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A PRELIMINARY STUDY ON THE PROBLEMS IN THE PREPARATION OF *ARTEMIA PARTHENOGENETICA* CHROMOSOMES FOR SCANNING ELECTRON MICROSCOPY

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ABSTRACT

The chromosomes of Crustacea, especially decapods, are usually numerous, small in size, and punctiform. These characteristics make it difficult for researchers to further investigate the chromosomes by light microscopy. As an alternative, the scanning electron microscopy (SEM) is recommended because it has higher resolution and is able to provide valuable three-dimensional data and X-ray microanalysis. The aim of this study is to obtain some fundamental knowledge for the preparation of the chromosomes of crustaceans for SEM observations. Since crustacean chromosomes are so small, impurities such as mucus and cytoplasm easily obscure the chromosome surface making it impossible for researchers to observe their fine structure. We used *Artemia parthenogenetica* as a model and confirmed impurities originating from the nauplii paste and the cytoplasm of the cells, and demonstrate that the nauplii paste can be removed by rinsing and the cytoplasm can be digested by trypsin.

INTRODUCTION

Crustacean chromosomes, especially those of decapods, are numerous, small, and either punctiform or rod-shaped. Therefore, it is difficult for researchers to investigate them

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by light microscopy (LM) (Lecher et al., 1995). Since scanning electron microscopy (SEM) has higher resolution and is able to provide valuable three-dimensional data and X-ray microanalysis, it is recommended as an alternative.

Over the last few decades, there has been remarkable progress in mammalian, particularly human, chromosome research by SEM (Kingsley-Smith, 1970; Iino, 1975; Harrison et al., 1987; Allen, 1988; Seed et al., 1988; Sanchez-Sweatman, 1993; Sharma and Sharma, 1994). Recent reports indicate that SEM has been used in combination with immunocytochemical methods to investigate the fine structure of human chromosomes (Sumner, 1996). As far as crustaceans are concerned, however, SEM is seldom used. To the best of our knowledge, only one study has been reported on the brachyuran crab *Eriocheir japonica* (Lee et al., 2004). Unfortunately, the fine structure of the chromosomes presented in that study is not very clear. This may be due to the fact that, in contrast to mammalian chromosomes, with the chromosomes of the crab impurities such as mucus and cytoplasm easily obscure the chromosome surface making it impossible for researchers to observe fine surface structure by SEM.

In this study, we used *Artemia parthenogenetica* in an attempt to confirm the existence of such impurities as seen in the crab and to show how the impurities can be removed. The objective of this investigation is to obtain some fundamental techniques

for the preparation of the chromosomes of crustaceans for SEM observations.

MATERIALS AND METHODS

The genus *Artemia* has a world-wide distribution and has been widely used for the basic and applied research of crustaceans for many years (Sorgeloos et al.,1987; Browne et al., 1991; Abatzopoulos et al., 2002). The dry cysts of *Artemia* are easy to preserve and their nauplii can be obtained simply by incubating the cysts in seawater for 24-30 hours.

The cysts of *Artemia parthenogenetic* (42 chromosomes) (Barigozzi et al., 1987) used in this study were from Qingdao, China. The nauplii of *A. parthenogenetic* were collected after the cysts had been incubated for 32 hours in 80% seawater at the temperature of 28°C.

Making of chromosome plates

Chromosome plates were made following the procedures modified from previous studies (Evans et al., 1964; Yamazaki et al., 1981; Murofushi and Deguchi, 1983).

1.) Nauplii were incubated in colchicine solution (4 mg/ml sea water) for 4 hours at the temperature of 28°C.

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2.) Nauplii were taken out of the colchicine solution and placed in distilled water for hypotonic treatment for one night at the temperature of 4°C.

3.) Nauplii were immersed in freshly prepared methanol /acetic acid (1:1) fixative, each lasting for 4 hours, at room temperature.

4.) Twenty individuals of the fixed nauplii were chopped into paste using a specially made “chopping machine” (Lee, 1990) on a glass slide coated with silicone. The paste was poured into a 1.7ml micro-tube and methanol /acetic acid (1/1) fixative was added up to 1ml.

5.) The cells were rinsed three times with methanol /acetic acid (1/1) fixative and twice with 45% acetic acid solution by centrifugal separation (1500×g, 5 min each time). Subsequently, the supernatant was decanted, 45% acetic acid was added, and the cells of the nauplii were re-suspended.

6.) Four to five drops of the cell suspension were put on a glass slide. The slide glass was then slanted slightly to remove the extra suspension. The slide was air-dried overnight.

7.) The metaphase chromosome plates were observed and photomicrogaphed with phase contrast microscopy (Olympus IMT-2; Olympus Optical, Tokyo, Japan).

8.) The glass slide with chromosome plates was cut into pieces of a suitable size

(about 5mm×5mm) for SEM (Hitachi S-2300; Hitachi, Tokyo, Japan) observations and photomicrography.

Cleaning

In order to confirm the existence of the impurities, after Step 7, parts of the chromosome plates were scraped by sliding the tip of a surgical blade lightly on the surface. In general, hydrochloric acid or other acids are effective in cleaning the cytoplasm (Sharma and Sharma 1994). Several enzymes, in addition to acids, have been used to clean the cytoplasm through digestion (Sharma and Sharma 1994). In this study, after Step 7, we used xylene, hydrochloric acid and trypsin for cleaning.

Xylene.— Plates were treated with 100% xylene for 5 min, 20 min, and 60 min, respectively, at room temperature for lipid removal. After the treatment, plates were air-dried overnight.

Hydrochloric acid.— Treatment employed 0.2N hydrochloric acid solution for 5min, 20 min, and 60 min respectively at room temperature. Susequently, plates were rinsed with distilled water three times and air-dried overnight.

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Trypsin.— Plates were treated with the 0.1% trypsin solution for 1 min, 5 min, and 30 min, respectively, at the temperature of 4°C, after which they were rinsed with distilled water three times and air-dried overnight.

RESULTS AND DISCIUSSION

Evidence of the existence of impurities

The chromosome plates prepared without rinsing (Step 5) showed that the chromosomes could be observed by LM (Fig.1a) without any problem. However, in our SEM observations (Fig. 1b, c), a layer of impurities was detected by scraping the slide glass lightly with a surgical blade (Fig. 1b, c). This layer of impurities was so thick that all the chromosomes were covered and could not be observed clearly by SEM. These impurities covered the slide, and we suspect that they were from the nauplii paste.

In contrast, while we could observe the chromosomes on the plates prepared with rinsing (Step 5) by SEM (Fig. 2a, b), but we nonetheless still had a layer of impurities over the chromosomes, observed by scraping a part of the chromosome plate (Fig. 2a, b). However, this layer was not so thick as the one mentioned above; it did not spread all

127 over the slide glass but was confined only within the chromosome plate. Therefore, we
128 believe that this layer of impurities came from the cytoplasm of the cell that belonged to
129 the chromosome plate itself.
130
131 How to remove the impurities
132 The chromosome plates prepared with rinsing and treated by xylene showed that the
133 impurities could not be removed even in the case of 60 min treatment (Fig. 3a),
134 suggesting that there was no lipid in them.
135 The chromosome plates prepared with rinsing and treated by hydrochloric acid
136 showed that most of the impurities could not be removed. Even in the case of the
137 treatment for 60 min, the surface of the impurities appeared to be a little bumpy (Fig.
138 3b), which means that this treatment failed to eliminate the impurities. The length of
139 the treatment and the density of the hydrochloric acid solution may be related to this
140 result.
141 The chromosome plates prepared with rinsing and treated by trypsin showed that the
142 impurities could be removed entirely after the treatment had been carried out for 60 min
143 (Fig. 4c, d), but not in the case of 1 or 5 min (Fig. 4a, b). Compared with the
144 chromosomes prepared without trypsin cleaning (Fig. 2a, b), the chromosomes could be

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clearly observed after the impurities had been removed (Fig. 4c, d).

Other observations

Some of the chromosomes appeared to have the shape of a horseshoe. This kind of phenomenon has also been observed in other studies of *Artemia* (Abatzopoulos et al., 1986; Barigozzi, 1994), and yet the mechanism or reason is still unknown.

Trypsin is a kind of enzyme that can digest proteins. The relation between trypsin and chromosomes has been well studied in G-banded chromosomes studies with LM and SEM (Seabright, 1971; Sumner et al., 1971; Harrison et al., 1985). However, the chromosomes we observed in our study did not show any damage or structural deformation. It is possible that the density of trypsin or the duration of treatment we used might have been too low or too short to cause such effects in the chromosomes

Conclusion

We have confirmed the existence of impurities in *Artemia* originating from the nauplii paste and the cytoplasm of the cells. These impurities seriously affect the observation of the fine surface structure of the chromosomes. We have shown that the impurities originating from the paste can be removed by rinsing and the impurities from cytoplasm

can be digested by trypsin.

Other problems remain to be solved in preparing *Artemia* chromosomes for SEM observations. For example, we were not able to observe the chromosome fiber in this study. For future research on the observation of the fine chromosome structure of crustaceans, we suggest that glutaraldehyde or osmium tetroxide fixation, critical point drying, and cytoplasm digestion by surfactant such as Triton X-100 be attempted in the preparation of the chromosomes.

REFERENCES

Abatzopoulos, J., J. A. Beardmore, J. S. Clegg, and P. Sorgeloos. 2002. *Artemia*: Basic and Applied Biology . Kluwer Academic Publishers.

———, C. D. Kastritsis, and C. D. Triantaphyllidis. 1986. A study of karyotypes and heterochromatic associations in *Artemia*, with special reference to two N. Greek populations. Genetica 71: 3-10.

Allen, T. D., E. M. Jack, C. J. Harrison, and D. Claugher. 1988. The three dimensional structure of human metaphase chromosomes determined by scanning electron microscopy, pp. 51-72. In, K. W. Adolph (ed.), Chromosomes and chromatin.

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Vol.2. CRC Press, Boca Raton.

Barigozzi, C., P. Valsasnini, E. Ginelli, G. Badaracco, P. Plevani, and L. Baratelli. 1987. Further data on repetitive DNA and speciation in *Artemia,* pp. 103-105. In, P. Sorgeloos, D. A. Bengtson, W. Decleir, and E. Jaspers (eds.), *Artemia* Research and applications Vol. 1. Universa Press, Wetteren, Belgium.

———. 1994. The chromosomes of the *Artemia* living in the Salt Lake of Larnaca, Cyprus. Rendiconti Fisica Accademia dei Lincei 5: 363-365.

Browne, R. A., P. Sorgeloos, and C. N. A. Trotman. 1991. *Artemia* Biology, CRC Press, Boca Raton.

Evans, E. P., G. Breckson, and C. E. Ford. 1964. An air-drying method for meiotic preparations from mammalian testes. Cytogenetics 3: 289-294.

Harrison, C. J., E. M. Jack, T. D. Allen, and R. Harris. 1985. Light and scanning electron microscopy of the same metaphase chromosomes. Journal Cell Science 77: 143-153.

———, ———, and ———. 1987. Light and scanning electron microscopy of the same metaphase chromosomes, pp. 189-249. In, M.A. Hayat (eds.), Correlative microscopy in biology: Instrumentation and methods. Academic Press, Orlando.

Iino, A. 1975. Human somatic chromosomes observed by scanning electron microscope. Cytobios 14: 39-48.

Kingsley-Smith, B. V. 1970. The application of scanning and transmission electron microscopy to a study of whole chromosomes. Micron 2: 39-57.

Lecher, P., D. Defaye, and P. Noel. 1995. Chromosomes and nuclear DNA of Crustacea. Invertebrate Reproduction and Development 27: 85-114.

Lee, T-H. 1990. Chopping machine for chromosome preparation. IDEN (Tokyo) 44**:** 75-76.

———, N. Naitoh and F. Yamazaki. 2004. Chromosome studies on the mitten crabs *Eriocheir japonica* and *E. sinensis*. Fisheries Science 70: 211-214.

Murofushi, M. and Y. Deguchi. 1983. A method for obtaining metaphase chromosomes from large shrimp-like crustaceans. Report of Mishima Research Institute of Science and Living, Nihon University 6: 31-34.

Sanchez-Sweatman, O. H., E. P. Harven, and I. D. Dube. 1993. Human chromosomes: Evaluation of processing techniques for scanning electron microscopy. Scanning Microscopy 7: 97-106.

Seabright, M. 1971. A rapid banding technique for human chromosomes. Lancet 11: 971-972.

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Seed, T. M., L. V. Kaspar, F. Domann, G. K. Niiro, and D. A. LeBuis. 1988. Developmental and radiobiologic characteristics of canine multinucleated, osteoclast-like cells generated in vitro from canine bone marrow. Scanning Microscopy 2**:** 1599-1611.

Sharma, A. K., A. Sharma. 1994. Chromosome Techniques: A Manual. Harwood Academic Publishers.

Sorgeloos, P., D. A. Bengston, W. Decleir and E. Jaspers. 1987. *Artemia* research and its applications. Vol. 1. Universa Press, Wetteren, Belgium.

Sumner, A. T. 1996. Problems in preparation of chromosomes for scanning electron microscopy to reveal morphology and to permit immunocytochemistry of sensitive antigens. Scanning Microscopy (Supplement) 10**:** 165-176.

———, Evans, H. J., and Buckland, R. 1971. New techniques for distinguishing between human chromosomes. Nature (London) New Biology 232: 31-32.

Yamazaki, F., H. Onozato, and K. Arai. 1981. The chopping method for obtaining permanent chromosome preparation from embryos of teleost fishes. Bulletin Japanese Society of Scientific Fisheries 47: 963.

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LEGENDS OF FIGURES

Fig. 1. *Artemia parthenogenetica* metaphase chromosome plate prepared without rinsing. a, Observed with phase contrast microscopy (Bar = 100 μ m); b, Observed with scanning electron microscopy (Bar = 100 μ m); c, Enlargement of b (Bar = 10 μ m). Arrows show the scrape formed by a surgical blade.

Fig. 2. *Artemia parthenogenetica* metaphase chromosome plate prepared with rinsing and observed with scanning electron microscopy. a and b, Arrows show the edge formed by scraping the impurities with a surgical blade. Black triangles show the range of the impurities. Bars = 5 μ m.

Fig. 3. *Artemia parthenogenetica* metaphase chromosome plate prepared with rinsing and observed with scanning electron microscopy. a, Treated with xylene for 60 min and the impurities remained; b, Treated with hydrochloric acid for 60 min and the surface of the impurities was only slightly bumpy. Black triangles show the range of

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the impurities. Bars = 5 μ m.

Fig. 4. *Artemia parthenogenetica* metaphase chromosome plate prepared with rinsing, treated with trypsin and observed with scanning electron microscopy. a, Treated for one min, almost all of the impurities remained (Bar = 5 μ m); b, treated for 5 min, the impurities decreased (Bar = 5 μ m); c, treated for 60 min, all of the impurities disappeared (Bar = 5 μ m); d, enlargement of c. Black triangles show the range of the impurities (Bar = 1.5 μ m).

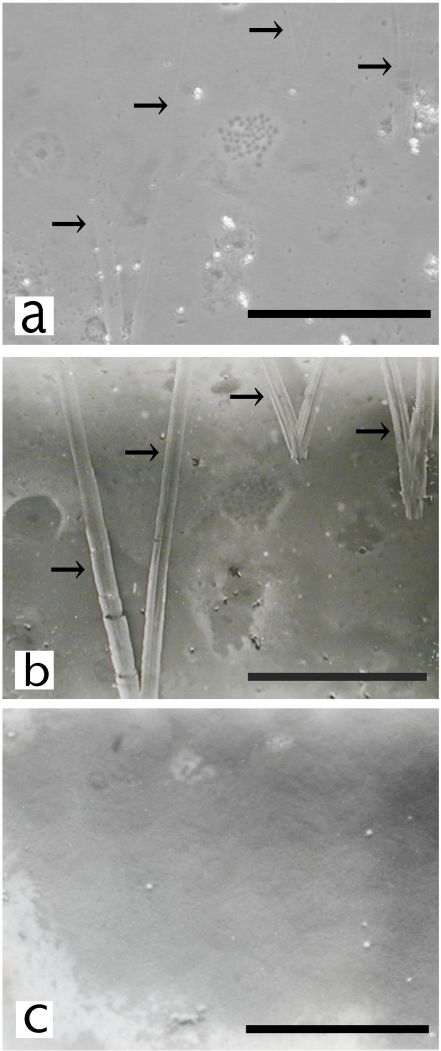

Figure 1

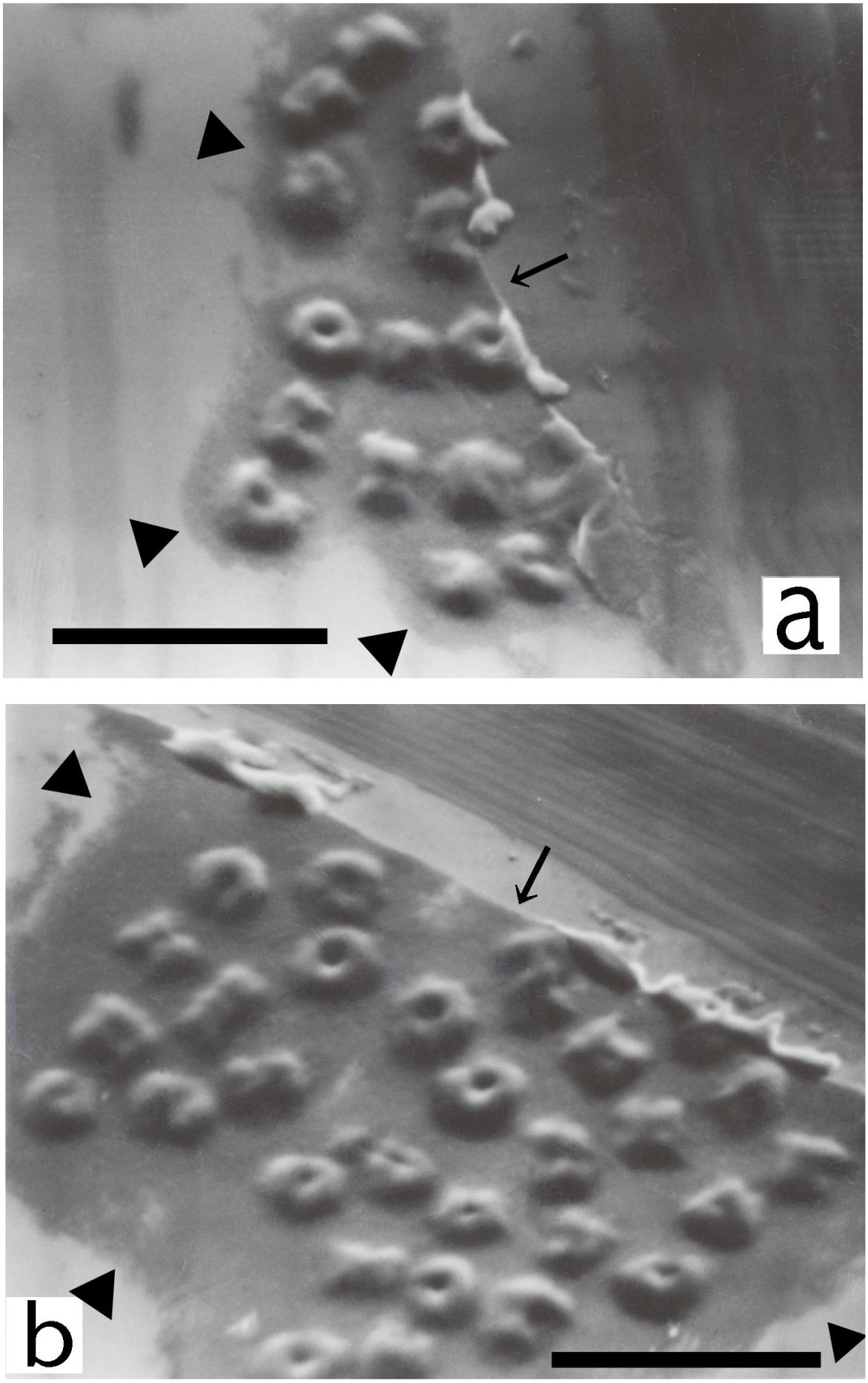

Figure 2

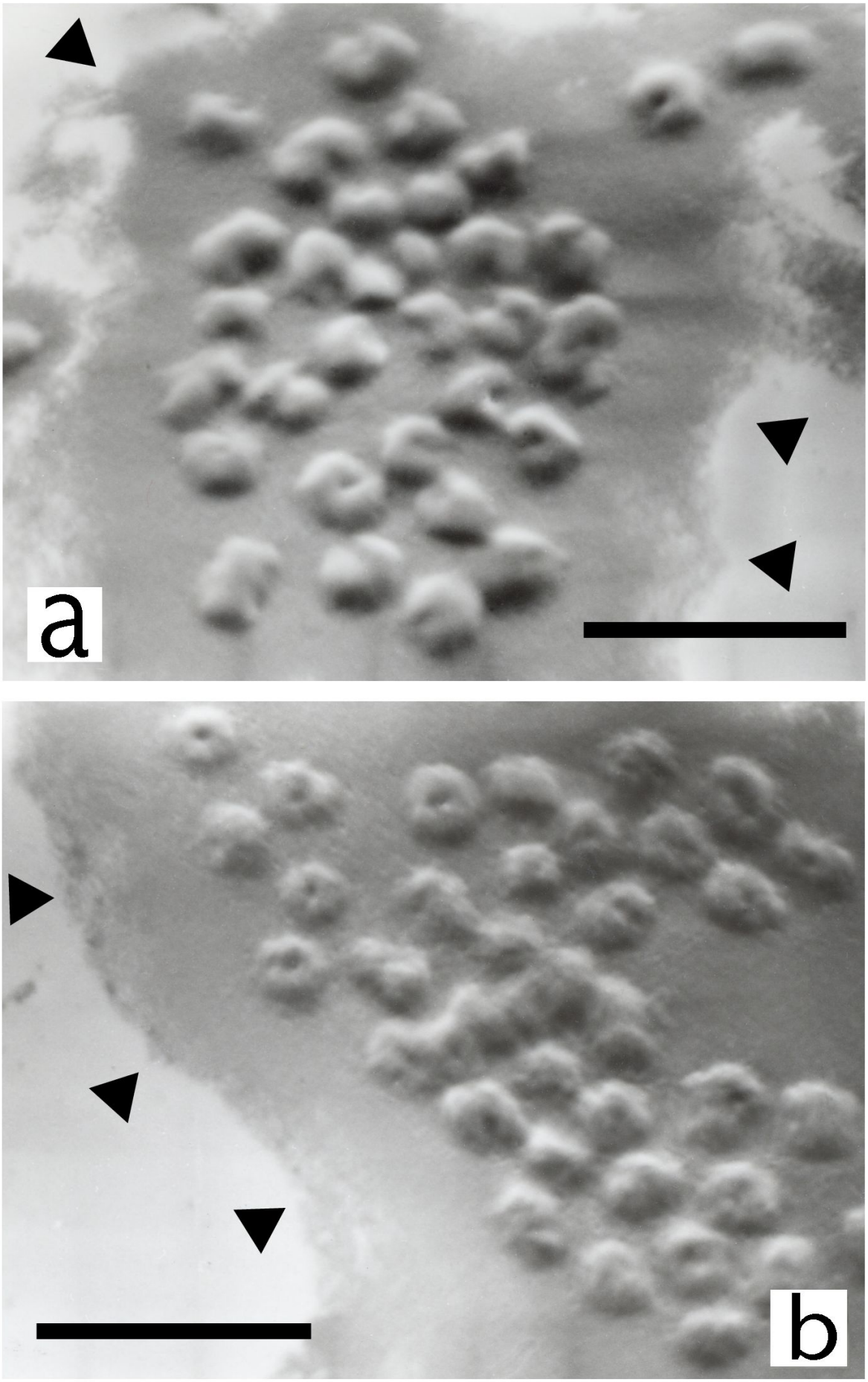

Figure 3

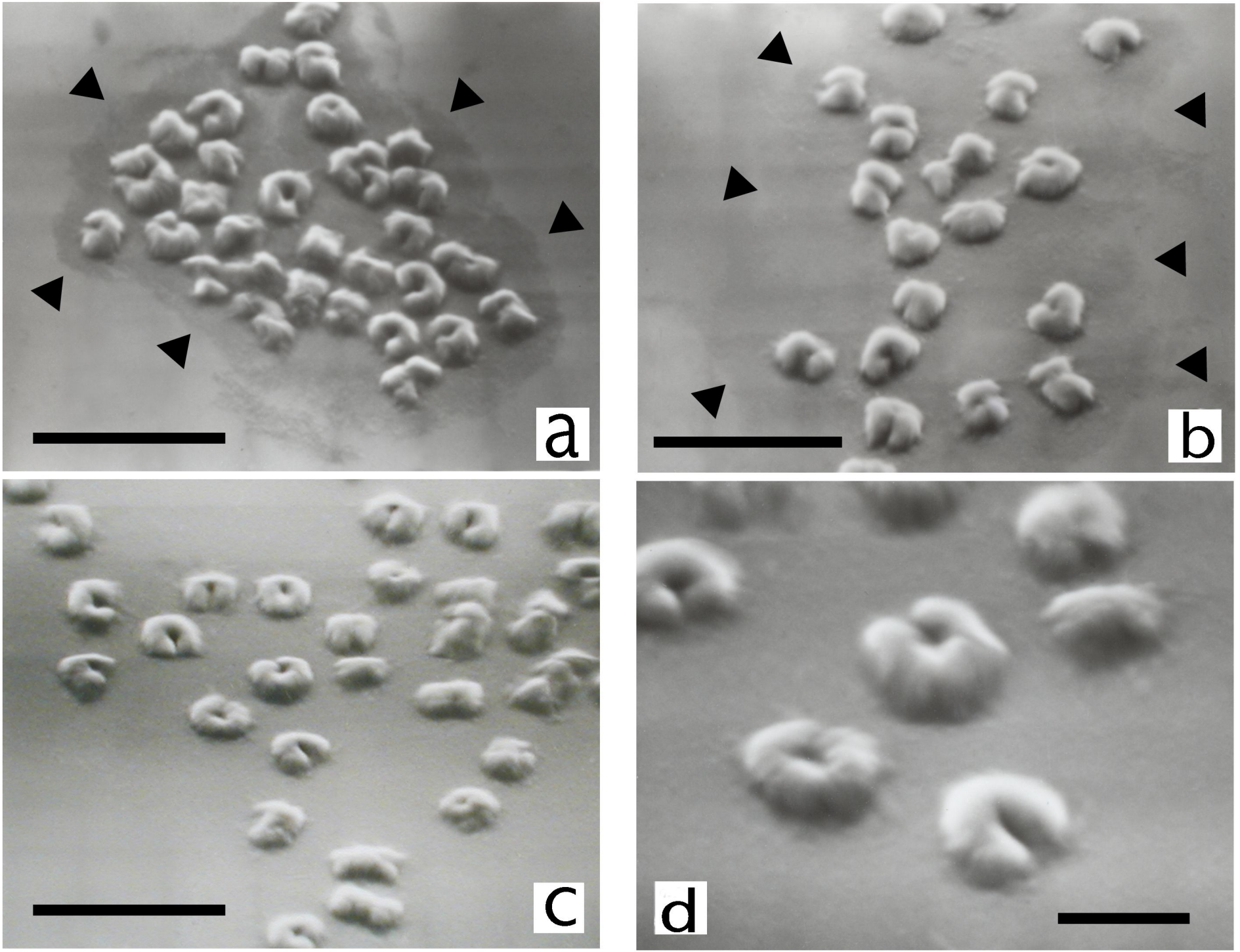

Figure 4