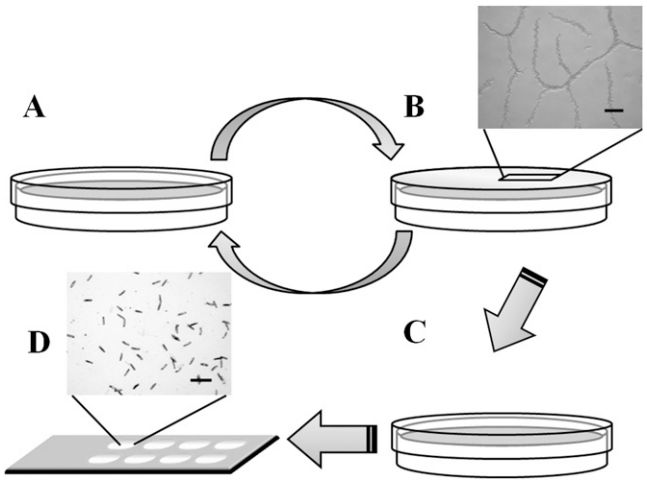

FIG. 1. Method for collecting early *A. besseyi* embryos. (A) *A. besseyi* is cultured on a fungal mat of *B. cinerea* grown on PDA for a few days at 30°C. (B) After a few days of culturing, the nematodes are reproducing and some of them have moved from the surface of the PDA to the lid of the Petri dish. Nematodes in web-like aggregations are visible (inset of B). (C) Nematodes are collected from the lid with sterile water, transferred to a new fungal mat of *B. cinerea* grown on 1/10 PDA, and incubated for 24 to 48 hours at 30°C. (D) After the incubation, many eggs are laid on the PDA plate. Many early embryos are collected and transferred to an 8-well slide (inset of D). Scale bar, B, 1 mm; D, 200 μm.

DAPI staining for chromosomes: To visualize the chromosomes during early embryogenesis, early *A. besseyi* embryos were collected from the PDA plate as described above, transferred into the wells of a poly-l-lysine-coated slide glass (Multitest slide 8-well, MP Biomedicals, LLC) (Fig. 1D), and allowed to adhere to the glass. The embryos were fixed, stained, and observed according to the procedures described by Hasegawa et al. (2006). Stained embryos were observed with a ZEISS Axiovert 200 microscope equipped with a confocal laser-scanning module (ZEISS LSM510).

Immunostaining for microtubules: Rabbit α-tubulin antibody staining was used to visualize the microtubules in the early *A. besseyi* embryos. Early *A. besseyi* embryos were collected from the PDA plate as described above, transferred into the wells of the poly-l-lysine coated slide glass (Multitest slide 8-well) (Fig. 1D), and allowed to adhere to the glass. Embryos were fixed and stained with monoclonal anti-α-tubulin (DM1α, Sigma) and FITC-conjugated anti-mouse antibodies (Sigma) according to the procedures described by Hasegawa et al. (2004). Stained embryos were observed with a ZEISS Axiovert 200 microscope equipped with a confocal laser-scanning module (ZEISS LSM510).

RESULTS

Pronuclear meeting and early embryogenesis: Although the sex ratio was biased (the number of females in the growing culture exceeded that of males), the *A. besseyi* used in this experiment could not propagate without mating (data not shown), which indicated that the mode of this reproduction system was sexual amphimictic, not parthenogenetic. After a few days of culturing, *A. besseyi* females migrated from the surface of the PDA and aggregated on the lid of the Petri dish (Fig. 1B). Almost all adult nematodes in the web-like aggregates were females, having sperm in their spermatheca but immature oocytes (Fig. 2A). These nematodes were then transferred to a new fungal mat of *B. cinerea* and incubated for 24 to 48 hours at 30°C. These transferred females fed on fungi, matured, and started laying eggs synchronously (Fig. 1C). A gravid adult *A. besseyi* female usually had only one fertilized egg in the uterus, whereas many sperm were observed in the spermatheca (Fig. 2B). After the oocyte moved through the spermatheca toward the uterus, the sperm fertilized the oocyte, the eggshell was formed, and then the two pronuclei, male and female, were reconstructed (Fig. 2B). The fertilized egg was laid around this period of pronuclear reconstruction, so we could observe the pronuclear meeting and early embryonic cell division.

Because the *A. besseyi* embryo is very long and transparent (about 20 × 80 μm), the relative positions and behavior of the male and female pronuclei could be clearly observed. The cortical membrane of a newly-laid

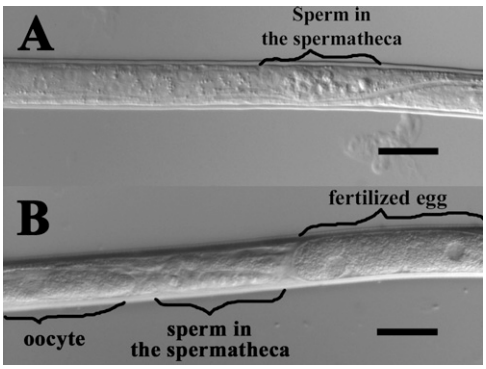

FIG. 2. (A) Spermatheca of an adult female collected from nematodes in the web-like aggregation. Although sperm are seen in the spermatheca, oocytes are not detectable. (B) Fertilized eggs in the uterus of an adult female. An oocyte in the oviduct and sperm in the spermatheca are visible. Anterior is left. Scale bar, 20 μm.

egg is smooth, and neither directed cytoplasmic streaming nor pseudo-cleavage was observed (Fig. 3A). At this time, one pronucleus appeared at one pole of the embryo and the other pronucleus emerged at a lateral position. The first and second polar bodies were extruded from the lateral pronucleus implying that it was female and the other male (Fig. 3A). The male pronucleus moved toward the female pronucleus, which remained at the center of the egg (Fig. 3B). They met and rotated 90° (Fig. 3C) and fused (Fig. 3D). The embryo then divided unequally (Fig. 3E) to form a larger cell, termed the AB cell, and a smaller cell, known as the P_1 cell (Fig. 3F). The next round of cell divisions began with the P_1 cell (Fig. 3G), followed by the larger AB cell (Fig. 3H) to form a linearly-arranged 4-cell stage embryo (Fig. 3I). It takes 123 ± 23 min (N = 4, at 24°C) from the appearance of the two pronuclei to the 4-cell stage. We continued to follow the embryogenesis and confirmed that all of the observed embryos hatched successfully (Fig. 3J, K, L). From the shape of the embryo at the tadpole stage (Fig. 3K), we could recognize the head and tail positions and discern that the pole of entry for the male pronucleus always became the future anterior pole (Fig. 3A).

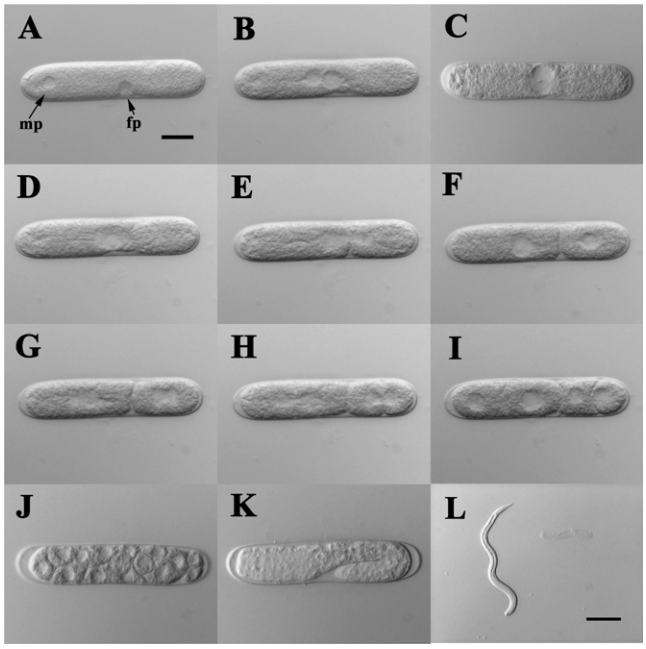

FIG. 3. Pronuclear meeting (A-D), early (E-J) and late (K) embryogenesis, and hatchee (L) of *A. besseyi* by Nomarski differential interference contrast optics. mp, male pronucleus; fp, female pronucleus. Anterior is left. Scale bar, A, 10μm; L, 50 μm.

Chromosome number: To clarify the reproductive strategy and chromosome number of *A. besseyi*, we observed the chromosomal structure and behavior during pronuclear meeting by DAPI staining. Soon after fertilization and before pronuclear reconstruction, the sperm appeared as a condensed dot and six chromosomes were visible during the 1st meiosis (Fig. 4A, B, C). After the completion of oocyte meiosis, the male and female membranous pronuclei were reconstructed and subsequently migrated. Pronuclear chromosomes became condensed, and each pronucleus contained three chromosomes (Fig. 4D, E, F). After the pronuclear meeting, the embryo started mitotic cell division (Fig. 3) and six diploid chromosomes were visible in each cell of the two-cell stage embryos (Fig. 4G, H). As these results indicate, the mode of reproduction for this nematode is amphimictic, with a haploid sperm (N = 3) fertilizing a haploid oocyte (N = 3) to form a diploid embryo (2N = 6). All three chromosomes look similar.

Microtubule formation and axis polarity from fertilization to 4-cell stage: In *C. elegans* the fertilizing sperm brings the centrosome into the oocyte and is thought to determine the anterior-posterior axis of the embryo (Goldstein and Hird, 1996; Galli and van den Heuvel, 2008). We analyzed the relationship of the microtubule formation and anterior-posterior axis in *A. besseyi* embryos following fertilization through the 2-cell stage.

After fertilization, the *A. besseyi* oocyte resumed its 1st meiosis, and the meiotic spindle segregated bivalent chromosomes (Fig. 5A, a). A green fluorescence dot associated with sperm stained with anti-α-tubulin antibody was seen at the future anterior pole of the embryo (Fig. 5A a). Two pronuclei were reconstructed and the duplicated centrosomes were nucleating microtubules around the male pronucleus moving toward the female pronucleus (Fig. 5 B, b). The two pronuclei met and rotated 90° at the center of the embryo such that the two nucleating centrosomes were symmetrically located anterior-posteriorly (Fig. 5C, c). The two pronuclei fused (Fig, 5D, d), the posterior centrosome moved posteriorly in anaphase (Fig. 5E, e), and the egg divided unequally to form the 2-cell stage (Fig. 5F, f). Subsequently, the smaller P_1 cell first divided anterior-posteriorly (Fig. 5G, g), followed by the larger AB cell dividing anterior-posteriorly (Fig. 5H, h) to form a linearly-arranged 4-cell stage embryo (Fig. 5I, i).

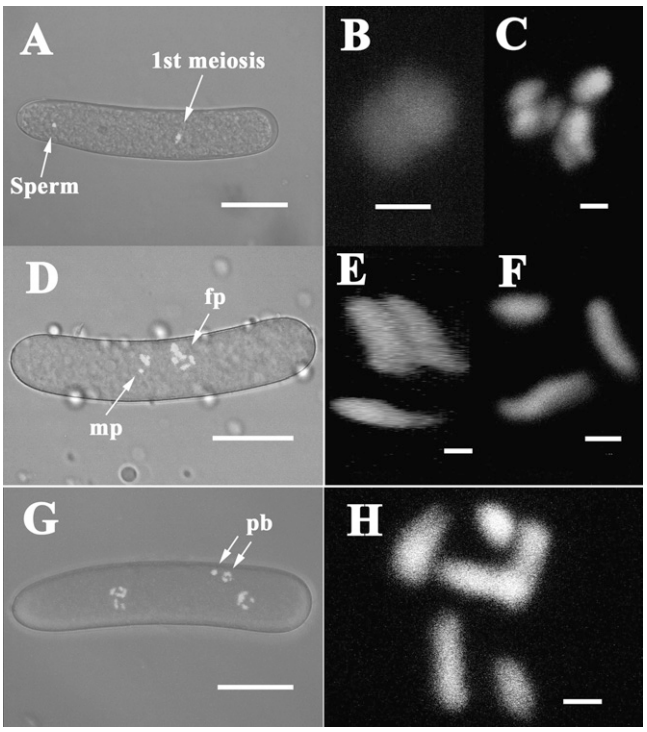

FIG. 4. Confocal laser-scanning microscope images wherein DNA is visualized by DAPI staining. (A) Fertilized egg, just after extrusion from the vulva (oviposition). The sperm is seen as a dot at the future anterior end of the embryo (left side in this photograph), and the 1st meiosis is located laterally in mid-embryo. Merged view of the bright field and fluorescence images. (B) High magnification of the sperm shown in the (A) fluorescence image. (C) High magnification of the first meiosis, shown in the (A) fluorescence image. (D) Rearrangement of male (mp) and female (fp) pronuclei. Merged view of the bright field and fluorescence images. (E) High magnification of male pronucleus and (F) Female pronucleus shown in (D) fluorescence image. (G) 2-cell stage embryo. Merged view of the bright field and fluorescence images. (H) High magnification of the chromosomes of the AB cell, shown in (G) fluorescence image. Anterior is left. Scale bar: A, D, G, 20 μm; B, C, E, F, H, 1 μm.

DISCUSSION

To analyze the reproductive strategy of *A. besseyi*, we observed its chromosome structure and behavior during pronuclear meeting and early embryogenesis as these stages provide easier access to many cellular events (Hasegawa et al., 2006). We conclude that the mode of this reproduction system is sexual amphimictic because *A. besseyi* could not propagate without mating; both haploid male and female pronuclei had three chromosomes (N = 3); and two pronuclei fused to form a diploid nucleus in the fertilized egg (2N = 6) followed by embryonic development. Nematodes employ a variety of reproductive strategies, such as asexual meiotic parthenogenesis, mitotic parthenogenesis, sexual amphimixis, hermaphroditism, and pseudogamy (Triantaphyllou and Hirschmann, 1964; Goldstein, 1981; Wright and Perry, 2006). Some *Aphelenchoides* species (B'Chir and Dalmasso, 1979) as well as some *A. besseyi* isolates (Fortuner and Williams, 1975; Gokte-Narkhedkar et al., 2001) were reported to reproduce parthenogenetically. Although the nematode presently studied reproduces amphimictically, male ratios in a population maintained under laboratory conditions were low (Huan et al., 1979; Jamali et al., 2008; Yoshida et al., unpublished data), and almost all adult nematodes collected from the web-like aggregated population were females (Fig. 2A).

In this study, the haploid chromosome number detected in all *A. besseyi* male and female pronuclei was three, and the morphology of all three chromosomes was apparently identical (Fig. 4). Thus, the sex determination system in *A. besseyi* does not appear to be of the XO type as in *C. elegans* (Meyer, 1997). The sex of *A. besseyi* might be determined by a male/female heterogamete system, by other multiple sex chromosome systems such as that for *Ascaris suum* (Goldstein, 1981), or by environmental control (Triantaphyllou, 1971). This sex ratio distortion might also be controlled by cytoplasmically inherited microorganisms, such as *Wolbachia* (Charlat et al., 2003), Microsporidia (Terry et al., 2004), or *Spiroplasma* (Montenegro et al., 2005).

The anterior-posterior axis formation marks the first apparent step in the establishment of the nematode body plan. In *C. elegans*, the anterior-posterior axis is reported to depend on the centrosome, which is brought into the egg by the sperm, the actin cytoskeleton, and such maternal elements as cortical protein and cytoplasmic determinants. The sperm entrance site on the egg eventually becomes the presumptive posterior pole (Galli and van den Heuvel, 2008).

The relationship of sperm entry to anterior-posterior axis formation is reported in some other nematode species. In some species, this axis was determined by the sperm, whereas in others it was not (Goldstein et al., 1998; Schierenberg, 2006). In *Acrobeloides* sp. (PS1146), the fertilizing sperm neither seems to bring the centrosome into the oocyte nor contributes to the anterior-posterior axis formation (Goldstein et al., 1998). In parthenogenetic nematodes, embryogenesis starts without fertilization. In the parthenogenetic *Acrobeloides nanus*, anterior-posterior polarity is defined by the mother's body axis; whereas in another parthenogenetic species, *Diploscapter coronatus*, anterior-posterior polarity is expressed at random (Schierenberg, 2006). These nematodes seem to utilize some other cues, such as maternally supplied materials, environmental conditions, or random decision (Goldstein et al. 1998; Schierenberg, 2006). Although directed cytoplasmic streaming and pseudo-cleavage were not observed, the pole where the male pronucleus appeared in all *A. besseyi* embryos examined presently became the future anterior pole (Fig. 3), and this axis formation is the same as that for the pine wood nematode *B. xylophilus* (Hasegawa et al., 2004; Hasegawa et al., 2006). Active nucleating centrosomes associated with the male pronuclei were also detected during the male pronuclear movement toward the

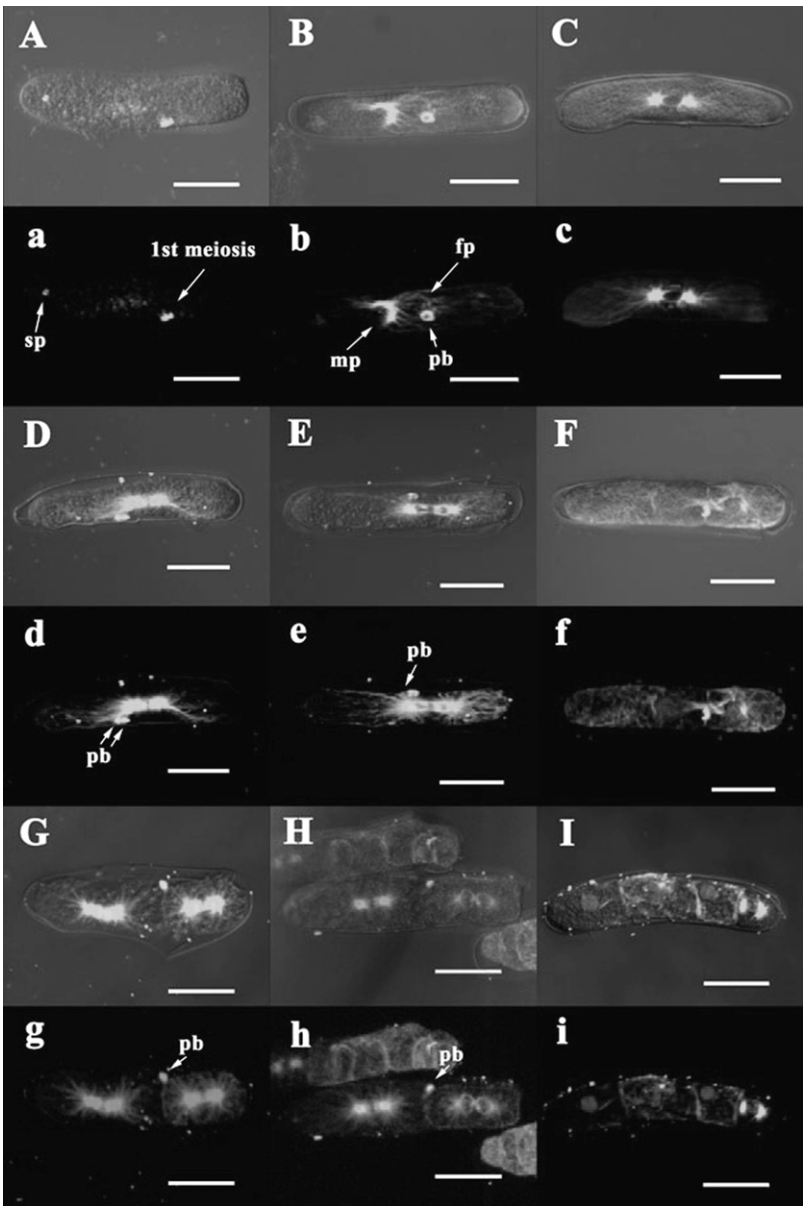

FIG. 5. Confocal laser-scanning microscope images where microtubules are visualized by antibody (Green) and DNA by DAPI staining (Blue). (A - I) Merged view of the bright field and fluorescence images, (a - i) fluorescence images. (A, a) Just after oviposition, the sperm (sp) is visible as a faint dot at the future anterior pole of the embryo and the meiotic spindle segregates bivalent chromosomes. (B, b) Two pronuclei are reconstructed, and the male pronucleus moves toward the female pronucleus while the duplicated centrosomes around it are nucleating microtubules. (C, c) Two pronuclei meet and rotate 90° at the center of the embryo and two nucleating centrosomes are symmetrically located anterior-posteriorly. (D, d) Metaphase stage embryo. (E, e) Anaphase stage embryo, posterior centrosome moved posteriorly. (F, f) 2-cell stage embryo. (G, g) First the smaller P_1 cell divides anterior-posteriorly (H, h), then the larger AB cell divides anterior-posteriorly. (I, i) Linearly-arranged 4-cell stage embryo. mp, male pronucleus; fp, female pronucleus; pb, polar body. Anterior is left. Scale bar, 20 μm.

female pronucleus (Fig. 5). This evidence suggests that the sperm entry position determines the anterior-posterior axis in this nematode.

In *C. elegans*, the AB and P_1 cells display different developmental fates and division patterns. The AB cell begins to cleave first, with the cleavage furrow parallel to the long axis (anterior-posterior axis), followed by the P_1 cell cleavage, parallel to the short axis (dorsal-ventral axis), and the embryo enters the rhomboid-shaped 4-cell stage. The position of ABp at this stage marks the future dorsal, thus forming the dorsal-ventral axis (Gönczy, 2005). Furthermore, signaling from the P_2 cell controls the EMS division plane and cell fate (Gönczy, 2005). The developmental timing and orientation of the *B. xylophilus* embryo, from 2-cell to 4-cell stage, were also similar to those of *C. elegans* (Hasegawa et al., 2004). In contrast to these nematodes, however, the embryogenesis of *A. besseyi* from the 2-cell to 4-cell stage was very unique in that the smaller P_1 cell divided anterior-posteriorly before the larger AB cell did (Fig. 5). Because of these cell divisions, the four cells are arranged linearly, making it difficult to even guess the

dorsal side from the ventral side at this stage (Fig. 5). Linear arrangement of the 4 cells was also observed in some other species (Goldstein, 2001). The nature of the mechanism of controlling the dorso-ventral axis determination in these species, which must be different from that for *C. elegans* or *B. xylophilus*, is a challenging problem to solve.

Now that we have developed an easy way of hitherto difficult mass culturing of *A. besseyi* in Petri dishes, a handy method to obtain a good number of pre-pronuclear fusion eggs, and synchronizing the developmental stages, the cellular embryology of this culturally important and yet peculiar nematode should be feasible, as we have demonstrated here.

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