The differentiation of cellular structure during encystment in the soil hypotrichous ciliate Australocirrus cf. australis (Protista, Ciliophora)

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ABSTRACT
Ciliates are able to form resting cysts as a survival strategy in response to stressful environmental factors. Studies on the characteristics of cellular structure during encystment may provide useful information for further understanding of the regulatory mechanism of cellular patterns and supply new clues regarding the phylogeny of ciliates. Scanning and transmission electron microscopies were used to observe the ultrastructure of cells during encystment of the soil ciliate Australocirrus cf. australis. The dedifferentiation of ciliature was revealed for the first time. Ciliary shafts first shortened, and the remaining ciliature, including basal bodies and the fibrillar cirral basket, retracted into the cytoplasm and was surrounded by the autophagic vacuoles and then gradually digested. A large number of autophagic vacuoles were observed in mature resting cysts. Autophagy might not only be necessary for the differentiation of cellular structures during encystment but might also be important to sustain the basic life activities in the resting stage. Australocirrus cf. australis formed a kinetosome-resorbing cyst and contained four layers in the cyst wall: the ectocyst, mesocyst, endocyst and granular layer. The ciliature resorbing state and the number of layers in the cyst wall were consistent with those found in other oxytrichous ciliates. However, the phenomenon wherein the two macronuclear nodules are not fused during encystment is not commonly observed among oxytrichids. Additionally, the octahedral granules in the mesocyst of this species exhibit different morphology from the congeners.

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Introduction
Ciliated protists are eukaryotic unicellular organisms with wide distributions in various habitats (Gu 1991; Berger 1999, 2006, 2011). Ciliates in many groups form resting cysts when cells are subjected to stressful environmental conditions, such as starvation, high population density, and salinity variation; whereas excystment occurs when environmental conditions become favorable. This process is typically regarded as a survival strategy of ciliates responding to their living conditions (Gu et al. 1996; Li et al. 2012). During such encystment and excystment, cellular structures undergo dedifferentiation and redifferentiation (Grimes 1973; Walker et al. 1975, 1980; Walker & Maugel 1980). Studying the resting cysts of ciliates provides evidence to further understand the adaptation of eukaryotic cells to their habitats, the function of organelles, and the regulatory mechanism of cellular patterns (Gu & Zhang 1992). Previous studies have mainly focused on the process of ciliature differentiation and cyst wall formation. However, the detailed mechanism of ciliature differentiation, especially focusing on the basal body units, is largely unknown (Grimes 1973; Walker et al. 1975, 1980; Walker & Maugel 1980; Matsu-saka 1984, 1989; Gu & Ni 1995; Gu & Xu 1995; Gu et al. 1996, 2002; Foissner et al. 2007; Müller et al. 2010; Benča-tová et al. 2016). Moreover, ultrastructural characteristics may also provide further insight to the taxonomy and phylogeny of hypotrichous ciliates (Berger 1999, 2006, 2011).

Australocirrus cf. australis is a large soil ciliate (Foissner 1995; Berger & Foissner 1997; Yang et al. 2015). It was utilized in the present study, because it can be easily cultured and induced to encyst. The ultrastructures of vegetative cells, encysting cells, and resting cysts were observed and discussed. The results provided new information for cell differentiation under particular physiological conditions.

Materials and methods

Materials and identification
We used the same population of A. cf. australis as described in Yang et al. (2015) with name of Cyrtohymena (Cyrtohymenides) australis, which was collected from the soil of farmland in Shandong, China, in 2012. For detailed light microscopic morphology, morphometry, and phylogeny see Yang et al. (2015). According to the new arrangement of Australocirrus species in Kumar and Foissner
our organisms are similar to both *Australocirrus shii* and *A. australis* but not identical to either species. Given that this species does not cluster with *A. shii* in phylogenetic trees as revealed in Yang et al. (2015), we therefore identified this species as *A. cf. australis*. The uniprotistan culture was maintained in Petri dishes with filtered, ciliate-free pond water at room temperature, and sterilized wheat grains were added weekly to support continuous bacterial food. After the cultured cells reached a relatively high density, the feed was reduced to induce the cells to form cysts (Gu et al. 1996).

**Scanning electron microscopy (SEM)**

Vegetative cells and encysting cells were fixed in a 1:6 mixture of 1% O$_2$O$_4$ and a saturated solution of HgCl$_2$ at room temperature for 10 min, whereas the resting cyst were fixed for 15 min in the same fixative. Then, the cells were rinsed with 0.1 M phosphate buffer, dehydrated in a graded series of ethanols, dried with a critical point dryer (Leica CPD300), and coated with gold in an ion coater (Leica ACE600). Observations were performed using a scanning electron microscope (Hitachi S-4800) at an accelerating voltage of 10 kV (Gu & Ni 1993).

**Transmission electron microscopy (TEM)**

Ciliates were prefixed in a 1:1 mixture of 2% O$_2$O$_4$ and 2.5% glutaraldehyde at 4°C for 10 min, and the resting cyst were fixed for 15 min. The fixed cells were then washed several times with 0.1 M phosphate buffer and postfixed in 1% O$_2$O$_4$ at 4°C for 1 h. The postfixed cells were washed again and then dehydrated through a graded series of acetones and embedded with Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (Hitachi 7700) at an accelerating voltage of 100 kV (Gu et al. 2002a).

**Results**

**Morphology of cells in vegetative and encysting stages**

The vegetative cells of *A. cf. australis* displayed an elongated oval shape, measuring approximately 100 to 250 µm × 60 to 80 µm. Adoral zone of membranelles (AZM) occupied 40% of the body length, containing approximately 50 membranelles and diaphragm structures between two adjacent membranelles (Figure 1(A)). Each membranelle was composed of 3 or 4 rows of basal bodies (Figure 2). An undulating membrane (UM) covered the buccal cavity and was distinctly curved at its anterior part. Eighteen frontal-ventral-transverse cirri were arranged following a typical ‘Oxytricha’ mode: three frontal cirri (FC), one buccal cirrus (BC), and four frontal-ventral cirrus (FVC) right of the adoral zone; three postoral-ventral cirri (PVC) and two pretransverse cirri (PTVC) located separately; and five transverse cirri arranged in an oblique row. The distance between the anterior pretransverse ventral cirrus and the anterior transverse cirrus as a percentage of body length was 1.90–6.84% (4.17% on average, n = 12). A single row of left and right marginal cirri (LMC, RMC) was located near the cell margins (Figure 1(A)). We observed 9–12 dorsal kineties (DK) and three caudal cirri (CC) (Figure 1(B)).

A locomotion change from swimming freely to circling in situ and slower contractile vacuole activity were indicative of the beginning of encysting. At the early stage, the posterior part of the cell bent backward, whereas the UM collapsed, and the ciliary shafts of membranelles near the posterior end of the AZM dedifferentiated first (Figure 3(A)). Later, the posterior part of the cell further twisted, and the two ends pushed toward to the middle. This action caused the cell to lose its normal shape, and a few marginal cirri and transverse cirri reversed to the dorsal side (Figure 3(B)). Then, the shape of the cells gradually changed into sphere, but the dorsal bristles were unchanged (Figure 3(C)). In the next stage, ventral cirri likely disassembled in a certain order because the five transverse cirri were observed to be unequal in

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**Figure 1.** Scanning electron micrographs of the ventral and dorsal surface of *A. cf. australis* in the vegetative stage. Scales are 10 µm. (A) Ventral surface showing the arrangement of cilia. (B) Dorsal surface showing dorsal kinetics and caudal cirri. AZM, adoral zone of membranelles; CC, caudal cirri; DK, dorsal kinetics; FVC, frontal-ventral cirri; LMC, left marginal cirri; PTVC, pretransverse ventral cirri; PVC, postoral-ventral cirri; RMC, right marginal cirri; TC, transverse cirri; UM, undulating membrane.
length (Figure 3(D)). As a consequence of the transformation, the location of the transverse cirri and the collar part of the AZM became the two poles of the sphere cell (Figure 3(D)). The spherical shaped cell further lost its dorsal bristles, and its surface formed a large number of irregular protuberances, possibly due to dehydration.

Figure 2. Images of the membranelles in SEM: in collar part of AZM were composed four basal body rows (left), those in lapel part comprised three rows (right) of basal bodies.

Figure 3. Scanning electron micrographs of the morphological changes and differentiation of ciliature during encystment. Scales are 10 µm. (A) Ventral view showing the disappearance of the undulating membrane (arrow) and the ciliary shaft dedifferentiation of membranelles near the posterior end of the dedifferentiated AZM (arrowhead). (B) Dorsal view showing the twisted posterior part of a cell with marginal cirri (arrowhead) and transverse cirri (arrow) that reversed to the dorsal side. (C) Dorsal view of a cell changing into a spherical shape, demonstrating the unchanged dorsal bristles. (D) Dorsal view of a spherical shaped cell, demonstrating the disassembly of the TC and the location of the TC and AZM after the cell transformation. (E) The dorsal bristles were absorbed, and the cell surface formed irregular protuberances (arrowhead). (F) The surface of a mature resting cyst. AZM, adoral zone of membranelles; DK, dorsal kineties; TC, transverse cirri.
during encysting and differentiation of the cyst wall (Figure 3(E)). The mature cysts were 42–70 µm (58.5 µm on average, \(n = 14\)) in diameter in SEM preparations, while 59–84 µm (69.2 µm on average, \(n = 13\)) in light microscopy, with flat wrinkles on the surface (Figure 3(F)).

**The dedifferentiation of ciliature during encystment**

In vegetative cells, the ciliary shafts of AZM were composed of central microtubules, peripheral microtubules, and plasma membranous structures. Basal bodies were underneath the pellicle accompanied by microtubules and abundant mitochondria (Figure 4(A)). During encysting, microtubules underneath basal bodies disintegrated into fragments, and the mitochondria decreased in number (Figure 4(B)). The plasma membrane around ciliary shafts inflated, and some of the connecting fibers between basal bodies disappeared (Figure 4(C)). The cirri of body ciliature of vegetative cells comprised dozens of aggregated cilia, and their basal bodies were located in the surrounding fibrillar cirral basket beneath the pellicle and associated with developed attached structures of the ciliature (Figure 4(D)). During encystment, the ciliary shafts became scattered, and the top of ciliary shafts was coated by the inflated plasma membrane, which formed a vesicle structure containing a low electron dense substance. The ciliary shafts were shortened and the apical end of cilia was inflated (Figure 4(E)). The bases of cirri retracted, and the associated microtubules detached. Then, the residual ciliature structures, that is, basal bodies, and part of the ciliary shafts surrounding the fibrillar cirral basket together with some of the cytoplasm were surrounded by a plasma membranous structure to form a vesicle. The surrounding fibrillar cirral basket attached to the inner side of the vesicle membrane as a type of skeleton (Figure 4(F, G)). In the following stage, the ciliary shafts were completely disintegrated, and the basal bodies were disorganized into short microtubules (Figure 4(H)). The vesicle separated from its originating site where the pellicle then fused. The inclusion of the vesicle was gradually digested as the vesicle migrated into the deep cytoplasm. However, the surrounding fibrillar cirral basket and incomplete basal bodies were observed before the cyst wall started to form (Figure 4(I, J)). Such vesicles were not observed in mature resting cysts, and no ciliature structures were noted.

**Cyst wall and changes in organelles in the cytoplasm and nuclei**

At the beginning of encystment, membranous vacuoles emerged in the cytoplasm, containing a highly electron dense substance, accompanied by a banded or tubular-shaped structure that was similar to the Golgi apparatus (Figure 5(A)). Then, the inclusions in the membranous vacuoles changed into dense granules, and the vacuoles gradually migrated towards the pellicle, which possibly released cyst wall material outward (Figure 5(B)). As the cyst wall material accumulated, a fibrous substance formed a lamellar structure, and the sub-pellicle microtubules disassembled (Figure 5(C, D)). The cyst wall of the mature cyst thoroughly enclosed the cell and comprised four distinct layers: ectocyst, mesocyst, endocyst, and the granular layer (Figure 5(E)). The ectocyst, 78 nm on average \((n = 16)\), was composed of relatively thin fiber layers, forming a slightly wrinkled cyst surface, which was also revealed by SEM (Figure 3(F)). The thickness of desmosome changed accompanied with the wrinkles, from 80 nm to 2.3 µm \((n = 16)\), with low electron density in most parts. A few highly electron dense polygonal granules were also found in the mesocyst (Figure 5(E, H)). When the ectocyst was dilacerated, the granules were revealed to be octahedrons distributed among the filamentary network (Figure 5(F, G)). The endocyst, 517 nm thick on average \((n = 16)\), exhibited a similar density to the ectocyst, displaying regular fine stripes (Figure 5(E, H)). The granular layer was relatively thin, 109 nm on average \((n = 16)\), and attached to the notched pellicle (Figure 5(E, H)). High-density granules could be observed underneath the pellicle, which were possibly the cortical granules (Figure 5(H)). The cytoplasm of the mature resting cyst was very condensed with high electron dense materials. The mitochondria were mainly located in the periphery, with a small number located in the center. They showed no significant structure changes except for decreased numbers (Figure 5(I)). Two macronuclear nodules were present, and their nuclear membrane was difficult to distinguish. The chromatin aggregated, and some nucleoli collapsed. Moreover, the cytoplasm contained many spherical autophagic vacuoles that included different materials and structures, such as the collapsed mitochondria, tightly twisted fibers, bacteria, and some dense granule materials. The micronucleus appeared to be unchanged (Figure 5(J)).

**Discussion**

**Characteristics of the cortical structure during encystment of A. cf. australis**

Numerous reports have focused on the morphology of cysts in different Hypotrichia (s. l.) ciliates. Based on the extent of resorption of the ciliature after encystment, resting cysts of hypotrichids are classified into three typical groups: (1) non-kinetosome-resolving cysts of
euplotids; (2) partial-kinetosome-resorbing cysts of urostylids; and (3) kinetosome-resorbing cysts of oxytrichids (Grimes 1973; Rios et al. 1985; Berger 2006; Zhao et al. 2009). However, observations focusing on the mechanism of ciliature disintegration are quite limited. A few ciliary shafts were dedifferentiated in autophagic vacuoles in cysts of *Euplotes* (Gu & Ni 1995). Matsusaka et al. (1984) revealed that the axonemes and basal bodies disintegrated after they retracted into the cytoplasm in *Histricus muscorum*. In the present study, the

Figure 4. Transmission electron micrographs of the dedifferentiation of the cortex in oral zone membranelles (A–C) and the dedifferentiation of cirri (D–J) during encystment. Scales are 1 µm (A, B, G, and J), 0.5 µm (C, E, F, H, and I), and 2 µm (D). (A) The cortex in oral zone membranelles of a vegetative cell, depicting the longitudinal section of membranelles, some microtubules (arrowheads), and abundant mitochondria. (B, C) The cortex in oral zone membranelles of an encysting cell, depicting the fragmentation of microtubules (arrowhead in B) and the inflated ciliary shafts (arrow) and the disappearance of the connecting fibers (arrowheads) between the basal bodies (C). (D) Cirri of vegetative cell showing the aggregated cilia, surrounding fibrillar cirral basket (arrowheads) and associated microtubules (arrow). (E) Image depicting the scattered cilia (arrowhead), the shortened ciliary shafts, and the inflated apical end of cilia (arrow). (F) Image depicting the retraction of cirri, the shortened ciliary shafts, and separation of associated microtubules (arrowhead). (G) Image depicting the vesicle formed by the cirri retraction, which includes basal bodies, part of the ciliary shafts, some cytoplasm, and the surrounding fibrillar cirral basket (arrowhead). (H) Image depicting vanishing ciliary shafts (arrow) and the basal bodies disorganized into short microtubules (arrowhead). (I) The vesicle separated from the pellicle (arrow). (J) The vesicle migrated into the deep cytoplasm and diminished, and the existence of basal bodies and the surrounding fibrillar cirral basket is shown (arrow). Mit, mitochondrion.
soil ciliate *A. cf. australis* resorbed all ciliature during encystment, forming kinetosome-resorbing cysts. This finding is consistent with the previous conclusion of hypotrichous ciliates (Berger 1999, 2006). During encystment, the dedifferentiation of ciliature occurred in a specific order. For oral ciliature, dedifferentiation started from the disassembly of the UM, and then the related microtubules gradually disintegrated. For body ciliature, ciliary shafts shortened first followed by the separation of associated microtubules and the retraction of the remaining ciliature into the cytoplasm. Then, the retracted structure formed a vesicle, which was

Figure 5. Transmission (A–E, H–J) and scanning (F, G) electron micrographs of cyst wall formation (A–D) and structures of mature resting cysts (E–J). Scales are 0.5 µm (A–D), 200 nm (E), 10 µm (F, G, and I), and 1 µm (H and J). (A) Image depicting a membranous vacuole (arrowhead) and the structure similar to the Golgi apparatus (arrow). (B) The inclusion of membranous vacuoles caused them to change into dense granules, and the membranous vacuoles fused with the pellicle (arrow). (C) Image depicting the accumulation of the cyst wall material (arrow); arrowhead indicates the microtubules under the pellicle. (D) Image depicting the stratification of cyst wall materials; arrowhead marks the microtubules absorbed. (E) Image depicting the four layers of the cyst wall in a mature resting cyst: ectocyst, mesocyst, endocyst, and the granular layer. The arrow depicts the polygonous granules in the mesocyst. (F and G) The granules were octahedral (arrows) and distributed among the filamentary network (arrowhead). (H) The structure of cyst wall and cytoplasm. (I) A cross-section of a resting cyst depicting the two macronuclear nodules and the cytoplasm that contained spherical autophagic vacuoles and mitochondria. (J) Image depicting the autophagic vacuoles in the cytoplasm (arrowheads) and the micronucleus. Ec, ectocyst; En, endocyst; Gr, granular layer; Ma, macronuclear nodules; Me, mesocyst; Mi, micronucleus; Mit, mitochondrion.
transported to the deeper cytoplasm and digested. In the present study, we first revealed the detailed digestion process of cortex ciliature in the encysting cells of *A. cf. australis*. Moreover, the vesicle which covered and gradually digested the residual ciliature (basal bodies, surrounding fibrillar cirral basket) performed an equivalent function of autophagy vacuole; thus, autophagy played an important role in the cortical ciliature dedifferentiation. This process may be regarded as an energy resource for encysting formation.

**The function of autophagy**

It was previously found that part of the food vacuoles were preserved from the trophic phase and digested during cyst phase in two colpodids, *Pseudomaryna australiensis*, and *Sandmanniella terricola* (Foissner 2003; Foissner & Stoeck 2009). However, autophagy in resting cysts was successively reported in hypotrichous ciliates (Gu & Ni 1995; Gutiérrez et al. 2001; Sun et al. 2014). In the present study, we demonstrated that autophagy also occurred in the resting cysts of *A. cf. australis*. Therefore, autophagy might not be the only process necessary for dedifferentiation of cellular structures as discussed above; autophagy is also important to sustain the basic life activities of hypotrichous ciliates with the capacity of encysting.

**The cyst wall and macronuclei**

Previous studies have accumulated evidence on the basic structures and the general formation mechanism of the cyst wall in Hypotrichia (s. l.) ciliates (Walker et al. 1980; Gutiérrez et al. 1983; Gu et al. 1996; Verni & Rosati 2011). It has been concluded that oxytrichids have four layers in their cyst wall, and that euplotids have two layers, and that urostyldids have three layers (Walker et al. 1975; Walker & Maugel 1980; Rios et al. 1985; Delgado et al. 1987). The wall of resting cysts is composed of ectocyst, mesocyst, endocyst, and granular layers in *A. cf. australis*, which is consistent with the characteristics of other oxytrichids that have been studied (Berger 1999; Foissner et al. 2007). Studies have also revealed that the ectocyst morphology of oxytrichids exhibits two different modes, that is, a spine-like protuberance (e.g. *Cyrtohymena tetracirrata*, *Laurentiella acuminata*, *Steinia sphagnicola*, *Kahlilera simplex*, and *Oxytricha bifaria*) or a slightly wrinkled surface (*Gastrostyla steinii*, *Oxytricha granulifera*, and *Parentocirrus hortualis*). *Australocirrus* *cf. australis* exhibited the later mode. Foissner et al. (2007) concluded that such ornamentations of ectocysts could not be used to distinguish the relationships of genera within the large family Oxytrichidae. However, this might be possible for the comparison of *Australocirrus* with its closely related genus, *Cyrtohymena*. *Australocirrus shii* and *A. australis*, which were recently transferred from *Cyrtohymena*, *A. oscitans*, and the present species all exhibit a slightly wrinkled ectocyst surface (Berger 1999; Singh et al. 2013; Kumar & Foissner 2015). In contrast, *Cyrtohymena tetracirrata* has spine-like protuberance (Foissner & Foissner 1987). Furthermore, we discovered that *A. cf. australis* also has granules in the mesocyst similar to its congeners, which were, however, not identical to globular granules of *A. australis* and crystals with six arms in *A. oscitans* (Foissner et al. 2005; Kumar & Foissner 2015). A *Rigidohymena* species was found to have osmiophilic crystalline structures of similar morphology in a cross-sectional view (Gu et al. 1996). The agglomerations of crystal-like particles were observed in *K. simplex* cysts, which were quite large and different from the octahedrons in *A. cf. australis* (Foissner & Foissner 1987). Therefore, the crystal granules in cyst walls might be a candidate characteristic for systematic analyses of hypotrichous ciliates.

Berger (2006) indicated that the state of macronuclear nodules, that is, fused or not fused, might serve as a clue regarding the phylogeny of the hypotrichs (s. l.). The macronuclei fuse during encystment in most oxytrichids (Berger 1999; Blatterer & Foissner 2003; Kumar & Foissner 2015; Benčaťová et al. 2016). Two macronuclear nodules were not fused in the resting cysts of *A. cf. australis*, which was similar to *A. shii* but differed from *A. australis* (Kumar & Foissner 2015). This finding implied that the state of the macronuclei was possibly not applicable for phylogenetic analysis in hypotrichous ciliates, even at the genus level.

**Disclosure statement**

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