Identification and Development of a Paramyxean Ovarian Parasite in the Pacific Oyster *Crassostrea gigas*

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(Received August 20, 2001)

ABSTRACT—The intracellular ovarian parasite causing unaesthetic appearance in the ovary of Pacific oysters *Crassostrea gigas* in Japan has remained unidentified since the first report of its occurrence in the 1930s. The parasite was observed through transmission electron microscopy, and identified as the paramyxean *Marteilioides chungmuensis*, which was first reported from Pacific oysters in Korea. In order to obtain information on the development of the parasite, the diameters of both the parasite and the infected ova were measured. The growth of the parasite was highly correlated with the growth and maturation of host gonad cells. Histological observations suggest that *M. chungmuensis* invades immature ova, which move to the center of the follicle along with its development, and infected ova are finally released outside through the genital canal.

Key words: *Marteilioides chungmuensis*, *Crassostrea gigas*, Pacific oyster, Paramyxaea, development, life cycle

An unidentified ovarian parasite of Pacific oysters *Crassostrea gigas* has been a serious pathogen for the oyster industry in Japan. The parasite causes nodule-like structures in the ovary of the oyster, and infected oyster becomes unmarketable because of the abnormal appearance. The economic loss caused by the disease was estimated to be a few hundred million Japanese yen in Okayama Prefecture, Japan (Oda, personal communication). This parasite has been detected widely in Japanese waters, and the prevalence of infection has been examined in four prefectures: Miyagi (Imai et al., 1968), Hiroshima (Matusato and Masumura, 1981), Mie (Imanaka et al., 2001) and Okayama (S. Imanaka, unpublished data). According to these studies, infection levels peaked in autumn, when oysters finished spawning. Concerning its taxonomic status, Matusato and Masumura (1981) considered that this parasite might be a coccidian, while Imanaka et al. (2001) postulated that it was assigned to the phylum Paramyxea. Similar disease was reported in Korea (Chun, 1979), and the causative parasite was identified as *Marteilioides chungmuensis*, a new genus and a new species (Comps et al., 1986). However, the parasite in Japan has yet to be identified to the species level.

As for the life cycle, Imanaka et al. (2001) described 8 developmental stages and postulated its developmental cycle in the host. They showed that the parasite develops with intracellular divisions, and suggested that it moves to the lumen of the genital tubule with the progress of the development. However, it has been unknown how the parasite leaves the host.

In this paper, we identify the parasite, based on transmission electron microscopic observations. Moreover, we give additional information on the life cycle of the parasite by histological observations.

Materials and Methods

**Development of the parasite**

Thirty-two infected cultured oysters (0-year old, mean shell length: 40.5 mm) were collected from July to November 1999 at Ushimado, Okayama Prefecture. Infected tissues were grossly detected and excised. Pieces of excised tissue were smeared on glass slides, air dried and stained with Diff-Quik (International Reagent Corp., Japan). Imanaka et al. (2001) described 8 developmental stages (A–H), of which A, B, C...
and D were mainly observed in the present study. The diameter of type A, B, C and D of the parasite and the infected ova of the host were measured (n=3561). The mean diameter of the parasite among each stage was then analyzed statistically with ANOVA ($P=0.05$) using StatView J-5.0. The correlation coefficient between the diameter of the parasite cells at different stages and ova were calculated ($P=0.01$).

**Transmission electron microscopic observations**

In order to observe the ultrastructure of the 4 developmental stages (type A to D) and identify this ovarian parasite, transmission electron microscopic observations were performed. One-year cultured Pacific oysters (mean shell length: 56.0 mm, n=10) were sampled in October 2000 at Ushimado, Okayama Prefecture. When nodule-like structures were grossly observed in the soft tissue, tissue pieces of about 1 mm$^3$ were dissected and fixed in 1% cold glutaraldehyde in 0.2 M PBS with 2% paraformaldehyde for 3 h. The specimens were then postfixed in 1% OsO$_4$ solution buffered with 0.2 M NaCl solution, dehydrated through a graded ethanol series, and embedded in Spurr resin. Ultrathin sections (60 nm in thickness) were stained with uranyl acetate and lead citrate. The sections were examined using an electron microscope (JEM 1010, JEOL) at 80 kV.

**Histological observations**

One-year cultured oysters (mean shell length: 53.9 mm, n=30) were sampled from July to November 2000 at Ushimado, Okayama Prefecture. The whole body of sampled oysters were fixed in Davidson's fixative (Shaw and Battle, 1957) or 10% buffered formalin. The tissues were dehydrated through a graded ethanol series, embedded in paraffin wax, and sectioned at 5 μm. The sections were stained with hematoxyline and eosin (H & E).

**Results**

**The development of the parasite**

The mean diameter of the parasite at each stage and that of the host cell are shown in Table 1. As the parasite developed from types A to D, the diameter of the primary cell increased. The diameter of the primary cell in types A to D was significantly different from each other ($P<0.05$). The relationship between the parasite cell diameter and the host ovum diameter at each parasite stages is presented in Figs. 1 to 4. The correlation coefficients between the diameter of primary cells and that of host egg cells are statistically significant for all types ($P<0.01$) except for type A ($P>0.01$).

**Transmission electron microscope observations**

Transmission electron microscope observations revealed the 4 developmental stages (A to D) of the parasite in host cells. Type A (5.9 μm × 5.1 μm in average size) consisted of a nucleus of the primary cell and one secondary cell (Fig. 5). The cytoplasm of the pri-

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**Table 1.** Relationship between the size of the ovarian parasite cell at developmental stages A–D and the size of infected oyster egg cell

<table>
<thead>
<tr>
<th>type</th>
<th>n</th>
<th>Parasite cell size ± S.D. (μm)</th>
<th>Host cell size ± S.D. (μm)</th>
<th>Correlation coefficient***</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>402</td>
<td>6.49 ± 1.39</td>
<td>32.45 ± 11.19</td>
<td>-0.014</td>
</tr>
<tr>
<td>B</td>
<td>403</td>
<td>10.36 ± 1.87</td>
<td>40.81 ± 8.50</td>
<td>0.262*</td>
</tr>
<tr>
<td>C</td>
<td>157</td>
<td>16.12 ± 3.00</td>
<td>43.74 ± 9.81</td>
<td>0.464*</td>
</tr>
<tr>
<td>D</td>
<td>2599</td>
<td>22.91 ± 2.19</td>
<td>44.85 ± 8.35</td>
<td>0.528*</td>
</tr>
</tbody>
</table>

*: significantly correlated
***: correlation coefficient between parasite cell and egg cell size

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**Type A (n=402)**

$r = -0.014$

$P > 0.01$

![Fig 1](image-url)
Figs. 1-4. Size relationship between each developmental stage of the ovarian parasite *Marteilioides chungmuensis* and infected ovum of Pacific oyster *Crassostrea gigas*. 1, Type A. Correlation coefficient is not significant ($P>0.01$). 2, Type B with a significant correlation coefficient ($P<0.01$). 3, Type C with a significant correlation coefficient ($P<0.01$). 4, Type D with a significant correlation coefficient ($P<0.01$).
Figs. 5–10. TEM observations of an ovum of Pacific oyster *Crassostrea gigas* infected with the ovarian parasite *Marteilioides chungmuensis*. 5, Pacific oyster ovum infected with type A. C1, primary cell; C2, secondary cell; N1, nucleus of primary cell; N2, nucleus of secondary cell. Scale bar = 500 nm. 6, Fine structures of a primary cell cytoplasm of type A. 6A: Vesicle in the primary cell cytoplasm. V, vesicle. Scale bar = 100 nm. 6B: Haplosporosomes in the cytoplasm of a primary cell. H, haplosporosome; R, ribosome. Scale bar = 200 nm. 7, Type B. C1, primary cell; C2, secondary cells; C3, tertiary cell; N1, nucleus of primary cell; N2, nuclei of secondary cells; N3, nucleus of tertiary cell (=spore cell). Scale bar = 1 μm. 8, Type C. C2, secondary cells; C3, tertiary cell (=spore cell); N3, nucleus of tertiary cell; C4, fourth cell (=secondary spore cell); N4, nucleus of fourth cell. Note that the nuclei of the primary cell and the secondary cells were not observed. Scale bar = 2 μm. 9, Type D. C2, secondary cells (=sporont); C3, tertiary cells (=spore cell); C4, fourth cells (=secondary spore cell). Note that the nuclei and organelle of secondary cells were not observed. Scale bar = 2 μm. 10, Ultrastructure of a spore cell in a sporont. SC1, spore cell; SC2, secondary spore cell; SC3, tertiary spore cell; SN2, nucleus of secondary spore cell; SN3, nucleus of spore tertiary cell. Scale bar = 1 μm.
mary cell contained haplosporosomes and vesicles (Figs. 6A and 6B). After division of the secondary cell, type A proceeded to type B (9.0 \( \mu m \times 6.3 \mu m \) in average), which contained 2 secondary cells inside (Fig. 7). In type C stage (12.5 \( \mu m \times 8.6 \mu m \) in average), the nucleus of the primary cell degenerated and disappeared, while 2 secondary cells, which included a tertiary cell, occupied most of its volume of the primary cell (Fig. 8). Figure 9 shows type D stage (20.2 \( \mu m \times 19.8 \mu m \) in average), in which one tertiary cell (=spore) was formed in each secondary cell (=sporont). In the sporont, a fourth cell (=secondary spore cell) and even a fifth cell (=tertiary spore cell) were recognized. No organelle is observed in the cytoplasm of the secondary cell (Fig. 10).

**Discussion**

Transmission electron microscope observations of the present parasite showed that internal cleavage in each secondary cell (sporont) formed one pluricellular spore. Moreover, the spore cell is enveloped by a cytoplasmic residue and the plasmalemma of the sporont. These observations are in accordance with the amended definition of the genus Marteilioides (Anderson and Lester, 1992). The genus Marteilioides includes two species, *M. chungmuensis* and *M. branchialis*, which are distinguished each other by the number of sporoplasm; *M. chungmuensis* spore consists of 3 sporoplasms, while *M. branchialis* spore consists of 2 sporoplasms. Moreover, primary cells degenerate during sporulation in *M. chungmuensis*, but not in those of *M. branchialis* (Anderson and Lester, 1992). In the present study, we confirmed that the spore consisted of 3 spore cells and that the primary cell degenerated during sporulation. Therefore, the present ovarian parasite is identified as *M. chungmuensis*, which was recorded from Pacific oysters in Korea (Comps et al., 1986). From the ultrastructure of each stage, the development of this parasite is postulated as follows: a secondary cell in type A is divided into two secondary cells in type B; each one enlarges in type C, and tertiary cells are budded endogenously in those secondary cells in type D. Moreover, haplosporosomes and multivesicular bodies were observed in the primary cell. Those characteristics are also in accordance with description of *M. chungmuensis* by Park and Chun (1989), and Comps et al. (1986).

Measurements of the parasite and host cell diameter elucidated that the parasite growth is closely associated with the degree of maturation of the host ovum. Histological sections showed that immature stages (types A, B and C) were observed on the lining of the follicle, while the large mature stage (type D) was observed in the lumen of the follicle and often in the genital canal. These observations suggest that *M. chungmuensis* invades an immature ovum on the lining of the follicle, moves to the lumen of the follicle with the development of the parasite, and the parasite-containing ovum is released from the host through the genital canal in the same way as uninfected ova are spawned.

In the present study, types E to H described by
Imanaka et al. (2001) were rarely observed. Moreover, type D was released from the host body. These suggest that the development of the parasite in the host can be completed without types E to H. Those types may be variations of type D, because the sizes of types E to H were very similar to that of type D (Imanaka et al., 2001). TEM observations could not identify types E to H.

The release of *M. chungmuensis* outside the host is in contrast with *Perkinsus* spp., other shellfish pathogens, which are released after death of the host (Auzoux-Borcenave et al., 1995). Imanaka et al. (2001) speculated that the ovarian parasite may kill wild oysters. Although the majority of sporonts of related pathogen, *Marteilia sydneyi*, are also shed intact into the water prior on the death of its host *Saccostrea glomerata* (see Roubal et al., 1989), *M. sydneyi* causes heavy mortality of the host (Adlard and Ernst, 1995). Further study will be needed to determine the pathogenicity of *M. chungmuensis*, but if the parasite did kill the host, post-mortem release of the parasite could be possible.

In all histological observations, *M. chungmuensis* was observed only in the ova, and all the development stages were in the sporogony. Therefore, after invasion of the parasite into host, multiplication stages may progress in some unidentified tissues other than ova. Molecular probes may be useful to detect such developmental stages in the oyster.

After release from the oyster, the life cycle of the parasite still remains unknown. Berthe et al. (1998) and Wesche et al. (1999) considered that other pramyxean *Marteilia refringens* and *M. sydneyi* may require alternate hosts in its life cycle. It seems possible that *M. chungmuensis* may also have a similar life cycle. Molecular detection of these parasites outside the host may be a strong tool for the elucidation of these unknown stages and their biology.

**Acknowledgements**

We sincerely thank the staff of Fisheries Experiment Station Okayama Prefecture, which helped our research and provided samples.

**References**


