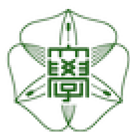

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5 A PRELIMINARY STUDY ON THE PROBLEMS IN THE PREPARATION OF
6 *ARTEMIA PARTHENOGENETICA* CHROMOSOMES FOR SCANNING ELECTRON
7 MICROSCOPY

8

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16 Key Words: *Artemia*, cell biology, genetics, morphology

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18

19 ## ABSTRACT

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

32

33 ## INTRODUCTION

34

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

3

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

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

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

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

56

57 ## MATERIALS AND METHODS

58

59 The genus *Artemia* has a world-wide distribution and has been widely used for the basic
60 and applied research of crustaceans for many years (Sorgeloos et al.,1987; Browne et al.,
61 1991; Abatzopoulos et al., 2002). The dry cysts of *Artemia* are easy to preserve and
62 their nauplii can be obtained simply by incubating the cysts in seawater for 24-30 hours.
63 The cysts of *Artemia parthenogenetic* (42 chromosomes) (Barigozzi et al., 1987) used
64 in this study were from Qingdao, China. The nauplii of *A. parthenogenetic* were
65 collected after the cysts had been incubated for 32 hours in 80% seawater at the
66 temperature of 28°C.

67

68 ### Making of chromosome plates

69 Chromosome plates were made following the procedures modified from previous
70 studies (Evans et al., 1964; Yamazaki et al., 1981; Murofushi and Deguchi, 1983).
71 1.) Nauplii were incubated in colchicine solution (4 mg/ml sea water) for 4 hours at
72 the temperature of 28°C.

5

73 2.) Nauplii were taken out of the colchicine solution and placed in distilled water for

74 hypotonic treatment for one night at the temperature of 4°C.

75 3.) Nauplii were immersed in freshly prepared methanol /acetic acid (1:1) fixative,

76 each lasting for 4 hours, at room temperature.

77 4.) Twenty individuals of the fixed nauplii were chopped into paste using a specially

78 made "chopping machine" (Lee, 1990) on a glass slide coated with silicone.

79 The paste was poured into a 1.7ml micro-tube and methanol /acetic acid (1/1)

80 fixative was added up to 1ml.

81 5.) The cells were rinsed three times with methanol /acetic acid (1/1) fixative and

82 twice with 45% acetic acid solution by centrifugal separation (1500×g, 5 min

83 each time). Subsequently, the supernatant was decanted, 45% acetic acid was

84 added, and the cells of the nauplii were re-suspended.

85 6.) Four to five drops of the cell suspension were put on a glass slide. The slide glass

86 was then slanted slightly to remove the extra suspension. The slide was

87 air-dried overnight.

88 7.) The metaphase chromosome plates were observed and photomicrogaphed with

89 phase contrast microscopy (Olympus IMT-2; Olympus Optical, Tokyo, Japan).

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

91 (about 5mm×5mm) for SEM (Hitachi S-2300; Hitachi, Tokyo, Japan)

92 observations and photomicrography.

93

94 Cleaning

95 In order to confirm the existence of the impurities, after Step 7, parts of the

96 chromosome plates were scraped by sliding the tip of a surgical blade lightly on the

97 surface. In general, hydrochloric acid or other acids are effective in cleaning the

98 cytoplasm (Sharma and Sharma 1994). Several enzymes, in addition to acids, have

99 been used to clean the cytoplasm through digestion (Sharma and Sharma 1994). In

100 this study, after Step 7, we used xylene, hydrochloric acid and trypsin for cleaning.

101

102 Xylene.— Plates were treated with 100% xylene for 5 min, 20 min, and 60 min,

103 respectively, at room temperature for lipid removal. After the treatment, plates were

104 air-dried overnight.

105

106 Hydrochloric acid.— Treatment employed 0.2N hydrochloric acid solution for 5min, 20

107 min, and 60 min respectively at room temperature. Susequently, plates were rinsed

108 with distilled water three times and air-dried overnight.

7

109

110 Trypsin.— Plates were treated with the 0.1% trypsin solution for 1 min, 5 min, and 30
111 min, respectively, at the temperature of 4°C, after which they were rinsed with distilled
112 water three times and air-dried overnight.
113

114 RESULTS AND DISCIUSSION

115

116 Evidence of the existence of impurities

117 The chromosome plates prepared without rinsing (Step 5) showed that the chromosomes
118 could be observed by LM (Fig.1a) without any problem. However, in our SEM
119 observations (Fig. 1b, c), a layer of impurities was detected by scraping the slide glass
120 lightly with a surgical blade (Fig. 1b, c). This layer of impurities was so thick that all
121 the chromosomes were covered and could not be observed clearly by SEM. These
122 impurities covered the slide, and we suspect that they were from the nauplii paste.
123 In contrast, while we could observe the chromosomes on the plates prepared with
124 rinsing (Step 5) by SEM (Fig. 2a, b), but we nonetheless still had a layer of impurities
125 over the chromosomes, observed by scraping a part of the chromosome plate (Fig. 2a, b).
126 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

9

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

146

147 Other observations

148 Some of the chromosomes appeared to have the shape of a horseshoe. This kind of

149 phenomenon has also been observed in other studies of *Artemia* (Abatzopoulos et al.,

150 1986; Barigozzi, 1994), and yet the mechanism or reason is still unknown.

151 Trypsin is a kind of enzyme that can digest proteins. The relation between trypsin

152 and chromosomes has been well studied in G-banded chromosomes studies with LM

153 and SEM (Seabright, 1971; Sumner et al., 1971; Harrison et al., 1985). However, the

154 chromosomes we observed in our study did not show any damage or structural

155 deformation. It is possible that the density of trypsin or the duration of treatment we

156 used might have been too low or too short to cause such effects in the chromosomes

157

158 CONCLUSION

159 We have confirmed the existence of impurities in *Artemia* originating from the nauplii

160 paste and the cytoplasm of the cells. These impurities seriously affect the observation

161 of the fine surface structure of the chromosomes. We have shown that the impurities

162 originating from the paste can be removed by rinsing and the impurities from cytoplasm

163 can be digested by trypsin.

164 Other problems remain to be solved in preparing *Artemia* chromosomes for SEM

165 observations. For example, we were not able to observe the chromosome fiber in this

166 study. For future research on the observation of the fine chromosome structure of

167 crustaceans, we suggest that glutaraldehyde or osmium tetroxide fixation, critical point

168 drying, and cytoplasm digestion by surfactant such as Triton X-100 be attempted in the

169 preparation of the chromosomes.

170

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233

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238 LEGENDS OF FIGURES

239 Fig. 1. *Artemia parthenogenetica* metaphase chromosome plate prepared without

240 rinsing. a, Observed with phase contrast microscopy (Bar = 100 μm); b, Observed

241 with scanning electron microscopy (Bar = 100 μm); c, Enlargement of b (Bar = 10 μm).

242 Arrows show the scrape formed by a surgical blade.

243

244 Fig. 2. *Artemia parthenogenetica* metaphase chromosome plate prepared with rinsing

245 and observed with scanning electron microscopy. a and b, Arrows show the edge

246 formed by scraping the impurities with a surgical blade. Black triangles show the

247 range of the impurities. Bars = 5 μm.

248

249 Fig. 3. *Artemia parthenogenetica* metaphase chromosome plate prepared with rinsing

250 and observed with scanning electron microscopy. a, Treated with xylene for 60 min

251 and the impurities remained; b, Treated with hydrochloric acid for 60 min and the

252 surface of the impurities was only slightly bumpy. Black triangles show the range of

1 5

253 the impurities. Bars = 5 μm.

254

255 Fig. 4. *Artemia parthenogenetica* metaphase chromosome plate prepared with rinsing,

256 treated with trypsin and observed with scanning electron microscopy. a, Treated for

257 one min, almost all of the impurities remained (Bar = 5 μm); b, treated for 5 min, the

258 impurities decreased (Bar = 5 μm); c, treated for 60 min, all of the impurities

259 disappeared (Bar = 5 μm); d, enlargement of c. Black triangles show the range of

260 the impurities (Bar = 1.5 μm).

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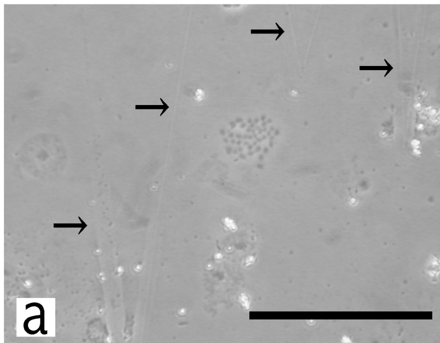
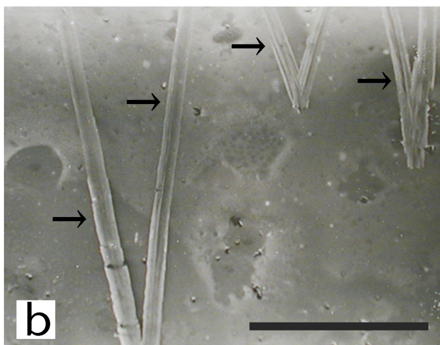




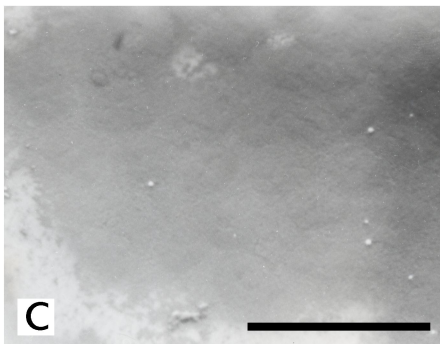


Figure 1

a

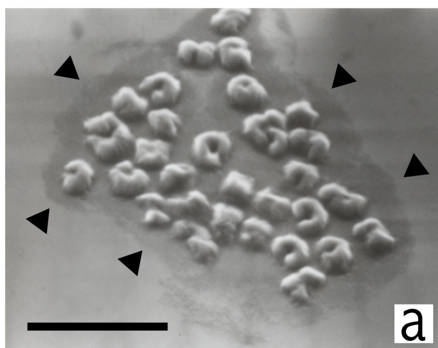
b

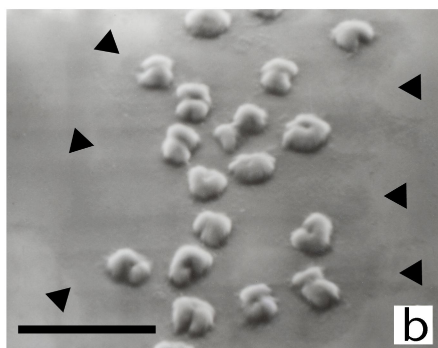
Figure 2

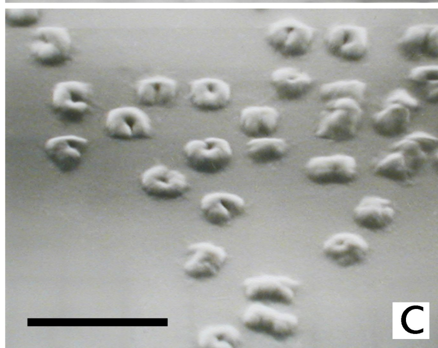
a

b

Figure 3







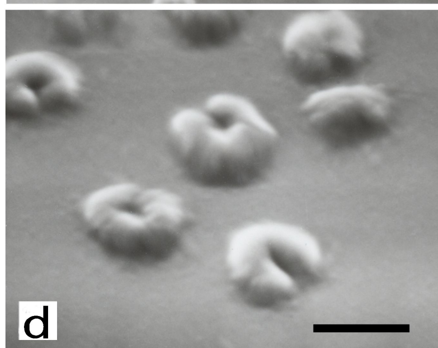


Figure 4