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Histopathological and Biological Survey on Marine Bivalve Parasites in Korean Waters

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국문 요약

1. 우리나라 바지락에 발병하는 기생충 및 병리현상 관찰

우리나라 수산 양식업에 있어 중요한 자원인 바지락을 대상으로 기생충 감염 및 병리현상을 관찰하기 위해 2007 년 4 월과 11 월에 서해안, 남해안, 동해안 그리고 제주도 총 23 지역에서 시료를 채집하였다. 지역별로 채집한 40 개체의 바지락을 조직학적 관찰에 사용하였으며, 지리학적 위치에 따른 기생충의 분포 및 감염 양상을 관찰하였다. 그 결과, 서해안과 남해안에서 채집된 바지락은 다른 지역에 비하여 기생충 감염률이 높게 나타났다. 계절별 기생충의 감염률은 차이를 보였으나, 지역에 따른 기생충 감염률의 차이는 없었다. 바지락 시료의 조직학적 관찰에 의해 확인된 기생충들 중, *Perkinsus*와 Trematode cercariae가 가장 많이 관찰되었다. *Perkinsus*는 바지락의 모든 기관에서 관찰되었고, 높은 감염률을 보인 개체에서는 혈구 침윤현상을 보였으며, Cercaria의 감염률이 높은 개체에서는 생식소 부분이 관찰되지 않았다. 이밖에 관찰된 기생충으로는 Turbellaria, gregarine, copepod, rickettsia-like organisms, *Martelioides*와 encysted larval cestode가 관찰되었으며 바지락에 심각한 영향을 끼치지는 않는 것으로 밝혀졌다. 기생충의 발병과 병리현상은 바지락 양식을 하지 않는 제주도와 동해안 지역보다 남해안과 서해안처럼 펄이 잘 형성되고 바지락을 상업적으로 양식하는 지역에서 채집된 개체군이 더 높게 나타났다. 기생충들의 공간적 분포는 서식지의 지형이나 바지락 양식의 밀도와 밀접한 관계가 있는 것으로 사료된다.

2. 난소기생충 (*Marteilioides chungmuensis*)에 감염된 참굴 (*Crassostrea gigas*)의 번식생태학적 특성

이 연구는 난소기생충인 *Marteilioides chungmuensis* 감염에 의한 참굴의 번식 생태학적 특징을 비교하기 위하여 2007 년 12 월 통영에서 채집된 참굴을 분석에 사용하였다. 감염된 개체의 외부 증상은 육질부에 불규칙한 난괴를 형성하고, 난괴 주위에는 투명한

물굴 상태를 나타냈다. 감염된 굴과 비감염된 굴의 습중량은 유의적 차이가 없었다. 조직학적 관찰 결과 *M. chungmuensis*는 난모세포 안에 기생하며, 초기 및 후기 발달단계 모두 발견 되었다. 감염된 개체는 혈구들의 응집현상도 확인하였다. 감염된 굴들의 성 성숙 관찰 결과, 후기발달단계 및 완숙단계를 나타냈지만, 비 감염 개채는 대부분이 소모기 또는 미분화 단계를 보였다. 감염된 알의 비율은 7.45-82.99%였으며, 알의 크기는 24.17±5.59 μm로 정상개체의 알 크기에 비하여 작은 것을 확인할 수 있었다. 감염된 개체를 대상으로 번식량을 측정하기 위하여, 개발된 참굴 알의 다클론항체를 이용하여 ELISA 분석을 실시하였고, 생식소중량지수 (GSI, Gonadosomatic index)는 평균 7.52로 매우 적은 값을 보였다. 감염된 굴의 소화맹낭은 수축된 상태였으나, 소화맹낭 위축도를 측정한 결과 비 감염 굴과 큰 차이를 보이지 않았다. 감염개체와 비 감염개체간의 체조성 성분은 유의적으로 차이가 있는 것을 확인하였다 ($p<0.05$). 총 단백질은 알이 존재하는 감염된 개체에서 비 감염 개체보다 유의적으로 높은 함량을 보였으나 ($p<0.05$), 총 탄수화물과 지질의 경우 비 감염개체에서 유의적으로 높게 나타났다 ($p<0.05$). 참굴 양식에 있어, *M. chungmuensis* 감염은 전체 생산량 감소 및 경제적 손실을 야기시키며, 지속적인 관찰을 통해 참굴의 건강도에 어떤 영향을 미치는지 확인해야 할 것으로 사료된다.

3. 우리나라 참굴 (*Crassostrea gigas*), 강굴 (*Crassostrea ariakensis*), 바지락 (*Ruditapes philippinarum*)에 발병된 난소기생충, *Marteilioides chungmuensis* (Paramyxea)

참굴의 주요 병원체인 *Marteilioides chungmuensis*는 난모세포에 감염하여 질병을 일으키는기생충성 포자충이다. 이 기생충의 진단은 현미경 관찰을 통하여 이루어진다. 2007년에 채집된 참굴, 강굴 그리고 바지락 시료의 조직학적 관찰을 통해 난모세포 안에 1-2개의 포자를 형성하는 단계의 *M. chungmuensis*를 발견하였다. 이 포자충의 정확한 동정을 위해 PCR 방법을 이용하여 난소포자충의 18s rDNA 유전자 서열을 증폭하였다. 증폭된 PCR 산물은 cloning과 염기서열 분석을 통해 염기서열을 결정하였고, 결정된 염기서열의

계통학적 유연관계를 분석하였다. 2007년 우리나라에서 채집된 참굴, 강굴 그리고 바지락 시료에서 관찰된 기생충의 조직학적 및 분자생물학적 분석 결과 *M. chungmuensis*로 확인되었다. 참굴과 바지락의 경우, 이미 우리나라와 일본에서 *M. chungmuensis*의 발병이 보고된 바 있지만, 강굴은 이 연구를 통해 처음으로 포자충 감염 발생을 확인하였다.

4. Fluid Thioglycollate Medium없이 바지락 (*Ruditapes philippinarum*)에서의 *Perkinsus olseni* 휴면포자의 유도

Fluid Thioglycollate Medium (FTM)을 이용한 *perkinsus* 배양법은 perkinsosis 진단에 널리 사용된다. FTM에서 배양 된 휴면포자는 뚜렷한 형태의 공포 (vacuole)와 세포질 가장자리가 Lugol's iodide에 검은색 또는 검은 갈색으로 염색된 반지형태를 특징으로 하는 구형의 단일핵 세포이다. *Perkisus*의 생활사 단계는 *Perkinsus marinus*에서 보고되었으나 *P. olseni*의 생활사 연구는 아직 미비한 상태로 대부분의 *perkinsus* 연구가 FTM 배양을 통한 휴면포자에 대한 연구가 주로 이루어졌다. 이 연구에서는 서로 다른 환경에서 바지락의 *P. olseni* 휴면포자의 형성 단계를 관찰하였다. *P. olseni*의 감염이 높은 선재와 황도 지역에서 채집된 바지락을 실험에 이용하여 휴면포자의 형성 단계를 조사하였다. 휴면 포자의 형성은 2 M의 NaOH 처리와 Lugol's iodide 염색으로 확인하였다. 살아있는 바지락 (4.29±0.55 um), conical tube와 petridish에서 배양한 바지락 조직, parafilm에 싼 바지락 (패각 포함)에서 작은 휴면포자를 발견하였다. 휴면포자의 성장은 감염된 바지락이 퇴적물에 묻혀 패각 내에서 죽는 현상을 모방한 parfilim에 싼 바지락에서 가장 높았다. 이 방법에 의해 생성된 휴면포자는 28 ℃에서 6 일 배양 후 70 μm까지 성장한 반면 낮은 온도 (4 ℃)에서는 휴면포자의 성장이 저하되었다. 무산소 환경과 부패된 죽은 바지락 조직은 휴면포자의 성장을 촉진시키는 인자인 것으로 사료된다. 휴면포자는 낮은 온도, 낮은 영양상태 및 무산소 환경에서 살아남을 수 있고 해수가 휴면포자의 성장에 필수적인 인자가 아님을 확인하였다. 이 연구에서 바지락의 휴면포자는 *perkinsus*의 생활사를 연장시키는데 중요

한 단계임을 알 수 있었다.

5. 한국 연안에 서식하는 바지락 (*Ruditapes philippinarum*)에서의 Paramyxean parasite *Marteilia* sp.의 첫 보고

Paramyxean 원생동물 *Marteilia*는 굴과 담치에서 Mateiliosis나 소화맹낭 질병을 일으키는 원인 생물로 보고되고 있다. 2009년 6월 전국 바지락 모니터링 중 통영 (40마리 중 1마리)과 고흥 (90마리 중 1마리)에서 *Marteilia* sp. 에 감염된 바지락이 조직학적 관찰을 통해 발견되었다. *Marteilia*는 한 세포 내에 2차 또는 3차 세포들이 증식된 형태로 소화맹낭 상피세포에 위치하였다. 기생충에 심하게 감염된 바지락은 소화맹낭 상피세포의 붕괴가 일어났다. *Marteilia*는 소화맹낭의 정상적인 기능에 영향을 주는 것으로 사료된다. 그러나 감염된 바지락에서 비만도의 뚜렷한 감소는 보이지 않았다. 이 연구는 우리나라 바지락에서의 *Marteilia* sp. 감염을 처음 보고하는 것이다.

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Part I

Pathologic survey on Manila clams *Ruditapes philippinarum* distributed along in Korean waters

Abstract

Parasite and pathologic conditions in Manila clam *Ruditapes philippinarum,* the economically important bivalve species in Korea fisheries, from 23 clam beds along west, south, east coast and Jeju Island were surveyed in April and November 2007. Forty Manila clams from each sampling sites were collected and processed by standard histological techniques. The variation of parasite distribution between geographic regions was analyzed. The north-west and south coast sampling sites showed higher prevalence of parasite infestations. There was a seasonal variation in parasites and pathological prevalence while the changes of parasite prevalence were not correlated with different sampling sites. Among the parasites observed, *Perkinsus* and trematode cercariae were the most common parasite in Manila clam. *Perkinsus* was observed in every organ of Manila clams and intensive hemocyte infiltrations were especially found in high infected clams. Gonad castration was observed in clams that were severely infected with cercaria. The other parasites (Turbellaria, gragarine, copepod, rickettsia-like organisms, Martelioides and encysted larval cestode) were minor pathogens of Manila clam due to low infestation and low pathological effect. Non infectious pathological condition observed in this study was digestive gland atrophy, non specific focal or systemic hemocyte accumulation and necrosis. The parasite infestation and pathological condition prevalence were much higher among the clams collected from commercial clam beds located in tidal flats along the west and south coast than clams from Jeju Island and east coast which were natural beds and sand beach. Spatial distributions of each parasite were possible associated with clam density and type of topography.

Introduction

Manila clam *Ruditapes philippinarum* (Japanese carpet shell, littleneck clam or shortneck clam) is wildly distributed in the coastal Yellow Sea from the Southern China Sea to South and East China Seas, Sea of Japan, Sea of Okhotsk, and around Southern Kuril Islands (FAO, 2009). Manila clams also introduced to several parts of the world, where they have become permanently established (FAO, 2009). In Korea, Manila clam is mainly distributed on the western and the southern coast and is often cultured at a commercial scale due to its fast growth and high productivity (Park et al., 2005). The severe low temperature in winter, the changes of sediment composition in clam habitat due to storm and wave and certain diseases are thought to be associated with the mortality. Several protozoan and metazoan pathogens have been observed from Manila clam in Korean waters (Park et al., 1999; Lee et al,. 2001;Ngo and Choi, 2004). *Perkinsus* sp. was identified from the Manila clam population inhabiting in the west coast where mass mortality has been reported and believed to be responsible for the mass mortalities (Park et al., 1999; Park and Choi, 2001). Ngo and Choi (2004) demonstrated that a high level of *Cercaria tapidis* infection cause gonad castration of Manila clams collected from a sand beach on the east coast of Jeju. The occurrence of *Vibrio tapetis*, the brown ring disease was first report in Asia from the clam collected from Anmyeondo Island, west coast of Korea (Park et al., 2006c). The routine histopathological survey along the south coast of Korea demonstrated *Perkinsus* sp., *Marteilioides*-like organism and *Cercaria tapidis* however the two latter parasites infected the gonad and did not evoke any significant host reaction (Lee et al., 2001).

Despite those of main parasites reported in Manila clam in Korea, many studies described different affects from several types of parasite and diseases to bivalve hosts.

Parasites of marine bivalves belong to several phyla and subtaxa including protozoan, helminthes, mollusks and arthropods (Cheng, 1988). The diseases caused by microorganisms: virus, bacteria, fungi, Rickettsia, Chlamydiae and Mycoplasma; also reports as an important disease of bivalve (Lauckner, 1983). To date, little is known about the parasite fauna of Manila clam in Korea waters and the pathologic affect of parasite to the clam was only well described in *Perkinsus* and the trematode *Cercaria tapetis* infection. Therefore the histopathological study was performed to provide reference information for the developing Manila clam culture industry in Korea.

The Korean peninsula is surrounded by three marine ecosystems which may affect the parasite community and Manila clam conditions. The East sea, to the east of Korea is a large, deep (>1000m) basin. The East China Sea, to the south of Korea, is larger in surface area, but shallower than the East Sea. The Yellow Sea to the west is a shallow, semi-enclosed shelf sea. The mean depth is 44 m, with a central trough reaching only about 90m. Tidal ranges are high. Shallow depth and strong tide result in high turbidity (Rebstock and Kang, 2003). In addition, as the factors influencing the geographic distribution of the host, such as temperature is also likely to affect that of the parasites (Kim and Powell, 2007). Therefore the survey study was established by collecting Manila clams from several clam beds from west, south, east coast and Jeju Island for two times in spring and autumn to compare the spatial and seasonal variation of parasite community and richness.

Recently, many diagnosis methods were develop however histological examination remains the best general approach for simultaneously evaluating a wide range of parasites, diseases and pathologies (Kim et al., 2006). Moreover the histopathological survey of parasites and pathologies can be an important component of a biomonitoring study of environmental pollution (Kim and Powell, 2006). The review by Sures (2004) indicated that

parasites are useful bioindicators for pollution. The increase of parasitism may cause by the negative effect of pollution to host defence mechanisms, thereby increasing host susceptibility, or by the increasing of suitable intermediate or final hosts. The decrease of parasitism by pollution coincided with the higher susceptible to pollutant of the parasites than host, decline of infected host or the extinction of intermediate or final host. The damage of digestive tract and digestive diverticular, increase of hemocyte and necrosis has been reported to relate with environmental factors such as salinity, petroleum hydrocarbons and cadmium (Gold-Bouchot et al., 1995).

Since the basis information concerning parasite fauna and pathological condition of Manila clam was scarce, this study aimed to assemble various types of parasite data and pathological condition to evaluate if they constituted a Manila clam health risk. Also the outcome from this study will be used as a background fundamental material in the future studies regarding the Manila clam health, production and aquaculture and marine pollution biomarker. The studies describe the distribution of parasites and pathologies in Manila clam from different geographic regions and evaluate if certain parasite was involve in unusual physical condition of the clams. Here is the first nationwide monitoring which the Manila clams were gathered from every different type of clam beds.

Materials and methods

1. Sampling efforts

Manila clams were collected from 23 clam habitats on the west, south and east coast of Korean peninsula (Fig 1-1). The bottom topography and sampling site condition were described in Table 1-1. The study was performed 2 times in spring (April or May) and autumn (November) 2007. The samples of 40 lived Manila clams at market size (shell length>30mm) from each sampling sites were selected to prepare histology slide. The clams were transported to the laboratory and directly processed or maintained in 10^{o}C less than 12 hours until processing. The shell length of each individual was measured with calipers. The soft parts were carefully removed from the shells and weighted. The shells were allowed to dried and weighted.

The condition index of individual was calculated as;

Tissue wet weight / Shell dried weight ……(1)

2. Histology slide preparation

The cross sections, approximately 5 mm thick which contain gonad, visceral organ, foot muscle, remnant of gill and mantle were taken from each clam (Fig. 1-2) and were fixed in Davidson's solution for 24 hours and preserved in 70% ethyl alcohol. The tissue was then embedded in paraffin and sectioned at 5 μm thickness. The tissue were placed on the glass slide and deparaffinized with xylene. Then the tissue was rehydrated with ethanol series and stained with Hematoxylin & Eosin, dehydrated with ethanol series and mounting.

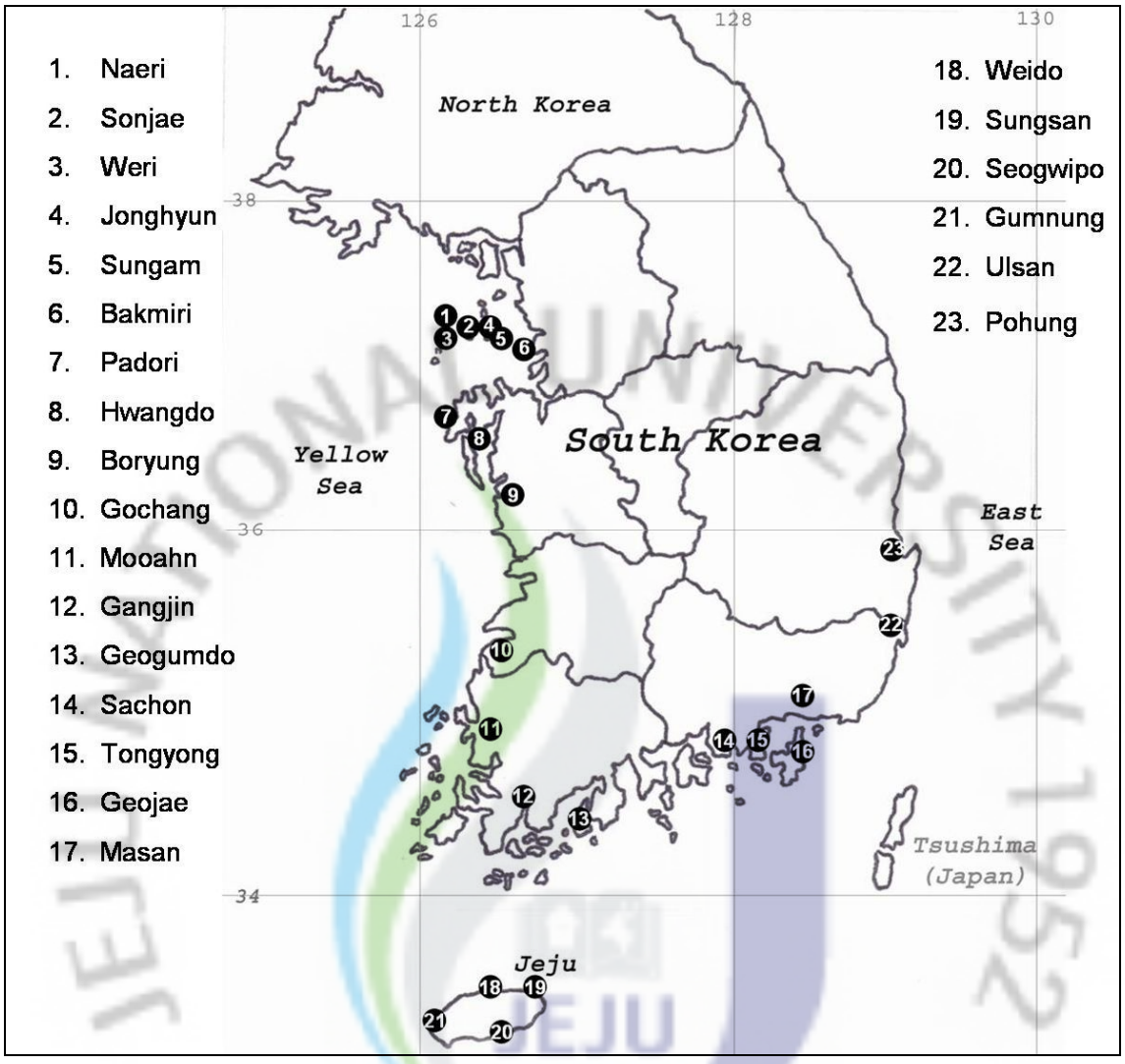

Figure 1-1 Map of Korea. The location of clam beds used for Manila clam collection was indicated by number 1-23.

Table 1-1 The location and characteristic of clam beds where the Manila clams were collected

	Site	Region	Topography	
1	Naeri	West coast	intertidal, sand mud tidal flat	commercial clam bed
2	Seonjae	West coast	intertidal, sand mud tidal flat	commercial clam bed
3	Weri	West coast	intertidal, sand mud tidal flat	commercial clam bed
4	Jonghyun	West coast	intertidal, sand mud tidal flat	commercial clam bed
5	Sungum	West coast	intertidal, sand mud tidal flat	commercial clam bed
6	Bakmiri	West coast	intertidal, sand mud tidal flat	commercial clam bed
7	Padori	West coast	Intertidal, sand mud tidal flat	commercial clam bed
8	Hwangdo	West coast	intertidal, sand mud tidal flat	commercial clam bed
9	Boryung	West coast	intertidal, sand mud tidal flat	commercial clam bed
10	Gochang	West coast	intertidal, sand mud tidal flat	commercial clam bed
11	Mooahn	West coast	intertidal, sand mud tidal flat	commercial clam bed
12	Gangjin	South coast	intertidal, sand mud tidal flat	commercial clam bed
13	Geogumdo	South coast	intertidal, sand mud tidal flat	commercial clam bed
14	Sachon	South coast	intertidal, sand mud tidal flat	commercial clam bed
15	Tongyong	South coast	intertidal, sand mud tidal flat	commercial clam bed
16	Geojae	South coast	intertidal, sand mud tidal flat	commercial clam bed
17	Masan	South coast	intertidal, sand mud tidal flat	commercial clam bed
18	Weido	Jeju island	intertidal, sandy beach with gravel	natural clam bed
19	Sungsan	Jeju island	Low subtidal, sand mud tidal flat	natural clam bed
20	Seogwipo	Jeju island	Intertidal, sand mud tidal flat	natural clam bed
21	Gumnung	Jeju island	intertidal, sandy beach with gravel	natural clam bed
22	Ulsan	East coast	subtidal, estuary	natural clam bed
23	Pohung	East coast	subtidal, estuary	natural clam bed

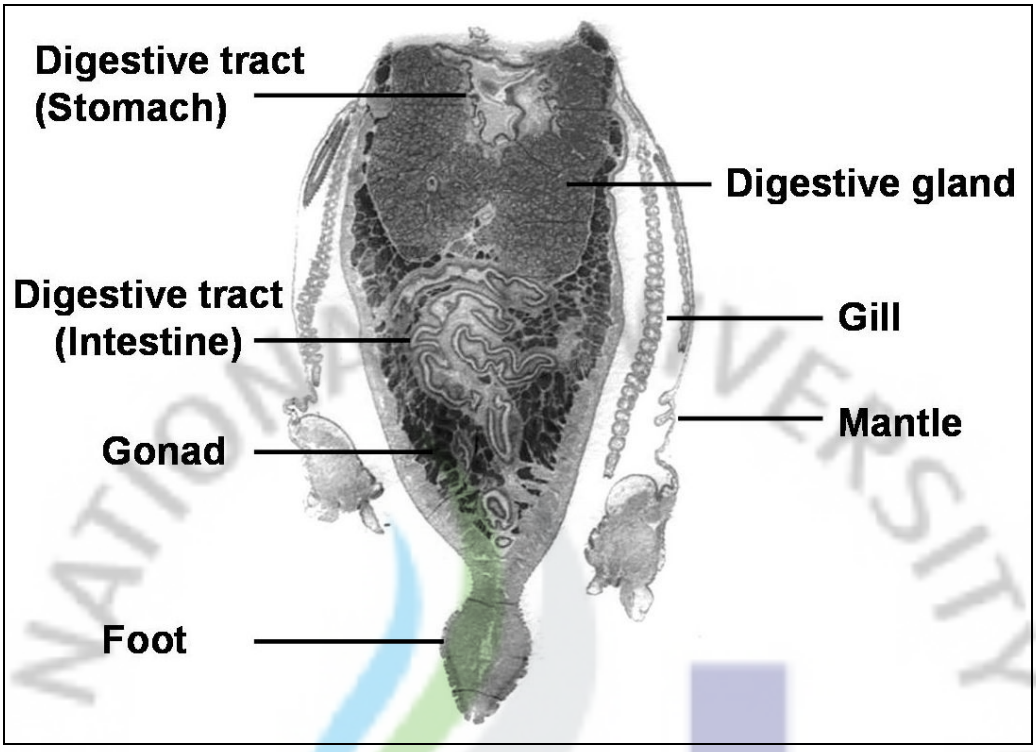

Figure 1-2 The cross section of Manila clam, *Ruditapes philippinarum*. The section used for histopathological observation contained digestive gland, stomach, intestine, gill, mantle and foot.

3. Lipofuscin histochemistry

Lipofuscin is a cell brown pigment which derives from damage cellular components through peroxidation (Dimitriadis et al., 2004). In order to confirm the pigment in H&E stained section, some tissue sections was used for Schmorl reaction (Moore, 1988). After the deparaffinized step, the tissue section was incubated in the reaction medium containing 1% ferric chloride and 1% potassium ferricyanide in a ratio of 3:1 v/v for 5 min and mounted with Canada balsam. The lipofuscin was detected as dark blue color granules.

4. Gonad development

The maturity of gonad developments of Manila clams were divided into six categories modified from Drummond et al. (2006): resting, early developing, late developing, partially spawning and spent stage as described in Table 1-2. Figure 1-3 and 1-4 demonstrated gametogenesis stage of Manila clams.

5. Prevalence and infection intensity

The histology slides were examined using microscope and the pathology of each individual was recorded. The parasitic infestation was identified by their characteristics in histological image which explained in result part. The non infectious pathological condition of digestive gland was categorized to 1) localized and 2) generalized necrosis, 3) localized and 4) generalized hemocyte infiltration, 5) vacuolization, 6) erosion or sloughing of digestive gland epithelium, 7) shrinkage of digestive gland tubule and 8) the occurrence of lipofuscin. Each digestive gland lesions were described in figure 1-5. The parasite infestation and pathologies were described in terms of *Prevalence* which calculated as;

Number of manila calms contained parasite or pathology / Total number of clams examined..(2).

Perkinsus, trematode cercariae and digestive gland atrophy were determined to semi-quantitative scales depending on the intensity or the distribution in the affected area. In case of *Perkinsus* infection, the intensity of infection was evaluated in individual part; gill, mantle, digestive gland, digestive tract, gonad and muscle, from score 0-3 depending on the number of trophozoites observed in tissue (Table 1-3). The criteria used for evaluate the numerical score of trematode infection and digestive gland atrophy was demonstrated in table 1-4 and 1-5 and figure 1-6. The degree of severity of infection was calculated using 2 equations. The weighted prevalence (Kim et al., 2006) demonstrates the average semi-quantitative score of parasite infection in each sampling sites which explains the relative severity of infection within the population. *Weighted prevalence* is calculated as;

Total score of parasite infection / Total number of clams examined …….(3).

Infection intensity demonstrated the severity of parasite infection in the parasitized animal only. *The infection intensity* was calculated in each sampling site as;

Total score of parasite infection / Number of clams infected ……..……..(4).

Table 1-2 Classification of reproductive stage of the Manila clam, *Ruditapes philippinarum* (Drummond et al., 2006)

Reproductive stage	Description
Resting	Gonad follicle compose of connective tissue. The follicle is empty, oogonia and spermatogonia cannot be observed.
Early developing	Gonad proliferation initiates; increasing number of oocytes at follicular wall, no free oocyte in the follicles. In spermatogenenic follicles, increase number of spermatogonia, spermatocytes present, no spermatids or spermatozoa.
Late developing	Free oocytes in the lumen but most oocytes attach on the follicular walls. Spermatogonia, spermatocytes, spermatids and spermatozoa coexist in follicles; in less developed specimens, there is no dominant cell type; in more developed specimens, the majority of the follicle is filled by spermatids and spermatozoa.
Ripe	Gonad filling large surface area, oogenesis follicle full with polygonal configuration oocytes. Spermatogenesis follicles predominantly compose of mature spermatozoa with their tail pointing towards the center of the lumen forming concentric bands or plugs; spermatozoa bands close to the follicle wall in very ripe specimens; follicles neat and orderly in appearance.
Partially spawning	Numbers of free oocytes in lumen are decrease, empty space in follicle can be observed. Spermatozoa clearly visible in a swirling shape and accounting for the greatest portion of cells in the follicle; empty space in some follicles due to release of mature spermatozoa.
Spent	Follicles appear broken, scatter and relatively empty, only residual oocytes or spermatozoa, numerous numbers of phagocytes.

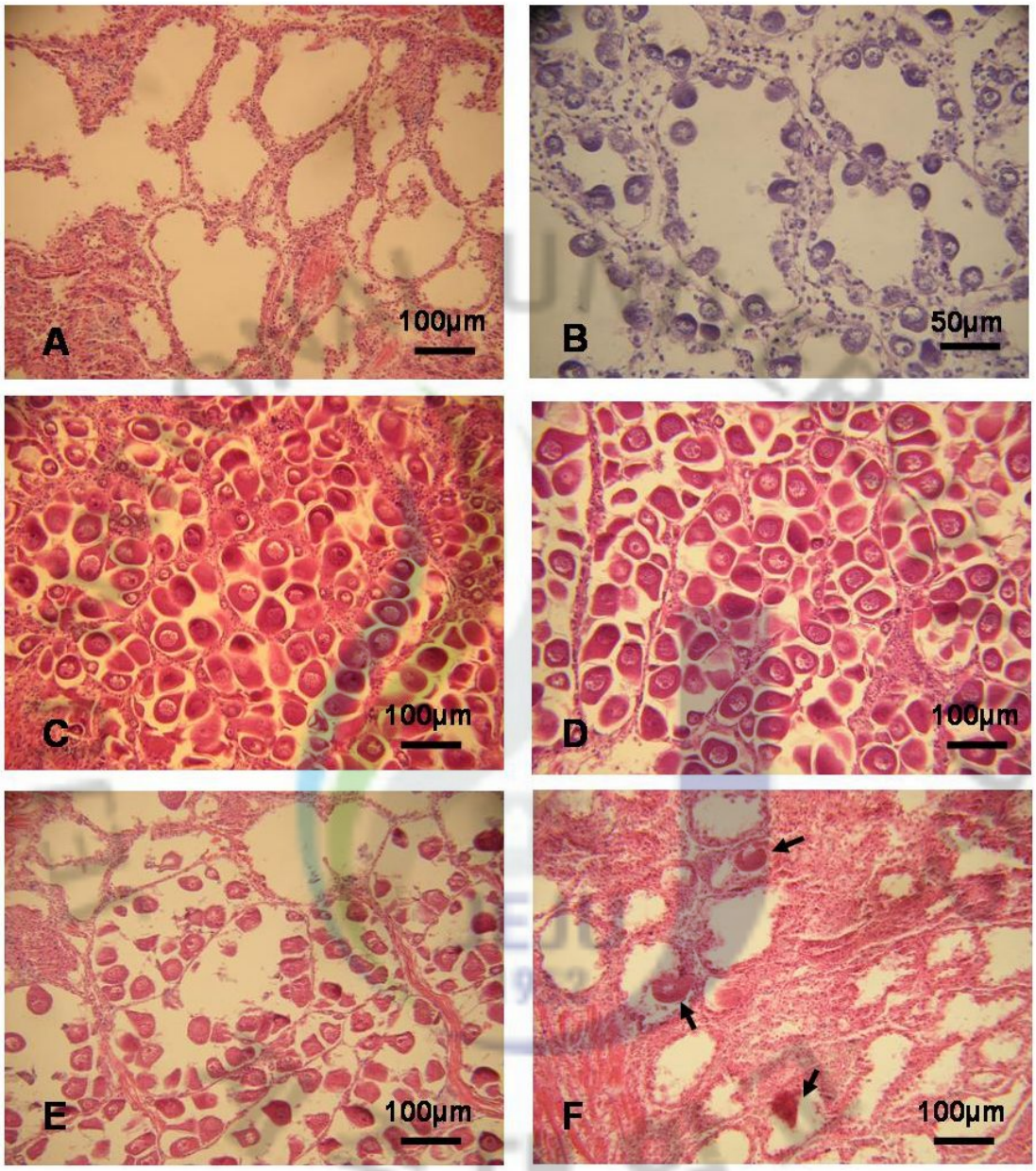

Figure 1-3 Histological pictures of Manila clam gonad. 1-3A: Resting stage, gonad follicle is empty. The development of female gonad was demonstrated in 1-3B-F. 1-3B: Early developing stage, the small oocyte developed along follicle wall. 1-3C: Late developing stage: The number of oocyte and the follicle size increase. 1-3D: Ripe stage, the follicle full with polygonal configuration oocytes. 1-3E: Partially spawning stage, number of eggs decrease and empty space in follicle was due to the releasing of eggs. 1-3F: Spent stage, the follicles appear empty and the absorbed residual oocytes (arrows) were observed.

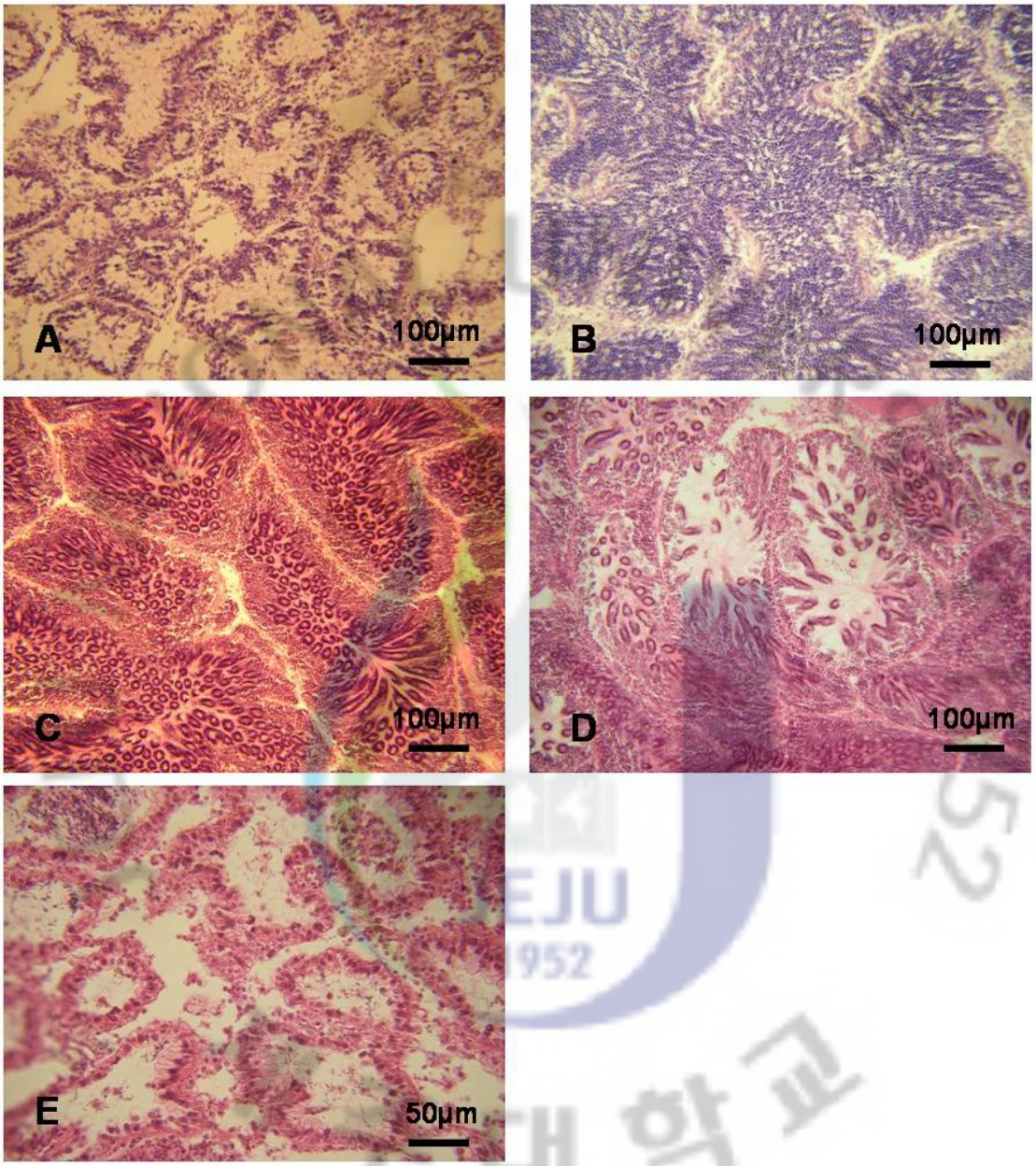

Figure 1-4 Histological pictures of male Manila clam gonad. 1-4A: Early developing stage, the spermatogonia and spermatocyte develop on the follicle wall. 1-4B: Late developing stage, the number of gamete cells and the follicle size increase. 1-4C: Ripe stage, the main population of gametes are spermatozoa which their tails pointing toward the center of lumen forming concentric bands. 1-4D: Partially spawning stage, the empty space in follicles was due to the releasing of mature spermatozoa. 1-4E: Spent stage, the follicle size was decrease and residual spermatozoa were observed.

Table 1-3 Semi-quantitative scale for *Perkinsus* intensity of infection

Score	Description
0	The *Perkinsus* trophozoite was not observed
1	Light infection; few number of *Perkinsus* was found in the tissue section. Trophozoite occupied in tissue section less than 5%
2	Moderate infection; the trophozoite distributed in tissue (5-20%) and easily to be observed
3	Heavy infection; the tissue was occupied by high number of *Perkinsus* (>20%), the group of *Perkinsus* can be observed in every high magnification (x400) microscopic field.

Table 1-4 Semi-quantitative scale for trematode sporocyst infection (Kim et al., 2006; Ngo and Choi, 2004)

Score	Description
0	Uninfected
1	Trematode found in some part of gonads, gametogenic follicles can be observed.
2	Trematode found in gonad and maybe in other tissue, <50% gametogenic follicle can be observed
3	Trematode completely fill in gonad, the gametogenic follicle cannot observed. Some trematode can be found in other tissue
4	Trematode completely fill in gonad, no gametogenesis follicle, severely invaded to other tissue

Table 1-5 Semi-quantitative scale for digestive gland atrophy (Kim et al., 2006)

Score	Description
0	Normal wall thickness in most tubules (0% atrophy), lumen nearly occluded, few tubules even slightly atrophied
1	Average wall thickness than normal, but greater than one-half normal thickness, most tubules showing some atrophy, some tubules still normal
2	Wall thickness averaging about one-half as thick as normal
3	Wall thickness less than one-half of normal, most tubules walls significantly atrophied, some walls extremely thin (fully atrophied)
4	Wall extremely thin (100 atrophied), nearly all tubules affected

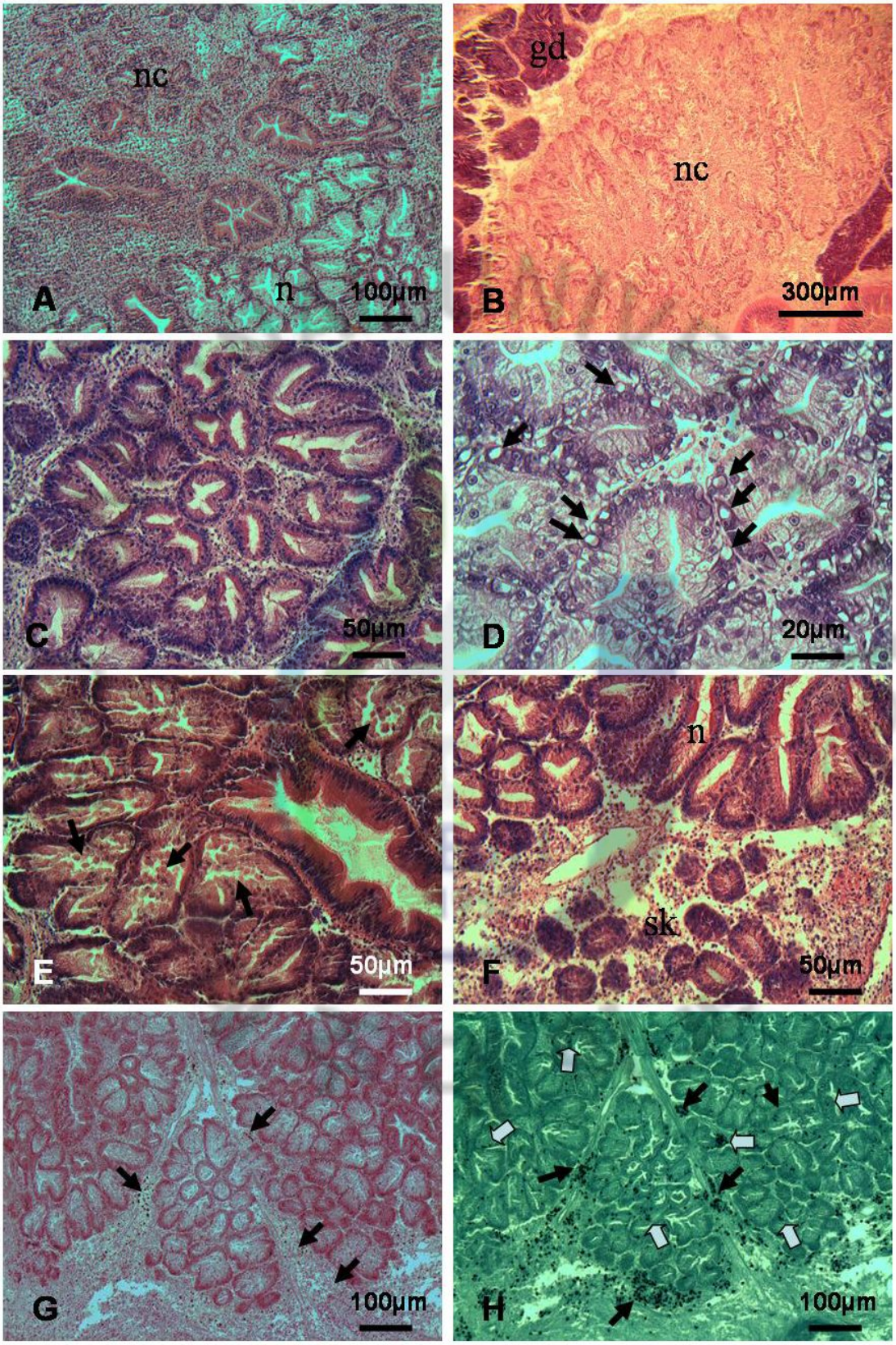

Figure 1-5

Figure 1-5 Digestive gland of Manila clam. 1-5A: Localized focal necrosis (nc) of digestive gland with hemocyte infiltration compare with normal digestive gland (n). 1-5B: Low magnification of digestive gland showed the generalized necrosis (nc) between the gonad (gd), the tubule can not identified, the necrosis tubule appeared eosinophilia. 1-5C: The hemocyte infiltration in connective tissue of digestive gland. 1-5D: Vacuolization (arrows) in digestive gland cells. 1-5E: The erosion of digestive gland cell (arrows) into the tubule lumen. 1-5F: The shrinkage of digestive gland tubule (sk) showed the smaller size of tubules to 1/2 to 1/3 compare with the normal one (n). 1-5G: The brown pigment granules in connective tissue of digestive glands (H&E). 1-5H: Schmorl's staining of digestive gland caompare with figure 1-5G showed the lipofuscin granules stained with dark blue color in connective tissue (black arrows) and digestive cells (white arrows).

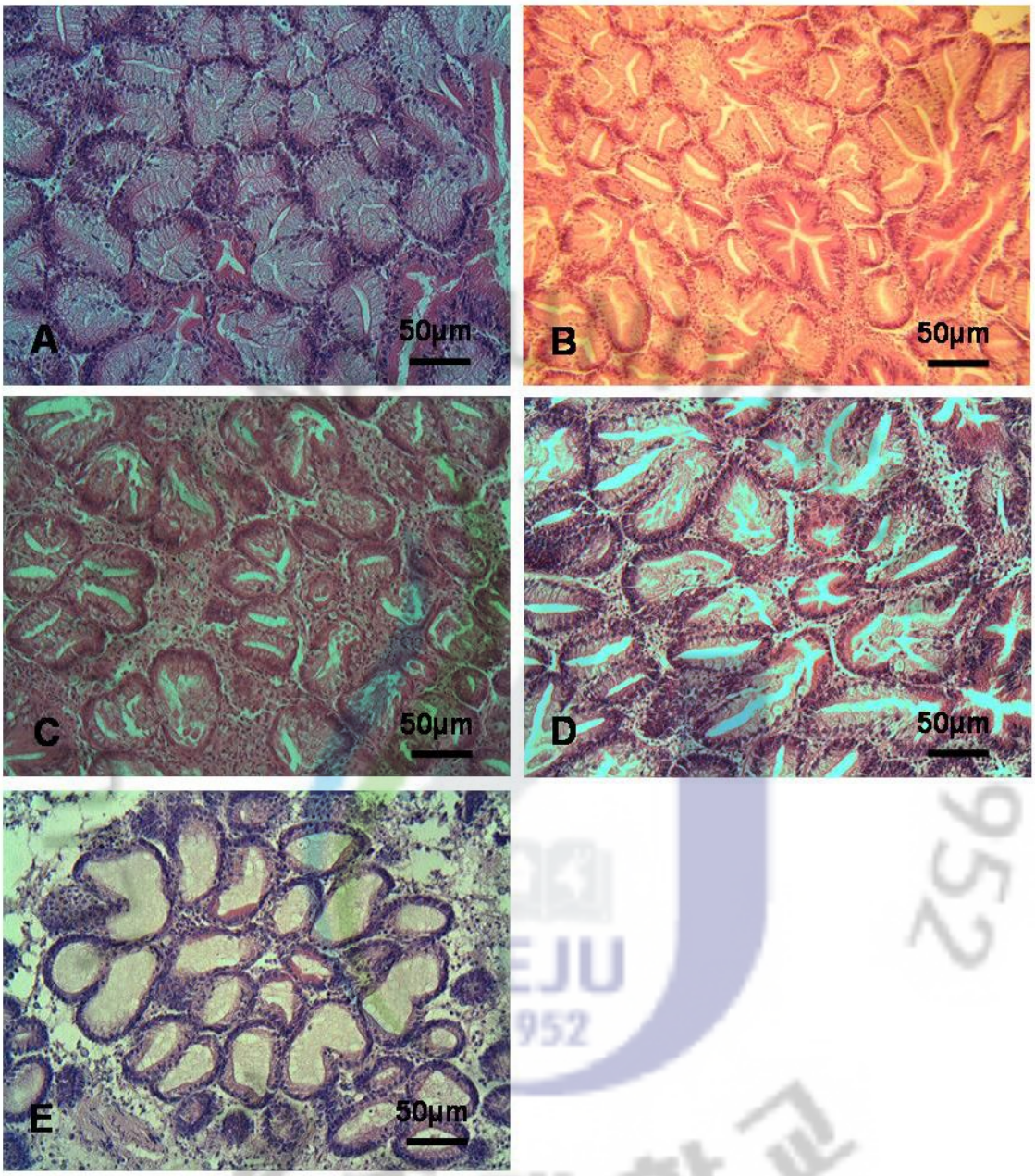

Figure 1-6 Digestive gland of Manila clam (H&E). 1-6A: Digestive gland without sign of atrophy, the well organized tall digestive cells was observed. 1-6B: Digestive atrophy score at 1, the thickness of digestive gland tube is normal but the small lumen appeared. 1-6C: Digestive gland atrophy score at 2, the columnar epithelium of digestive gland was observed with wider lumen. 1-6D: Digestive gland score at 3, the decrease in of columnar epithelium to almost cuboidal, the lumen was obvious. 1-6E: Digestive gland atrophy score at 4, the digestive gland tube is extremely thin. The large lumen was observed.

Results

1. Condition Index

Total of 1,840 Manila clams used in this study were 25.70-57.50 mm in shell length and the condition index (CI) were between 0.14-0.9. Shell length, tissue wet weight, shell weight and CI of Manila clam in each sampling site and sampling period were shown in Table 1-6. In April the highest mean CI was observed in Hwangdo followed by Gochang and Boryung. The lowest CI was observed in Ulsan and Sachon. In November, Manila clams from Hwangdo were in the highest CI followed by Boryung and Gangjin. Ulsan was the sampling site where Manila clams were in the lowest CI. The average CI of Manila clams from every sampling site collected in spring (0.50±0.18) was higher than autumn (0.39±0.09). Except for Padori, Sachon, Weido, Sungsan and Ulsan, the other sampling sites showed the lower condition index in November than in April or May (Fig. 1-7).

2. Reproductive stage

The sex ratio of Manila clams in each sampling site was shown in table 1-6. In April, the ratio between male and female clams was equal or closed to 1:1 with some variation in certain sites. Few numbers of clams were found to be indifferent. The majority of clams became indifferent in November in many sampling sites (12 in 23 sites) especially in the north west region. The percentage of reproductive in each sampling site in April/May and November was shown in figure 1-8 and 1-9. In April, most of Manila clams from west coast were in developing stage while in the south coast were in ripe and started partially spawning stage. The gametogenesis of Manila clams from Jeju Island which sampled in May was varied between sites. Weido and Gumnung were in late developing or ripe stage while Sungsan and

Seopwipo were in early developing stage. In east coast sampling sites, Ulsan clams were in early developing stage while Pohung started spawning. In November, most of the clams in every sampling sites were in inactive stage of gametogenesis (resting or spent). Spawning clams could be observed in some sampling sites from south coast, Jeju inland and east coast.

Table 1-6 The sampling sites, Sex ratio of male:female:indifferent clam (M:F:I), Number of samples (N), Shell length (mm), tissue wet weight (g), shell weight (g) and condition index of Manila clam *Ruditapes philippinarum*

Site	Sampling period	N	M:F:I	Shell length (mm)			Tissue weight (g)			Shell weight (g)			Condition Index		
				Mean	SD	Min-Max	Mean	SD	Min-Max	Mean	SD	Min-Max	Mean	SD	Min-Max
1. Naeri	Apr 07	40	19:18:3	40.33	3.71	35.80-54.20	4.50	1.60	2.99-10.89	7.10	2.53	4.08-16.77	0.66	0.12	0.35-0.89
	Nov 07	40	10:11:19	40.22	2.03	36.10-44.60	2.77	0.62	1.91-4.90	6.78	1.01	4.78-9.45	0.41	0.06	0.30-0.57
2. Seonjae	Apr 07	40	23:17:0	37.35	3.15	33.30-49.50	3.04	0.78	1.86-6.56	4.84	1.73	3.11-11.72	0.65	0.10	0.42-0.86
	Nov 07	40	4:1:35	34.18	1.33	31.40-38.40	1.59	0.22	1.24-2.30	4.12	0.81	2.57-6.33	0.39	0.07	0.22-0.54
3. Weri	Apr 07	40	17:20:3	39.02	2.39	34.70-44.30	3.27	0.63	2.29-4.96	5.53	1.10	3.67-8.32	0.60	0.07	0.37-0.74
	Nov 07	40	7:5:28	38.15	1.89	36.00-45.60	1.89	0.47	1.09-3.64	5.01	1.10	3.76-9.48	0.38	0.07	0.26-0.53
4. Jonghyun	Apr 07	40	22:12:6	40.41	2.21	37.70-49.60	3.55	0.88	2.51-7.02	6.25	1.41	4.73-11.02	0.57	0.10	0.44-1.05
	Nov 07	40	12:7:21	38.70	1.58	35.90-42.90	2.55	0.33	1.96-3.31	5.62	0.79	4.38-7.64	0.46	0.06	0.32-0.55
5. Sungum	Apr 07	40	16:22:2	37.16	2.22	30.80-43.00	2.32	0.63	1.29-4.23	5.36	1.08	4.01-9.54	0.44	0.12	0.18-0.67
	Nov 07	40	6:3:31	34.50	1.92	30.50-39.30	1.53	0.26	1.04-2.12	3.92	0.86	2.73-6.15	0.40	0.06	0.28-0.55
6. Bakmiri	Apr 07	40	18:13:9	37.08	2.11	33.50-41.80	2.51	0.57	1.32-3.89	4.94	1.09	3.33-7.10	0.52	0.10	0.31-0.70
	Nov 07	40	6:3:31	37.19	2.02	32.90-41.20	1.69	0.34	0.99-2.33	4.90	0.94	3.18-6.75	0.35	0.05	0.22-0.46
7. Padori	Apr 07	40	16:15:9	36.56	1.89	33.90-42.90	1.98	0.32	1.46-3.06	4.66	0.77	3.46-7.15	0.43	0.05	0.28-0.54
	Nov 07	40	3:5:32	38.78	1.20	37.00-41.30	2.58	0.44	1.78-4.05	5.76	0.61	4.46-7.31	0.45	0.06	0.36-0.67
8. Hwangdo	Apr 07	40	21:16:3	36.65	3.19	31.10-47.80	3.54	0.70	2.02-5.03	4.38	1.31	2.46-10.02	0.83	0.13	0.34-1.04
	Nov 07	40	6:10:24	35.69	2.41	30.90-43.40	2.18	0.64	1.15-3.96	4.24	1.20	2.97-8.44	0.52	0.09	0.33-0.70
9. Boryung	Apr 07	40	17:23:0	30.86	1.09	28.50-34.20	1.98	0.22	1.56-2.51	2.68	0.39	1.77-3.46	0.75	0.07	0.61-0.90
	Nov 07	40	13:11:16	37.49	1.90	34.20-43.70	2.31	0.41	1.69-3.57	4.78	0.48	3.49-6.85	0.48	0.06	0.34-0.65

Table 1-6 (Continued) The sampling sites, Sex ratio of male:female:indifferent clam (M:F:I), Number of samples (N), Shell length (mm), tissue wet weight (g), shell weight (g) and condition index of Manila clam *Ruditapes philippinarum*

Site	Sampling period	N	M:F:I	Shell length (mm)			Tissue weight (g)			Shell weight (g)			Condition Index		
				Mean	SD	Min-Max	Mean	SD	Min-Max	Mean	SD	Min-Max	Mean	SD	Min-Max
10. Gochang	Apr 07	40	18:20:2	33.85	3.08	29.00-43.90	2.89	0.94	1.15-5.83	3.82	1.39	1.95-9.33	0.78	0.19	0.28-1.03
	Nov 07	40	14:17:9	38.73	2.48	34.60-45.20	2.48	0.62	1.51-4.74	5.37	1.07	3.88-8.10	0.46	0.06	0.34-0.59
11. Mooahn	Apr 07	40	20:18:2	36.76	1.51	33.90-40.70	2.40	0.39	1.76-3.44	4.89	0.98	3.30-7.06	0.50	0.10	0.35-0.98
	Nov 07	40	4:9:27	33.78	1.64	31.10-39.40	1.02	0.18	0.71-1.43	3.25	0.52	2.34-4.55	0.31	0.04	0.24-0.41
12. Gangjin	Apr 07	40	22:17:1	38.84	1.86	36.00-43.10	3.53	0.55	2.59-4.87	6.59	1.09	1.66-9.59	0.54	0.08	0.39-0.73
	Nov 07	40	17:17:6	39.62	1.89	35.20-42.80	2.70	0.48	1.90-3.88	5.61	0.74	4.20-6.98	0.48	0.07	0.37-0.61
13.Geogumdo	Apr 07	40	21:18:1	41.11	2.34	36.40-47.60	3.84	1.16	1.65-6.08	8.00	1.59	5.39-11.92	0.48	0.13	0.21-0.80
	Nov 07	40	19:20:1	41.69	1.70	38.80-46.60	3.07	0.63	2.11-5.31	7.93	1.44	6.10-12.27	0.39	0.07	0.24-0.61
14. Sachon	Apr 07	40	22:18:0	41.13	2.63	35.40-49.20	2.07	0.59	1.17-4.05	7.62	1.60	4.60-12.36	0.27	0.04	0.18-0.38
	Nov 07	40	22:13:5	40.71	4.91	33.50-53.30	2.81	0.88	1.10-5.07	7.24	2.16	3.92-14.64	0.39	0.09	0.20-0.60
15. Tongyong	Apr 07	40	18:20:2	45.78	3.07	41.10-54.80	4.30	0.99	2.39-6.81	10.81	1.87	8.64-17.02	0.40	0.06	0.25-0.54
	Nov 07	40	14:20:6	44.28	5.39	35.50-59.50	3.34	1.07	1.85-7.10	9.21	3.38	3.60-18.75	0.38	0.09	0.23-0.58
16. Geojae	Apr 07	40	13:14:12	42.28	1.84	38.50-46.00	2.83	0.67	1.56-5.00	8.91	1.53	6.29-12.36	0.32	0.05	0.22-0.48
	Nov 07	40	11:14:15	36.99	2.01	30.90-41.10	1.67	0.31	0.93-2.32	5.70	1.00	4.00-8.86	0.30	0.04	0.18-0.41
17. Masan	May 07	40	16:15:9	38.64	1.59	36.30-43.10	2.60	0.35	1.66-3.59	6.26	1.01	4.86-9.12	0.42	0.07	0.28-0.58
	Nov 07	40	19:10:11	36.57	1.95	33.20-40.90	1.57	0.33	0.77-2.31	5.53	1.16	3.58-8.52	0.29	0.05	0.14-0.41
18. Weido	May 07	40	20:20:0	28.25	1.11	26.30-31.60	0.80	0.12	0.51-1.06	2.19	0.27	1.78-2.76	0.37	0.05	0.25-0.50
	Nov 07	40	19:18:3	28.04	1.12	25.70-31.50	0.86	0.10	0.68-1.06	2.02	0.32	1.43-2.61	0.43	0.04	0.34-0.53

Table 1-6 (Continued) Shell The sampling sites, Sex ratio of male:female:indifferent clam (M:F:I), Number of samples (N), Shell length (mm), tissue wet weight (g), shell weight (g) and condition index of Manila clam *Ruditapes philippinarum*

Site	Sampling period	N	M:F:I	Shell length (mm)			Tissue weight (g)			Shell weight (g)			Condition Index		
				Mean	SD	Min-Max	Mean	SD	Min-Max	Mean	SD	Min-Max	Mean	SD	Min-Max
19. Sungsan	May 07	40	21:12:7	38.45	3.13	32.60-44.40	2.21	0.60	1.12-3.45	7.77	5.57	2.90-13.51	0.30	0.07	0.14-0.44
	Nov 07	40	6:7:27	38.67	2.27	35.20-45.80	2.68	0.55	1.94-4.22	7.59	1.46	4.63-11.71	0.35	0.04	0.29-0.45
20. Seogwipo	May 07	40	17:16:7	30.09	3.84	25.70-38.00	1.33	0.51	0.81-2.66	3.56	1.70	1.69-7.70	0.39	0.07	0.26-0.60
	Nov 07	40	16:24:0	32.43	3.86	28.20-45.80	1.23	0.49	0.60-3.04	3.85	1.74	2.06-9.14	0.33	0.05	0.24-0.43
21. Gumnung	May 07	40	18:22:0	30.64	1.61	27.70-35.00	1.32	0.24	0.93-1.85	2.53	0.50	1.71-3.47	0.53	0.05	0.40-0.64
	Nov 07	40	13:23:4	31.98	1.96	28.50-36.00	1.21	0.31	0.71-2.20	2.97	0.77	1.82-5.37	0.41	0.04	0.30-0.53
22. Ulsan	Apr 07	40	17:6:17	41.14	2.63	35.40-49.20	2.08	0.59	1.17-4.05	7.62	1.60	4.60-12.36	0.27	0.04	0.18-0.38
	Nov 07	40	20:18:2	37.73	2.87	32.90-45.30	1.68	0.46	0.86-3.25	6.31	1.75	3.49-9.85	0.27	0.06	0.18-0.40
23. Pohung	Apr 07	40	21:18:1	50.49	2.73	46.50-57.50	8.49	1.63	5.76-12.39	15.21	3.71	9.40-23.50	0.57	0.08	0.42-0.75
	Nov 07	40	14:26:0	49.77	2.90	45.20-57.20	4.65	0.99	3.21-6.94	12.95	2.67	7.71-17.70	0.36	0.05	0.24-0.56

SD = standard deviation

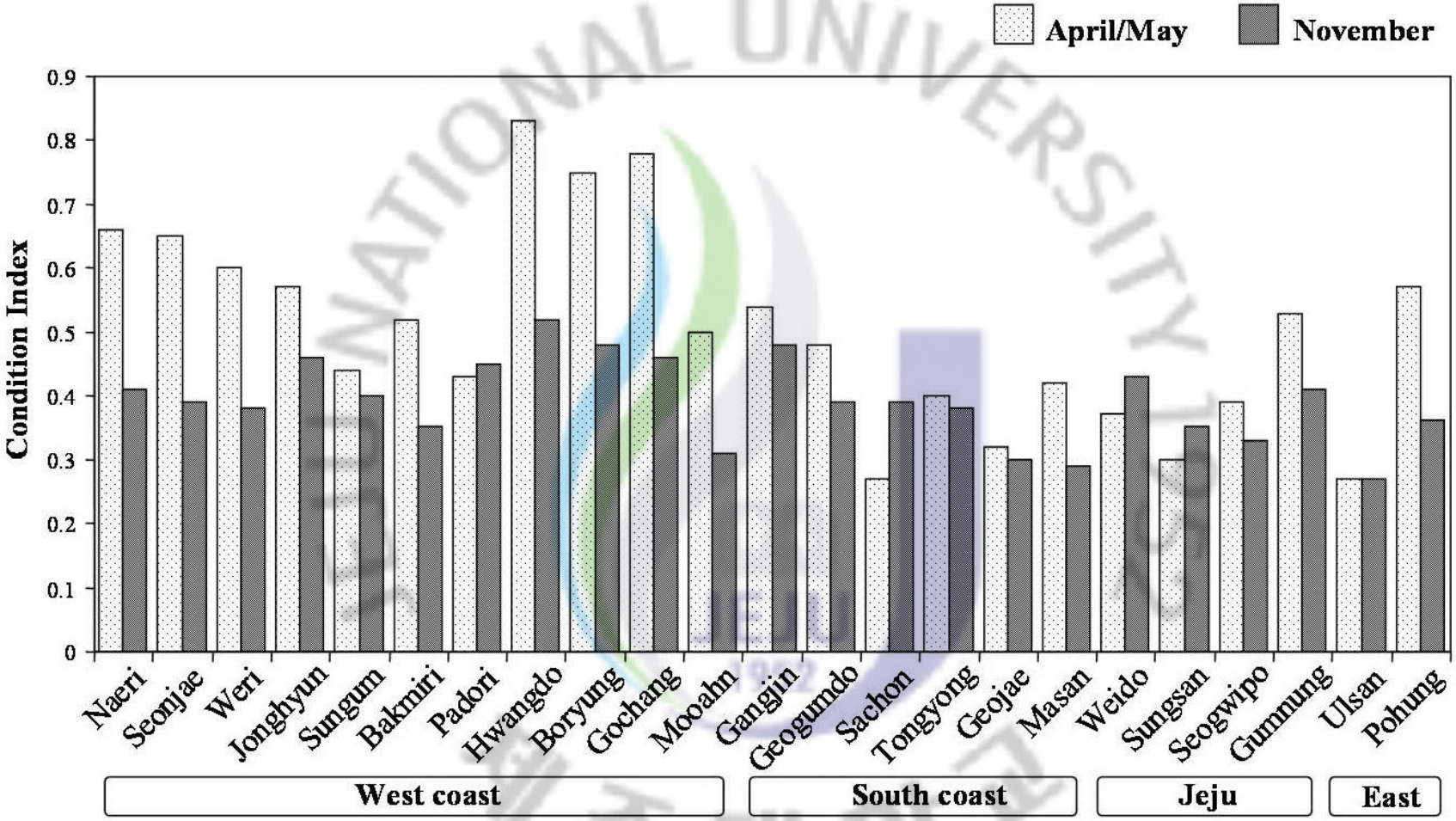

Figure 1-7 The condition index of Manila clam *Ruditapes philippinarum* collected in April or May and November from 23 sampling site.

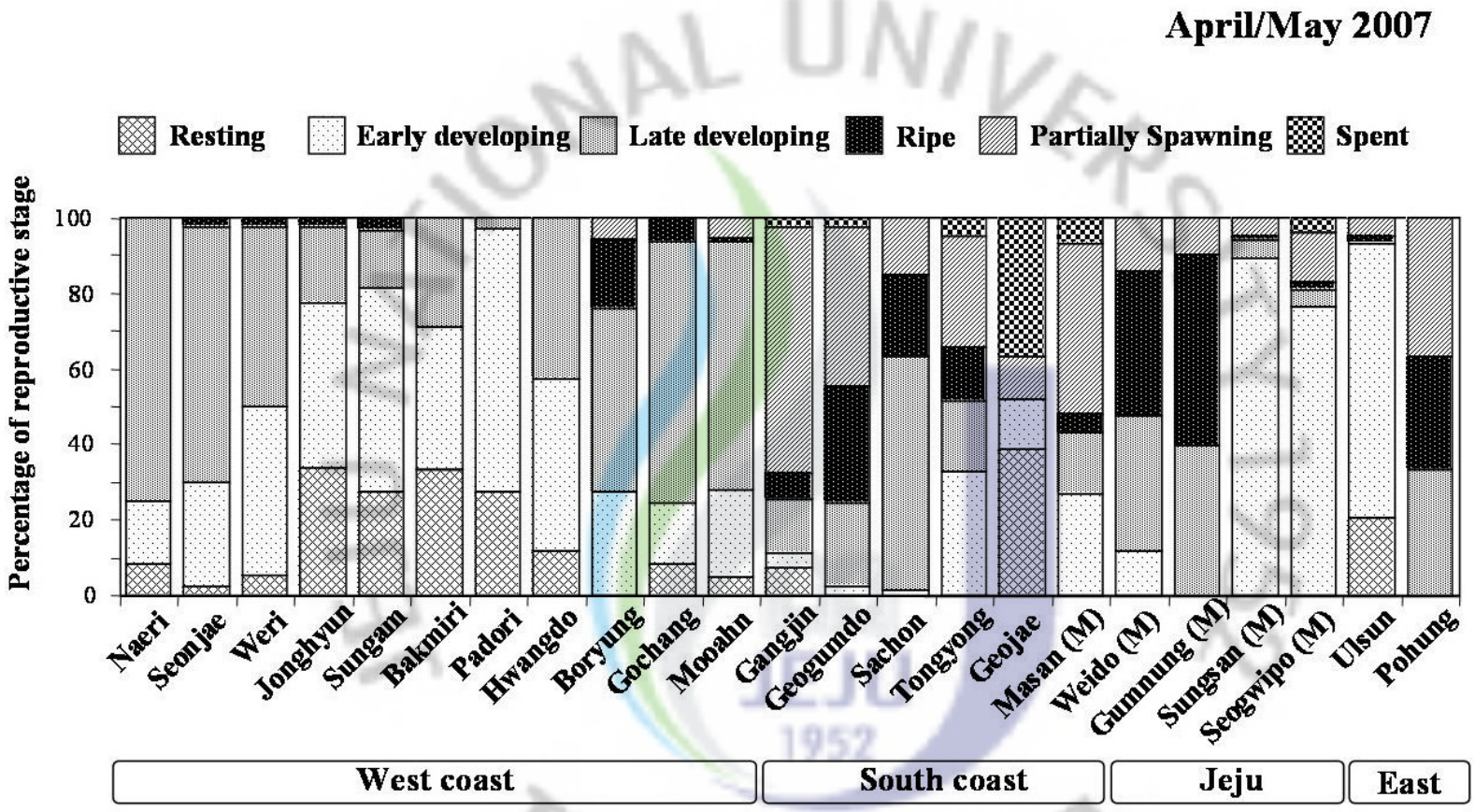

Figure 1-8 The percentage of reproductive stage of Manila clam *Ruditapes philippinarum* collected from 23 sampling sites in Korea during April or May (M) 2007.

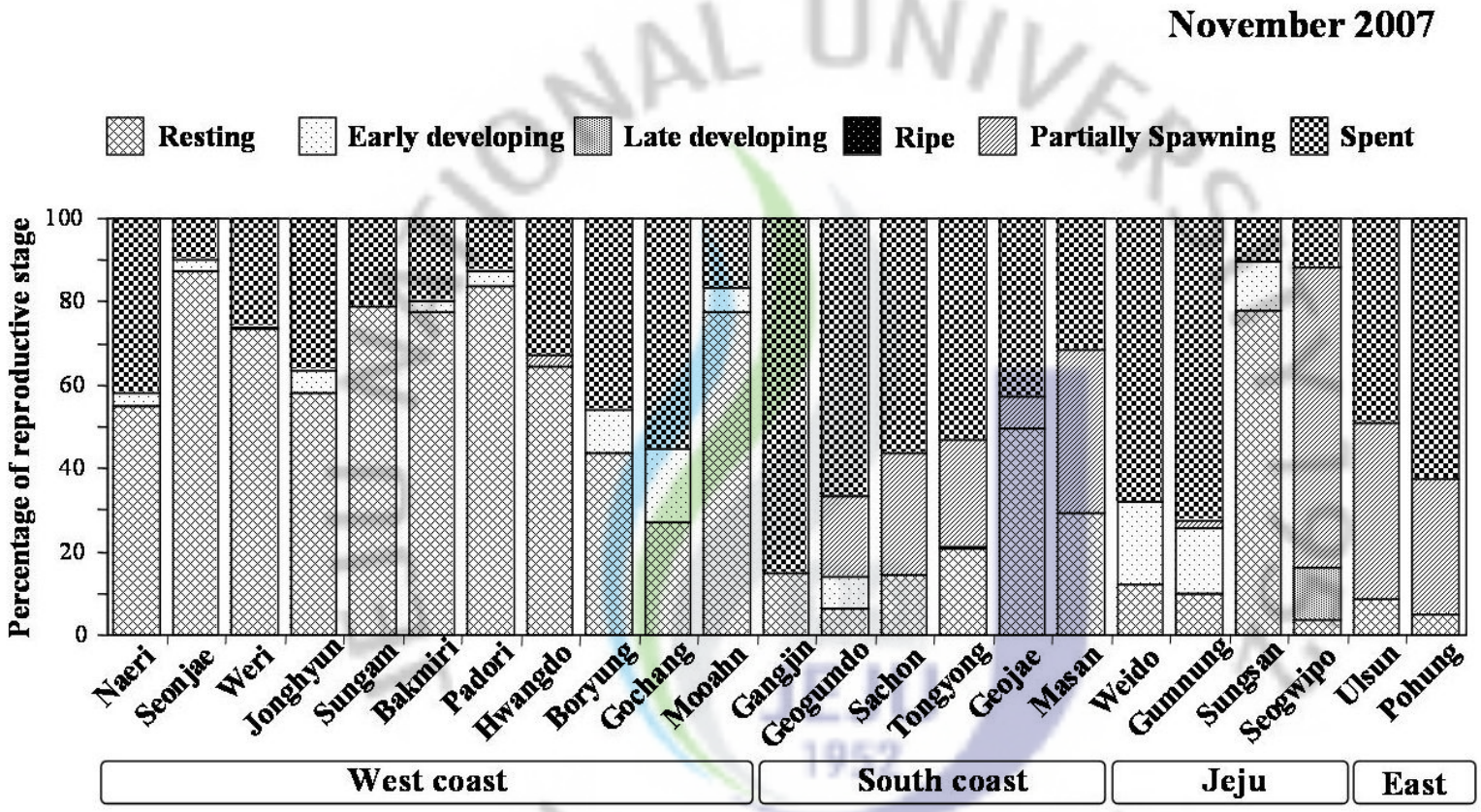

Figure 1-9 The percentage of reproductive stage of Manila clam *Ruditapes philippinarum* collected from 23 sampling sites in Korea during November 2007.

3. Parasite infestation

The examination of histological sections reveals the occurrences of microorganism, protistan and metazoan parasites of Manila clams. A summary of parasites observed in Manila clam inhabited in Korean waters during the year 2007 and the organ or tissue parasitized were demonstrated in table 1-7. The commonest parasites found in the histological samples were *Perkinsus* sp. and gonad cercaria. The prevalence (%) of parasite infection was varied in each sampling site and sampling period (Table 1-8).

Perkinsus

In histological sections, the trophozoite of *Perkinsus* sp. was distinguished by the spherical uninucleated cells with an obvious large eccentric vacuole occupying most of cell volumn whereas the basophilic nucleus was peripheral (Fig. 1-9A). The number of trophozoites which cell division was frequently observed was enclosed in the capsule. The parasite capsule normally was surrounded by the layers of hemocyte that was different in each sampling site. The host cellular response occasional occupied a large area of tissue. The distribution of the trophozoites was generally found in connective tissue of gill lamella, mantle, digestive gland, digestive tract and gonad (Fig 1-9B-F). From table 1-9, among the organ observed, the number of *Perkinsus* was found in higher density in gill and mantle. The heavy infiltration of *Perkinsus* in gill cause a deformation of gill lamella and fusion of gill lamella was observed. The trophozoite and hemocyte accumulation caused increasing of mantle tissue thickness. In most case, the varied size of *Perkinsus* capsule in digestive gland connective tissue were diffused and generalized hemocyte infiltration was common. The large group of *Perkinsus* and the host defense reaction sometime compressed the digestive gland tubule and cause the tubule deformation. *Perkinsus* trophozoite can be found both in gonad follicle and in

the interfollicular connective tissue and took place the gametogenesis follicles. In case of heavy infection the trophozoite could be observed in foot muscle (Fig. 1-10A). High number of *Perkinsus* and hemocyte accumulation between muscle fiber underlying the integument epithelium induced the elevation of integument surface thus the nodule can be observed even in gross examination (Fig. 1-10B). Beside of the connective tissue, the trophozoite was also rarely located extracellularly in the digestive tract epithelium (Fig. 1-10C). In acute phase of infection, the distribution of trophozoite capsule in Manila clam tissue was found scatter in connective tissue surrounded by generalized hemocyte infiltration (Fig. 1-10D). While in chronic inflammation, focal accumulation of hemocytes was form. Large encapsulation composed of numerous trophozoite inside due to continuous cell division was observed (Fig. 1-10E). The eosinophilic ground substance was often observed especially in chronic infestation. A granuloma-like lesion was formed by an arrangement of several layers of hemocyte which developed epithelial-like appearance and fibrous tissue (Fig. 1-10E-F). The tissue damage associated with large size granuloma was often observed (Fig. 1-10F).

The prevalence of *Perkinsus* infection observed in this study was varied from 0-100% (Table 1-9 and Fig. 1-11). Gumnung and Ulsan were the locations which not observed the trophozoite in histology. Among the sampling sites in the west coast region, Padori was the only one site which has low prevalence of *Perkinsus* infection. In south coast area, every sampling site showed high prevalence while low prevalence of *Perkinsus* was detected from Jeju Island except in Sungsan. There was no clear seasonal difference of *Perkinsus* prevalence in April or May and November. Gangjin was the only one site which had a big difference of *Perkinsus* prevalence of in April and November. The highest prevalence in April was found in Hwangdo and Geogumdo (100%) followed by Sachon and Mason. In November the highest prevalence was found in Boryung, Mooahn and Sungsan (100%) followed by Masan and

Gochang.

The intensity of *Perkinsus* infection was described in individual organ, gill, mantle, digestive gland, digestive tract, gonad and foot muscle were shown in table 1-9. The average of infection intensity was shown in figure 1-12. The high prevalence sampling site was also detected high intensity of infection except in Sungsan. Manila clams from Padori, Weido, Seogwipo and Pohung showed low intensity of infection. Among the individual organs which determined the infection intensity (gill, mantle, digestive gland, digestive tubule, gonad and foot muscle), foot muscle were an area where less *Perkinsus* trophozoite could be found while gill and mantle seem to be observed the higher number of *Perkinsus*. Most of the clams which had low intensity of infection were not observed *Perkinsus* in foot muscle.

Cercaria infestation in gonad

The trematode observed in gonad follicle used Manila clams as and first intermediate host. The intensity of infection was highest in Jonghyun and Seonjae in April and November respectively (Fig. 1-15). The growth stage of trematode observed in this study was germ ball, sporocyst and cercaria (Fig. 1-13A-D). The individual sample can be observed more than one stage of parasite. Most of the infected clam contained with sporocyst which contained germ ball or cercaria inside while the germ ball without mother sheath was the stage that rarely found in our histology slides (Table 1-10).

The gonad trematode at least 3 species were observed in this study by needle biopsy from gonad of fresh Manila clam. Even though the trematode species was not identified but the obvious 3 different types of cercariae was detected (Fig. 1-14). The Gymnocephalous cercaria (Fig. 1-14A) had long straight slender tails, the body part consisted of 2 suckers and the 2 eye spots were easily observed. The Gymnocephalus cercaria develop within radiae which

the shape was sausage-like and had blunt end (Fig. 1-14B). The trichocercous cercariae also had long tail that the integument composed of many long thin spines or bristles. The tail that connected to the body part was larger and smaller to the end. The two suckers, oral and ventral, were observed (Fig. 1-14C). The radia of this cercaria type was similar to Gymnocephalous cercaria. The Manila clams infected with this cercaria type showed the orange colour on the body due to the parasite pigment (Fig. 1-14D). Furcocercous type of cercariae was the smallest cercariae among 3 types of cercaria found in our study. The tails are forked at the end which each side was short and thin (Fig. 1-14E). The radia of furcocercous cercaria was conical shape which has one blunt end and one sharp end on the mouth part (Fig 1-14F). As the size of furcocercous cercaria was small and short tail, in one radia there was much more number of cercaria compare with the other 2 cercaria types.

From histology, the sporocysts of trematode were distributed in interfollicular space of gonad tissue. As the sporocyst grew, the follicles adjacent to the parasites were collapsed from the mechanical pressure (Fig. 1-13B-E). The small irregular shapes of follicles were observed (Fig. 1-13D). Heavy sporocyst infiltration caused complete castration of the clams therefore the gonad follicle was not observed. The eosinophilic old lesion of dead parasite can be founded with hemocyte aggregation (Fig. 1-13E). In heavy infestations, trematode can be found in other organs such as gill (Fig. 1-13F), foot muscle (Fig. 1-13G), digestive gland (Fig. 1-13H) and digestive tract connective tissue. The variations of the occurence of trematode in each developing stage and the distribution to other tissues were described in Table 1-10. The prevalence of infection was varied from 0-42.5% (Table 1-10). Weido and Gumnung were the sites which cercaria infestation in both sampling periods was not observed. The highest prevalence was found in Weri and the second was Bakmiri in November. Most of sampling site, the prevalence of cercaria was increase in November (Fig. 1-15).

Table 1-7 The parasites observed in Manila clam *Ruditapes philippinarum* landing in Korea collected in spring and autumn 2007.

Parasite	Organ or tissue parasitized	Host defense reaction and pathological affect
Perkinsus sp.	Mainly in connective tissue of gill, mantle, digestive gland, digestive tract, also between foot muscle fiber. Rare in intestinal epithelium	Various degree of hemocyte infiltration. Granuloma-like lesion and nodule formation of the integument
Trematode sporocyst and cercaria	Mainly in both male and female gonad and also connective tissue of digestive gland, digestive tract and gill. Rare in intestinal epithelium	Hemocyte infiltration of death absorbed parasite. Gonad castration and disruption of surrounding tissue by parasite growth
Trematode metacercaria	Mantle cavity	Mantle hyperplasia
Turbellarian	Stomach or intestinal lumen	No sign of host defense
Rickettsia-like organism	Digestive gland epithelium	No sign of host defense, Rupture of epithelium cells
Gregarine-like organism	Digestive tract epithelium and connective tissue	No sign of host defense
Marteilioides chungmuensis	Oocyte cytoplasm	Hemocyte infiltration was questionable due to host defense or normal phenomenon of spent stage
Copepod	Stomach and intestinal lumen	No sign of host defense, local damage of digestive tract epithelium
Larval cestode	Connective tissue around digestive tract, between muscle fiber	Encapsulation
Unidentified encysted parasite	Connective tissue around digestive tract, between muscle fiber	Encapsulation, hemocyte infiltration

Table 1-8 Prevalence (%) of parasites of Manila clam *Ruditapes philippinarum* collected in April/May and November 2007

Sampling site	Sampling period	*Perkinsus*	Trematode Cercaria	Trematode Metacercaria	Turbellarian	Rickettsia-like organism	Gregarine-like organism	Marteilioides	Copepod	Larval cestode	Unidentified encysted parasite
1. Naeri	Apr 07	77.5	15.0	5.0	35.0	12.5	2.5	0	2.5	0	2.5
	Nov 07	95.0	15.0	12.5	5.0	2.5	0	0	12.5	0	0
2. Seonjae	Apr 07	82.5	7.5	5.0	27.5	0	0	0	0.0	0	2.5
	Nov 07	60.0	15.0	5.0	1.0	0	1.0	0	1.0	0	0
3. Weri	Apr 07	48.7	10.3	12.5	20.5	0	2.5	0	2.5	0	0
	Nov 07	60.0	42.5	17.5	0	2.5	0	0	10.0	0	0
4. Jonghyun	Apr 07	69.2	5.1	23.1	20.5	7.7	2.6	0	10.3	0	2.6
	Nov 07	40.0	25.0	22.5	0	2.5	0	0	5.0	0	0
5. Sungum	Apr 07	80.0	2.5	25	27.5	10.0	2.5	0	2.5	0	10.0
	Nov 07	62.5	20.0	30	0	0	2.5	0	0	0	2.5
6. Bakmiri	Apr 07	60.0	30.0	5	15	5.0	5.0	0	0	0	0
	Nov 07	75.0	32.5	20	0	2.5	0	0	0	0	2.5
7. Padori	Apr 07	15.0	0	5	5	0	15.0	0	0	0	0
	Nov 07	22.5	7.5	12.5	5	0	0	0	12.5	0	0
8. Hwangdo	Apr 07	100.0	5.0	17.5	0	2.5	2.5	0	0	5.0	2.5
	Nov 07	90.0	17.5	32.5	0	2.5	0	0	0	0	0
9. Boryung	Apr 07	97.5	0	17.5	0	17.5	0	0	0	0	2.5
	Nov 07	100.0	12.5	37.5	2.5	0	2.5	0	2.5	0	5.0

Table 1-8 (continued) Prevalence (%) of parasites of Manila clam *Ruditapes philippinarum* collected in April/May and November 2007

Sampling site	Sampling period	*Perkinsus*	Trematode Cercaria	Trematode Metacercaria	Turbellarian	Rickettsia-like organism	Gregarine-like organism	Marteilioides	Copepod	Larval cestode	Unidentified encysted parasite
10. Gochang	Apr 07	82.5	10.0	5	0	2.5	0	0	2.5	0	0
	Nov 07	97.5	5.0	30	2.5	2.5	0	0	5.0	0	0
11. Mooahn	Apr 07	90.0	7.5	15	0	0	0	0	0	10	17.5
	Nov 07	100.0	2.5	10	0	5.0	10.0	0	0	0	5.0
12. Gangjin	Apr 07	90.0	10.0	5	0	2.5	0	2.5	2.5	0	10.0
	Nov 07	27.5	5.0	22.5	0	2.5	0	0	0	0	0
13.Geogumdo	Apr 07	100.0	7.5	5	0	2.5	5.0	0	10.0	0	2.5
	Nov 07	90.0	5.0	20	0	5.0	0	2.5	0	0	2.5
14. Sachon	Apr 07	97.5	2.5	10	5	2.5	0	0	0	0	0
	Nov 07	80.0	10.0	10	0	17.5	0	2.5	2.5	2.5	0
15. Tongyong	Apr 07	52.5	20.0	2.5	2.5	5.0	0	0	0	0	7.5
	Nov 07	80.0	5.0	0	5	0	0	5	15	0	0
16. Geojae	Apr 07	74.4	0	0	7.5	0	0	0	2.5	0	5.0
	Nov 07	77.5	2.5	5	15	10.0	5.0	5	12.5	0	2.5
17. Masan	May 07	97.5	17.5	5	2.5	5.0	7.5	0	0	0	2.5
	Nov 07	97.5	22.5	7.5	5	2.5	17.5	0	7.5	0	0
18. Weido	May 07	2.5	0	0	7.5	0	0	0	0	0	0
	Nov 07	0	0	0	0	0	0	0	0	0	0

Table 1-8 (continued) Prevalence (%) of parasites of Manila clam *Ruditapes philippinarum* collected in April/May and November 2007

Sampling site	Sampling period	*Perkinsus*	Trematode Cercaria	Trematode Metacercaria	Turbellarian	Rickettsia-like organism	Gregarine-like organism	Marteilioides	Copepod	Larval cestode	Unidentified encysted parasite
19. Sungsan	May 07	95.0	7.5	5	0	12.5	0	0	0	0	0
	Nov 07	100.0	10.0	5	2.5	12.5	2.5	0	0	0	0
20. Seogwipo	May 07	15.0	2.5	0	2.5	2.5	0	0	2.5	0	0
	Nov 07	12.5	0	0	5	10	0	0	5	0	0
21. Gumnung	May 07	0.0	0	0	10	0	0	0	0	0	0
	Nov 07	0.0	0	0	2.5	5	0	0	5	0	0
22. Ulsan	Apr 07	0.0	7.5	2.5	2.5	12.5	0	0	0	0	0
	Nov 07	0.0	0	7.5	5	7.5	0	0	2.5	0	0
23. Pohung	Apr 07	0.0	2.5	0	0	5	0	0	7.5	0	0
	Nov 07	2.5	2.5	0	0	5	2.5	0	2.5	0	0

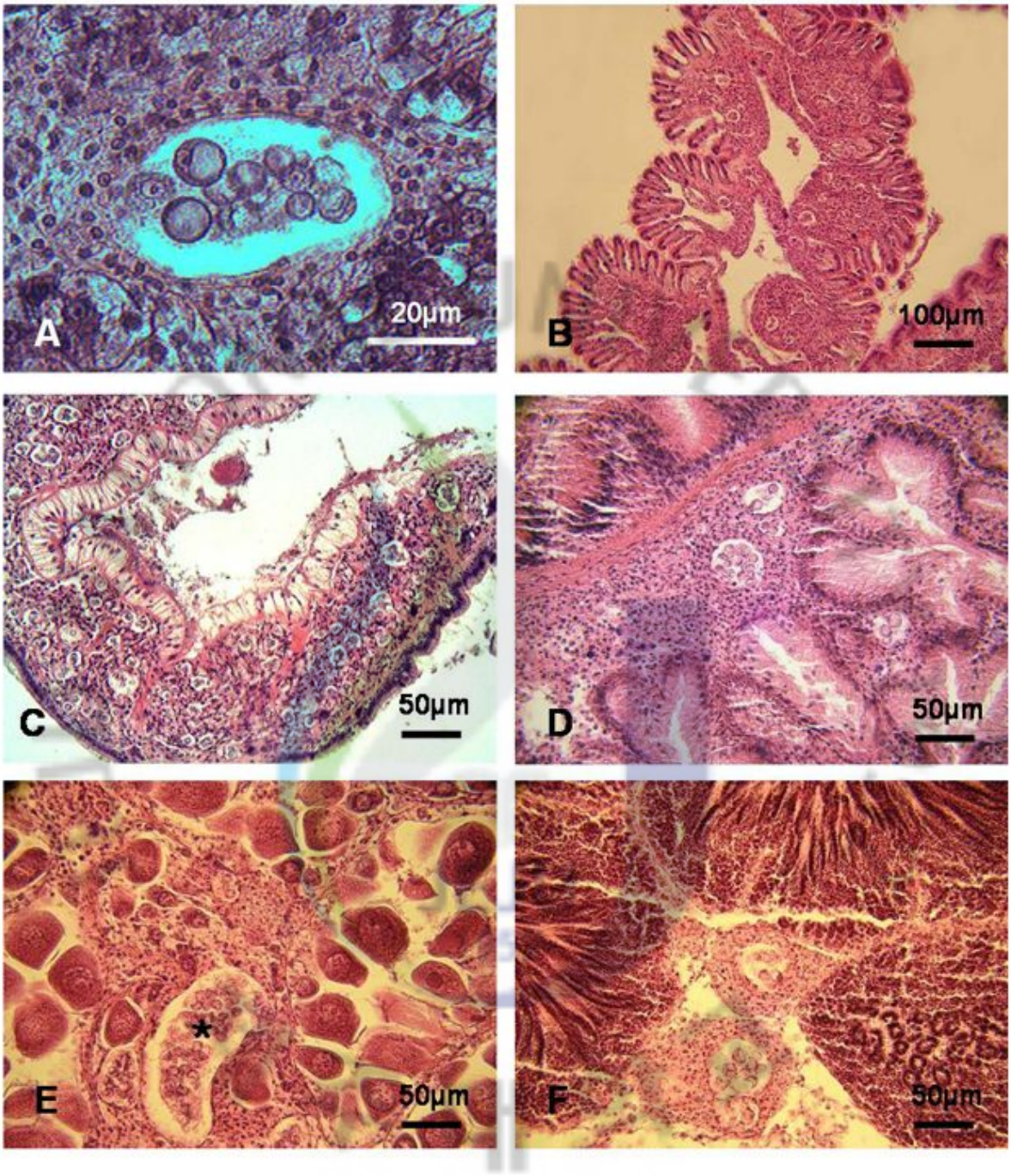

Figure 1-10 Histological sections of Manila clam infected by *Perkinsus* trophozoite (H&E). 1-10 A: *Perkinsus* trophozoite characterized by the spherical uninucleated cells with large eccentric vacuole. 1-10B: *Perkinsus* trophozoite in gill connective tissue. 1-10C: *Perkinsus* infiltrated in mantle, noted that the thickness of mantle was increase. 1-10D: *Perkinsus* in digestive gland connective tissue. 1-10E: Large group of *Perkinsus* (*) in female gonad follicle. 1-10F: *Perkinsus* was surrounded by several layers of hemocyte in interfollicular connective tissue of male clam.

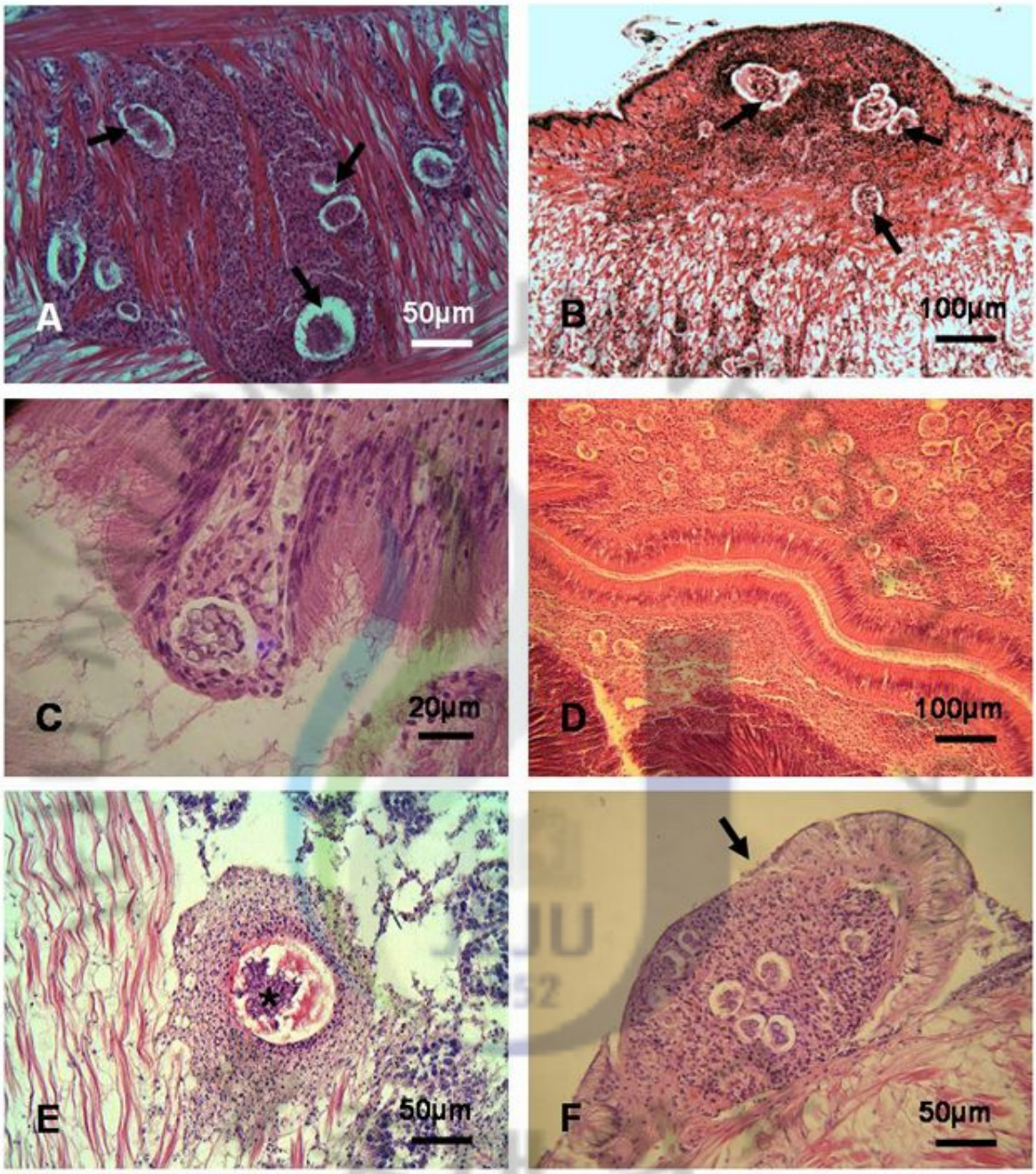

Figure 1-11 Histological sections of Manila clam infected by *Perkinsus* trophozoite (H&E). 1-11A: The lysed *Perkinsus* cells (arrow) infiltrated in foot muscle caused necrosis of muscle fibers. 1-11B: The nodule formation due to high number of Perkinsus (arrows). 1-11C: Trophozoite in extracellular of digestive tract epithelium. 1-11D: Acute *Perkinsus* infection, noted the diffuse of trophozoite in tissue and generalized hemocyte infiltration. 1-11E: Chronic infection, noted the large encapsulation contained with numerous trophozoites (*). 1-11F: The disruption of mantle epithelium (arrow) by the granuloma.

Table 1-9 Prevalence, Weighted prevalence and Infection intensity of *Perkinsus* sp. infection in Manila clams from 23 sampling site in Korea
Prevalence=no. of clams infected by *Perkinsus*/Total no. of clam, Weighted prevalence=Total score of *Perkinsus* infection/ Total no. of clam
Infection intensity=Total score of *Perkinsus* infection/Number of clams infected

Sampling site	Sampling period	Prevalence	Weighted prevalence						Infection intensity						
			gill	mantle	DG	DT	gonad	muscle	gill	mantle	DG	DT	gonad	muscle	mean*
1. Naeri	Apr 07	77.5	1.1	1.1	0.7	0.5	0.6	0.0	1.57	1.73	1.35	1.25	1.56	-	1.49
	Nov 07	95.0	1.2	1.5	1.3	0.9	0.8	0.1	1.33	1.69	1.51	1.24	1.22	1.00	1.33
2. Seonjae	Apr 07	82.5	1.2	1.5	0.7	0.5	0.4	0.1	1.54	2.00	1.38	1.40	1.45	2.00	1.63
	Nov 07	60.0	0.8	0.7	0.7	0.5	0.4	0.0	1.50	1.71	1.50	1.31	1.31	1.00	1.39
3. Weri	Apr 07	48.7	0.5	0.6	0.4	0.3	0.3	0.0	1.20	1.40	1.50	1.11	1.09	-	1.26
	Nov 07	60.0	0.6	0.0	0.5	0.2	0.4	0.0	1.00	-	1.11	1.00	1.25	-	1.09
4. Jonghyun	Apr 07	69.2	0.5	0.7	0.5	0.4	0.3	0.1	1.20	1.60	1.45	1.15	1.20	1.00	1.27
	Nov 07	40.0	0.5	0.4	0.3	0.3	0.3	0.0	1.56	1.22	1.50	1.10	1.50	1.00	1.31
5. Sungum	Apr 07	80.0	0.8	1.4	0.9	0.7	0.7	0.1	1.47	1.88	1.52	0.89	0.95	2.00	1.45
	Nov 07	62.5	0.5	0.8	0.8	0.6	0.5	0.0	1.25	1.29	1.50	1.35	1.43		1.36
6. Bakmiri	Apr 07	60.0	1.1	1.6	0.7	0.6	0.6	0.2	1.94	2.55	1.61	1.38	1.69	1.20	1.73
	Nov 07	75.0	1.4	1.8	1.1	0.8	0.7	0.3	2.47	2.58	1.83	1.72	1.81	1.67	2.01
7. Padori	Apr 07	15.0	0.1	0.1	0.1	0.1	0.1	0.0	1.00	1.00	1.00	1.00	1.00	-	1.00
	Nov 07	22.5	0.0	0.2	0.1	0.0	0.0	0.0	1.00	1.00	1.00	1.00	-	-	1.00
8. Hwangdo	Apr 07	100.0	2.9	2.9	2.4	1.9	2.2	1.1	2.90	2.90	2.38	1.95	2.35	1.54	2.33
	Nov 07	90.0	2.3	2.3	1.6	1.2	1.3	0.2	2.31	2.46	1.91	1.53	1.79	1.33	1.89
9. Boryung	Apr 07	97.5	1.9	2.4	1.0	0.7	0.7	0.3	2.00	2.40	1.18	1.08	1.22	1.22	1.52
	Nov 07	100.0	2.5	2.8	2.4	1.9	2.3	1.1	2.48	2.81	2.43	1.97	2.30	1.83	2.30

Table 1-9 (continued) Prevalence, Weighted prevalence and Infection intensity of *Perkinsus* sp. infection in Manila clams from 23 sampling site in Korea. Prevalence=no. of clams infected by *Perkinsus*/Total no. of clam, Weighted prevalence=Total score of *Perkinsus* infection/ Total no. of clam Infection intensity=Total score of *Perkinsus* infection/Number of clams infected

Sampling site	Sampling period	Prevalence	Weighted prevalence						Infection intensity						
			gill	mantle	DG	DT	gonad	muscle	gill	mantle	DG	DT	gonad	muscle	mean*
10. Gochang	Apr 07	82.5	1.4	1.6	0.6	0.4	0.3	0.0	1.68	2.10	1.41	1.31	1.33	1.00	1.47
	Nov 07	97.5	2.1	2.4	1.9	1.5	1.7	0.5	2.09	2.43	2.00	1.59	1.89	1.62	1.94
11. Mooahn	Apr 07	90.0	2.0	2.2	1.2	1.0	0.9	0.3	2.17	2.43	1.60	1.65	1.75	1.71	1.89
	Nov 07	100.0	2.6	2.5	2.5	2.4	2.6	1.5	2.63	2.53	2.50	2.40	2.63	1.90	2.43
12. Gangjin	Apr 07	90.0	1.6	1.7	0.9	0.9	0.8	0.2	1.88	2.21	1.38	1.33	1.48	1.00	1.54
	Nov 07	27.5	0.1	0.2	0.2	0.1	0.1	0.0	1.00	1.00	1.29	1.00	1.00	-	1.06
13.Geogumdo	Apr 07	100.0	2.3	2.7	1.9	2.0	2.0	0.9	2.28	2.68	2.10	2.11	2.13	1.71	2.17
	Nov 07	90.0	2.4	2.4	1.8	1.7	1.9	1.2	2.50	2.52	2.09	1.97	2.11	2.28	2.24
14. Sachon	Apr 07	97.5	1.4	1.6	1.2	0.8	0.6	0.1	1.55	1.82	1.34	1.27	1.14	1.25	1.40
	Nov 07	80.0	1.0	1.4	0.8	0.5	0.6	0.1	1.30	1.83	1.22	1.00	1.21	1.25	1.30
15. Tongyong	Apr 07	52.5	0.3	0.3	0.5	0.3	0.3	0.0	1.11	1.17	1.33	1.22	1.25	1.00	1.18
	Nov 07	80.0	0.8	1.3	1.0	0.6	0.5	0.1	1.56	1.85	1.70	1.16	1.43	1.33	1.50
16. Geojae	Apr 07	74.4	1.0	1.4	0.6	0.3	0.2	0.0	1.60	2.29	1.11	1.10	1.13	1.00	1.37
	Nov 07	77.5	0.9	1.8	0.9	0.6	0.7	0.2	1.44	2.35	1.57	1.20	1.22	1.17	1.49
17. Masan	May 07	97.5	2.3	2.6	1.9	1.8	1.7	0.8	2.34	2.63	1.92	2.03	1.89	1.63	2.07
	Nov 07	97.5	2.4	2.5	1.9	1.4	1.6	0.9	2.37	2.67	2.06	1.49	1.97	1.65	2.03
18. Weido	May 07	2.5	0	0.4	0	0	0	0	-	1.00	-	-	-	-	1.00
	Nov 07	0	0	0	0	0	0	0	-		-	-	-	-	-

Table 1-9 (continued) Prevalence, Weighted prevalence and Infection intensity of *Perkinsus* sp. infection in Manila clams from 23 sampling site in Korea. Prevalence=no. of clams infected by *Perkinsus*/Total no. of clam, Weighted prevalence=Total score of *Perkinsus* infection/ Total no. of clam Infection intensity=Total score of *Perkinsus* infection/Number of clams infected

Sampling site	Sampling period	Prevalence	Weighted prevalence						Infection intensity						
			gill	mantle	DG	DT	gonad	muscle	gill	mantle	DG	DT	gonad	muscle	mean*
19. Sungsan	May 07	95.0	1.8	2.2	1.7	1.1	1.2	0.5	1.97	2.47	1.94	1.40	1.84	1.43	1.84
	Nov 07	100.0	1.3	1.6	1.2	0.9	1.0	0.2	1.94	1.94	1.45	1.44	1.52	1.29	1.60
20. Seogwipo	May 07	15.0	0	0.4	0	0.1	0	0	1.00	1.67		1.00	-	-	1.22
	Nov 07	12.5	0.1	0.3	0.1	0.1	0	0	1.00	1.00	1.25	1.00	-	-	1.06
21. Gumnung	May 07	0	0	0	0	0	0	0	-	-	-	-	-	-	-
	Nov 07	0	0	0	0	0	0	0	-	-	-	-	-	-	-
22. Ulsan	Apr 07	0	0	0	0	0	0	0	-	-	-	-	-	-	-
	Nov 07	0	0	0	0	0	0	0	-	-	-	-	-	-	-
23. Pohung	Apr 07	0	0	0	0	0	0	0	-	-	-	-	-	-	-
	Nov 07	2.5	0	0	0	0	0	0	-	-	-	1.00	-	-	1.00

* The average of infection intensity from every observed organ

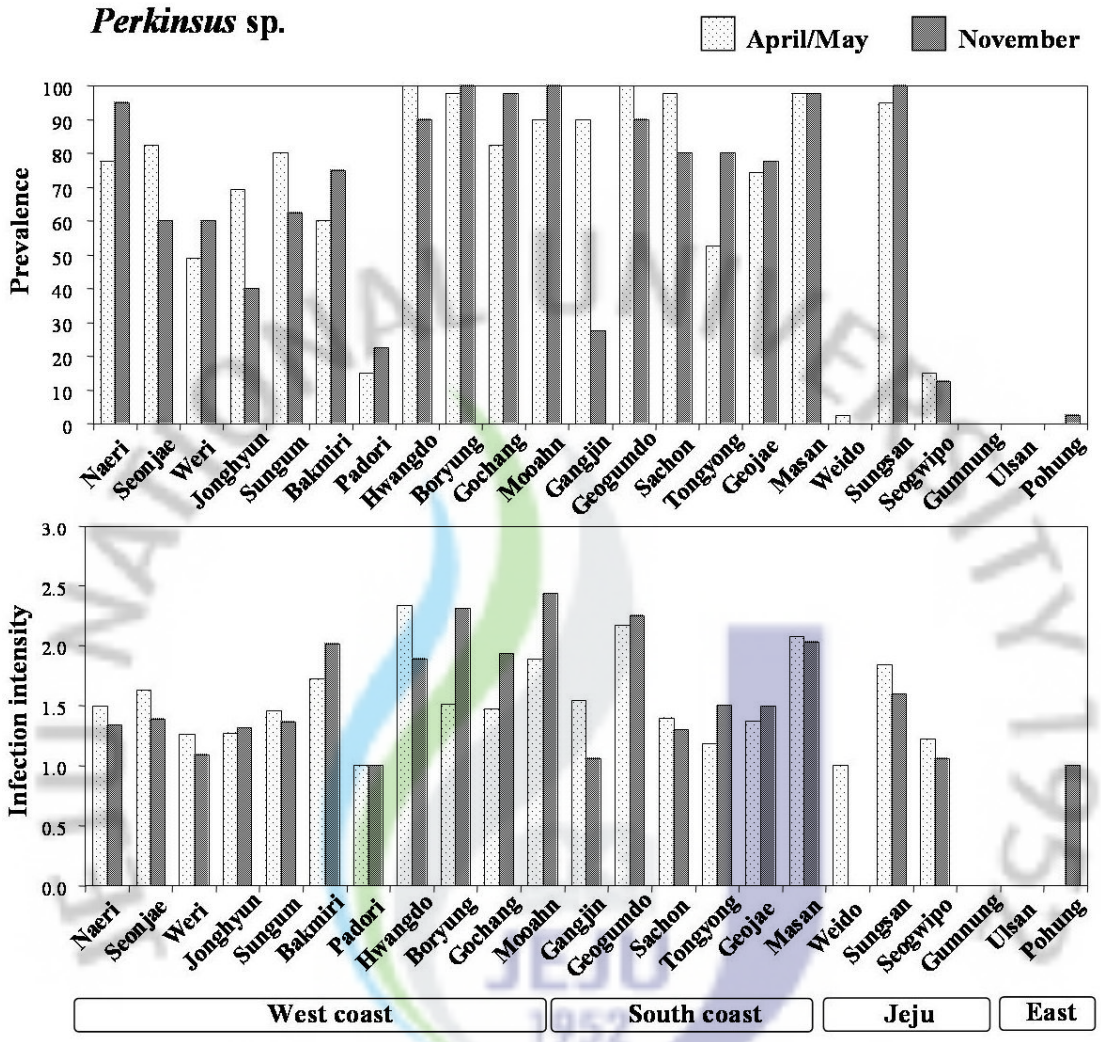

Figure 1-12 The prevalence and intensity of *Perkinsus* infection in Manila clams collected from 23 clam beds in Korea

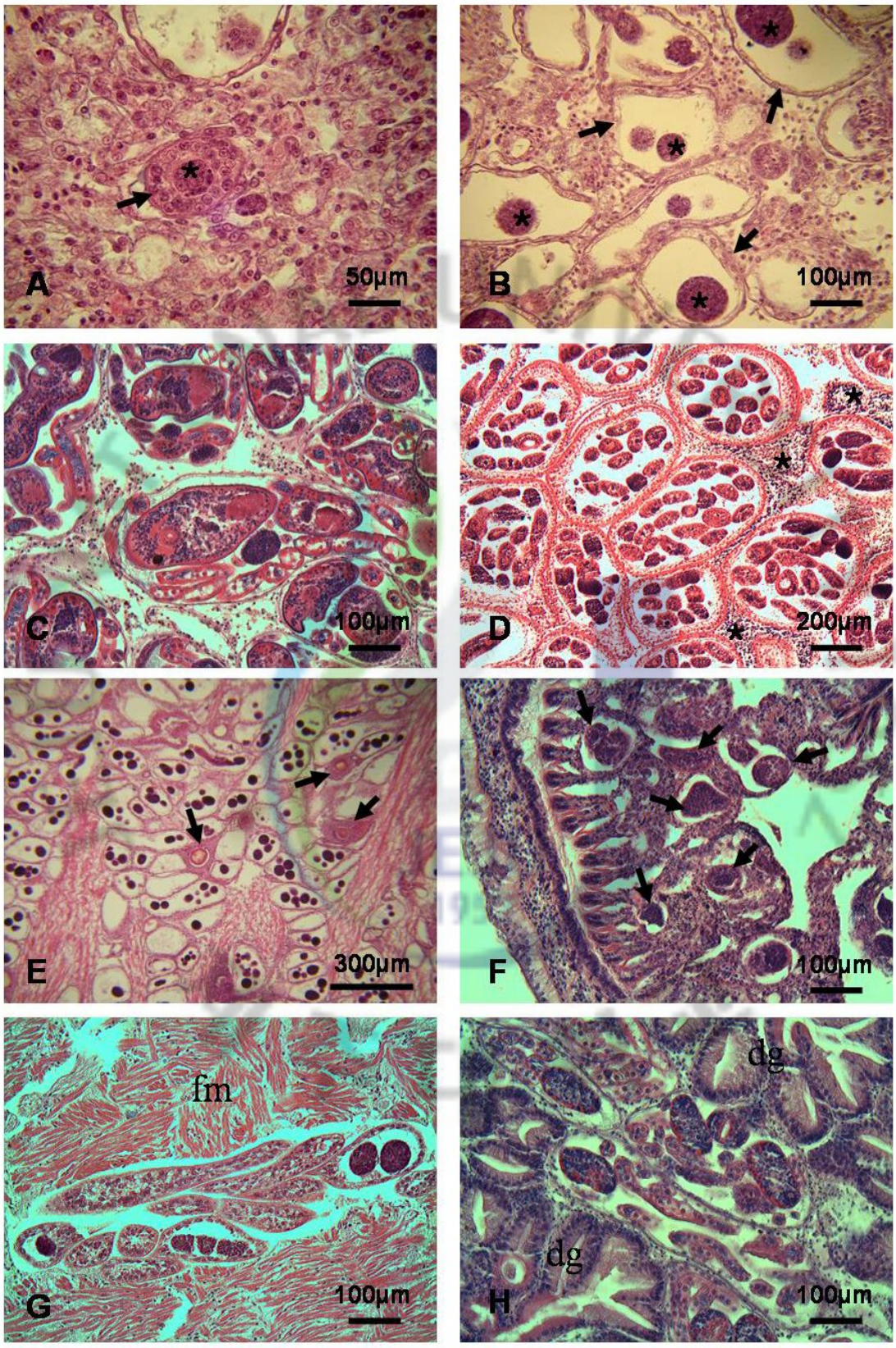

Figure 1-13

Figure 1-13 Histological sections of Manila clam infected by cercaria in gonad (H&E). 1-13A: The transverse section through germ tube shows the germ ball (*) inside the cuticle (arrow) which lined with muscle fiber. 1-13B: The early develop of sporocyte (arrow) showed the growing germ balls (*) inside. 1-13C: The mature cercaria, noted the body and tail part inside the mother sac. 1-13D: The heavy infestation of cercaria caused gonad castration, only the small irregular shape of gonad follicle was observed (*). 1-13E: The eosinophilic lesion of death parasite (arrows) was surrounded by hemocyte accumulation. The sporocyst of trematode can be found in other organs of high infected clam. 1-13F: Sporocysts (arrows) in gill connective tissue. 1-13G: Sporocysts between muscle fibers. 1-13H: Mature cercaria in digestive gland (dg) connective tissue.

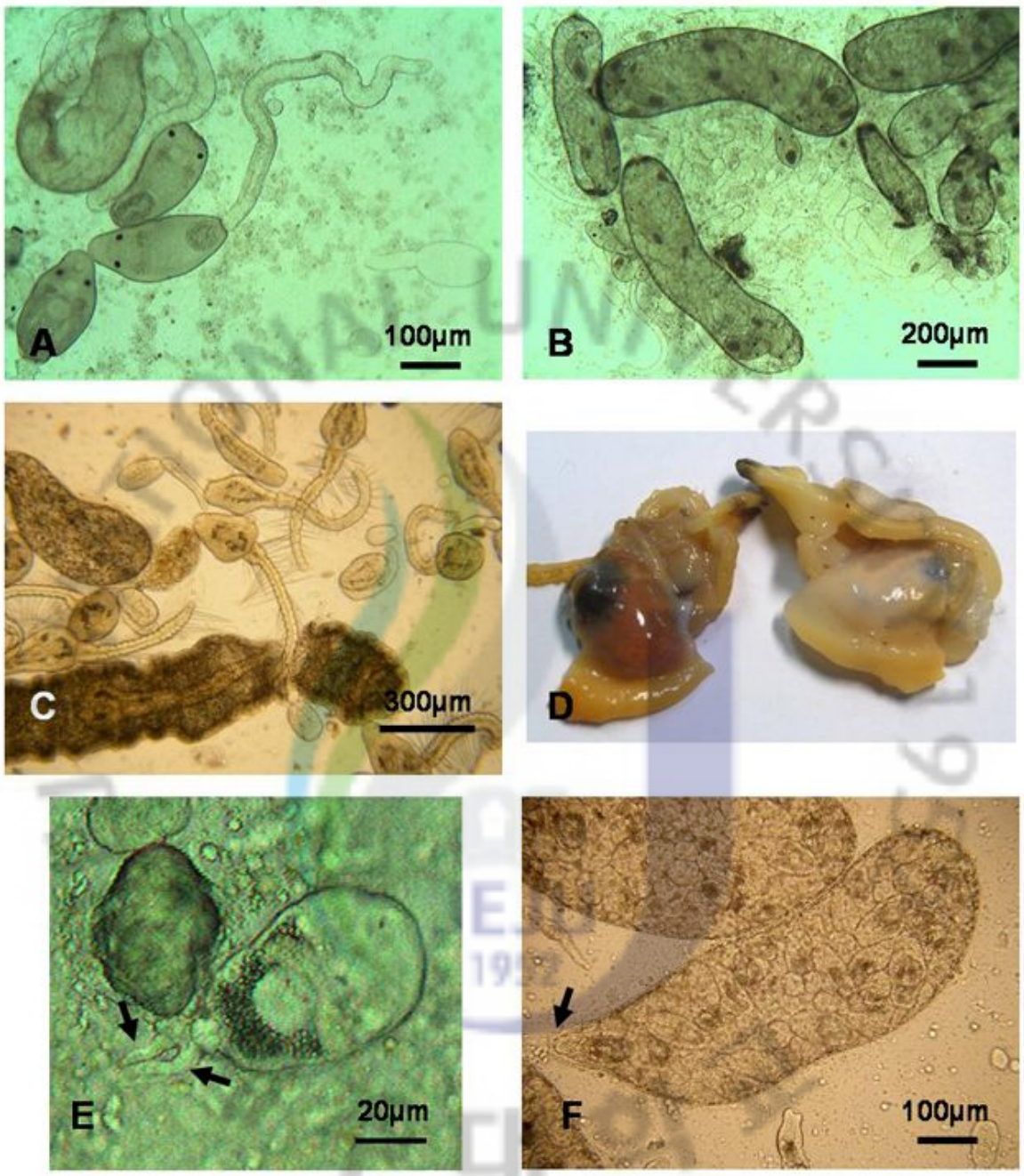

Figure 1-14 The needle biopsy of Manila clam gonad shows different type of cercaria. 1-14A: The Gymnocephalous cercaria, noted the straight long tail and two eye spots. 1-14B: The radia of Gymnocephalous cercaria was in sausage shape and blunted end. 1-14C: The trichocerrous cercaria has the long tail with thin spines along the tail. 1-14D: The Manila clam infested with trichocercous cercaria (left) showed the orange color of the body compared with normal clam (right). 1-14E: The furcocercous cercaria with the forked tail (arrows). 1-14F: The radia of furcocercous cercaria, noted the mouth part on the shape end (arrow).

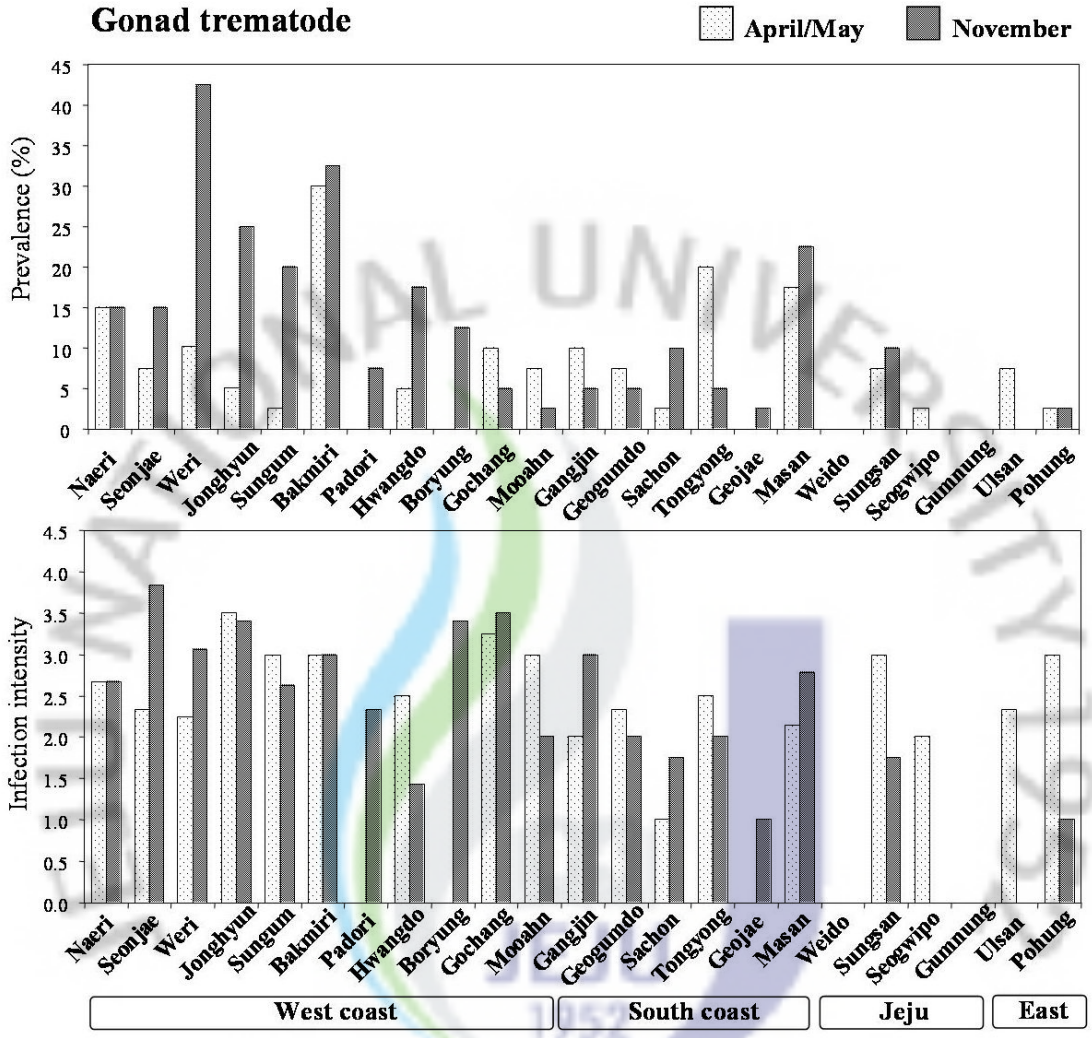

Figure 1-15 The prevalence and intensity of gonad trematode infection in Manila clams collected from 23 clam beds in Korea

Table 1-10 Prevalence, Weighted prevalence, Infection intensity, occurrence of each trematode stage and distribution in other organs of gonad trematode infection.

Sampling site	Sampling period	Prevalence	Weighted prevalence	Infection intensity	% Occurrence in infected clams			
					germ ball	sporocyst	cercaria	other tissues
1. Naeri	Apr 07	15.0	0.40	2.67	16.7	66.7	66.7	16.7
	Nov 07	15.0	0.40	2.67	0	66.7	33.3	83.3
2. Seonjae	Apr 07	7.5	0.17	2.33	0	100.0	100.0	66.7
	Nov 07	15	0.57	3.83	0	50.0	100.0	83.3
3. Weri	Apr 07	10.3	0.22	2.25	25.0	100.0	75.0	75.0
	Nov 07	42.5	1.30	3.06	0	35.3	76.5	64.7
4. Jonghyun	Apr 07	5.1	0.17	3.50	0	100.0	50.0	100.0
	Nov 07	25.0	0.85	3.40	0	10.0	90.0	60.0
5. Sungum	Apr 07	2.5	0.07	3.00	0	100.0	100.0	0
	Nov 07	20.0	0.52	2.63	0	62.5	75.0	50.0
6. Bakmiri	Apr 07	30.0	0.90	3.00	0	25.0	91.7	41.7
	Nov 07	32.5	0.97	3.00	15.4	30.8	61.5	53.9
7. Padori	Apr 07	0	0	-	-	-	-	-
	Nov 07	7.5	0.17	2.33	33.3	33.3	33.3	0
8. Hwangdo	Apr 07	5.0	0.12	2.50	0	100.0	50.0	0
	Nov 07	17.5	0.25	1.43	0	100.0	0	28.6
9. Boryung	Apr 07	0	0	-	-	-	-	-
	Nov 07	12.5	0.42	3.40	0	20.0	80.0	60.0
10. Gochang	Apr 07	10.0	0.32	3.25	0	50.0	100.0	50.0
	Nov 07	5.0	0.17	3.50	0	0	100.0	50.0
11. Mooahn	Apr 07	7.5	0.22	3.00	0	100.0	33.3	0
	Nov 07	2.5	0.05	2.00	0	100.0	0	100.0
12. Gangjin	Apr 07	10.0	0.20	2.00	0	100.0	0	0
	Nov 07	5.0	0.15	3.00	0	50.0	50.0	50.0
13.Geogumdo	Apr 07	7.5	0.17	2.33	0	100.0	33.3	66.7
	Nov 07	5.0	0.10	2.00	0	100.0	0	100.0
14. Sachon	Apr 07	2.5	0.02	1.00	0	100.0	0	0
	Nov 07	10.0	0.17	1.75	25.0	75.0	0	100.0
15. Tongyong	Apr 07	20.0	0.50	2.50	0	100.0	62.5	62.5
	Nov 07	5.0	0.10	2.00	0	100.0	0	100.0

Table 1-10 (continued) Prevalence, Weighted prevalence, Infection intensity , occurrence of each trematode stage and distribution in other organs of gonad trematode infection

Sampling site	Sampling period	Prevalence	Weighted prevalence	Infection intensity	% Occurrence in infected clams			
					germ ball	sporocyst	cercaria	other tissues
16. Geojae	Apr 07	0	0	-	-	-	-	-
	Nov 07	2.5	0.02	1.00	0	100.0	0	0
17. Masan	May 07	17.5	0.37	2.14	14.3	100.0	14.3	57.1
	Nov 07	22.5	0.62	2.78	11.1	77.8	0	44.4
18. Weido	May 07	0	0	-	-	-	-	-
	Nov 07	0	0	-	-	-	-	-
19. Sungsan	May 07	7.5	0.22	3.00	0	100.0	66.7	33.3
	Nov 07	10.0	0.17	1.75	25.0	75.0	25.0	50.0
20. Seogwipo	May 07	2.5	0.05	2.00	0	100.0	0	0
	Nov 07	0	0	-	-	-	-	-
21. Gumnung	May 07	0	0	-	-	-	-	-
	Nov 07	0	0	-	-	-	-	-
22. Ulsan	Apr 07	7.5	0.17	2.33	0	100.0	33.3	0
	Nov 07	0	0	-	-	-	-	-
23. Pohung	Apr 07	2.5	0.07	3.00	0	100.0	100.0	0
	Nov 07	2.5	0.02	1.00	0	100.0	0	0

Prevalence=no. of clams infected by gonad trematode/Total no. of clam,
Weighted prevalence=Total score of trematode infection/ Total no. of clam,
Infection intensity=Total score of trematode infection/Number of clams infected

Metacercaria infestation in mantle cavity

The metacercaria of trematode were observed in mantle cavity of Manila clam and not found in other tissues. The metacercaria was not encysted but found free in the cavity (Fig. 1-16A) or attached on the mantle epithelium with large strong oral sucker covered with gelatinous substance (Fig. 1-16B). The accumulation of hemocyte around the metacercaria was not found but metacercariae seem to induced mantle hyperplasia as the metacercariae was enveloped by abnormal expansion of mantle tissue (Fig. 1-16C). The fresh metacercaria was accidental observed during random gross examination. The body was ovoid, the two suckers, oral and ventral sucker were observed by the oral sucker was larger and well developed (Fig. 1-16D).

The prevalence of metacercaria evaluated by histology (Table 1-8) was highest in winter in Boryung (37.5%) and Hwangdo (32.5%). The range of prevalence was from 0-23.1% in April by Jonghyun was the sampling site where highest prevalence observed. The occurrence of metacercaria seemed to be higher in November than in April in most of sampling sites which the range of prevalence was 0-37.5%. The sampling sites which free from metacercariae infection observed by histology were Weido, Seogwipo, Gumnung and Pohung.

Rickettsia-like organism

The characteristic of rickettsia-like organism was roundish basophilic intracytoplasmic inclusions which the diameter was about 10-26 μm. The rickettsia-like organisms were found in digestive gland epithelium (Fig. 1-16E) and in only one case, the parasites were found in digestive tract epithelium (Fig. 1-16F). In some case, the inclusions can also be observed in the digestive gland lumen. No tissue reaction associated with rickettsia-like organism was observed. The pathologic lesion was the disruption of epithelium cells caused by the large size of parasite (Fig. 1-16G). The prevalence of rickettsia was recorded between 0 - 17.5% as

shown in table 1-8. The prevalence was not correlation with the sampling time.

Gregarine

The pear-shape gregarine-like organism was found in extracellular of the digestive tract epithelium and connective tissue. The characteristic of the gregarine-like organism in this study was in single cell with no septate or dividing form (Fig. 1-16H). The tissue reaction around the parasite was not observed. Only few number of parasite cells were found in one histological section. The highest prevalence was found in Masan (17.5%) during November while in other sampling sites the occurrence of this parasite was rare (Table 1-8).

Copepod

The copepod was found in stomach or intestinal lumen attached to the epithelium wall (Fig. 1-17A-E). Small segment of copepod sometime completely obstructed in the secondary duct of digestive gland (Fig. 1-17E). The localized rupture of the epithelium caused by copepod appendage was observed without tissue reaction (Fig. 1-17B). The appendage of copepod sometime protruded from the lumen to subepithelial connective tissue of the intestine (Fig. 1-17D). The prevalence of copepod was found from 0-12.5%. The high prevalence (≥10%) was observed in Jonghyun and Geogumdo in April and in Naeri, Padori and Geojae during November (Table 1-8).

Marteilioides chungmuensis

Marteilioides chungmuensis was the parasited of the oocyted observed in only female clams. Only 7 clams from 1840 clams collected during our study was found to be infected by Marteilioides. One infected Manila clam was found from Gangjin in April, one clam from

Geogumdo and Sachon each and 2 clams from Tongyong and Geojae each. *M. chungmuensis* located in oocyte cytoplasm and the growth of parasite pressed the nucleus to the margin and caused deformation (Fig. 1-17F and 1-17G). The sporulation stage of parasite contained with 1-2 sporonts. The clams infected with Marteilioides were in spawning or spent reproductive stage. The gonad was not fully contained with eggs.

Turbellaria in digestive tract

The flat worm turbellaria was observed in the digestive tract lumen (Fig. 1-18A and 1-18B). The body wall of turbellarian observed in our study was thick and the entire body surface epithelium was covered by cilia. The mouth position was in anterior part of the body. In some of histological slides, the turbellaria contained with embryo and 2 conspicuous eye spots were observed. The host reaction related to turbellaria was not observed. Prevalence of turbellaria observed in digestive tract was 0-35%. The highest prevalence was detected in April from Naeri. Prevalence of turbellaria of the sampling site from north-west coast was higher than other regions. The seasonal dependent of turbellaria occurrence was observed. In April the prevalence of turbellaria was higher than in November.

Larval cestode

The encysted larval cestode or metacestodes was observed in connective tissue around digestive tract (Fig. 1-18C) or between the muscle fiber (Fig. 1-18D). The encapsulation of larval cestodes by layers of connective tissue fibers was common. Encapsulated metacestodes in some samples were in the process of resorption. The cestode that encysted in Manila clam was rare and the highest prevalence (10%) was also found in Mooahn.

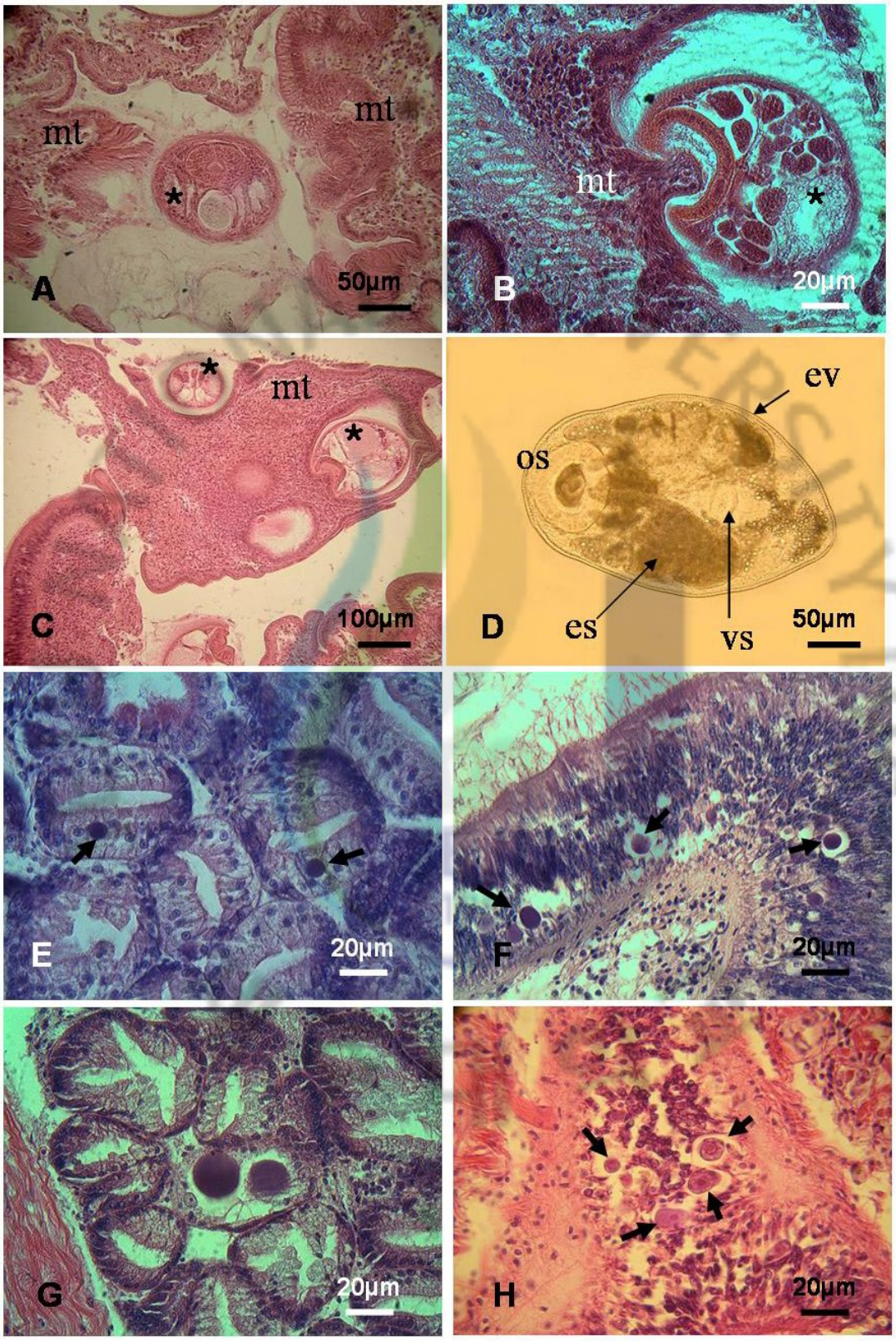

Figure 1-16

Figure 1-16 Histological section of Manila clams infected with parasites (H&E). 1-16A: The metacercaria (*) in mantle (mt) cavity. 1-16B: Metacercaria (*) attached the mantle (mt) epithelium with strong sucker. 1-16C: The metacercaria (*) enclosed by hyperplasia mantle tissue (mt). 1-16D: The metacercaria characterized by the oval shape, with oral (os) and ventral (vs) suckers, thick envelop (ev) and excretory vesicle (es). 1-16E: The basophilic intracytoplasmic inclusions of rickettsia-like organism (arrows) in digestive gland epithelium. 14F: The rickettsia-like organism in digestive tract epithelium. 1-16G: The large size of rickettsia-like organism cause digestive gland cell rupture. 1-16H: The pear-shape gregarine-like organism (arrows) in digestive tract connective tissue.

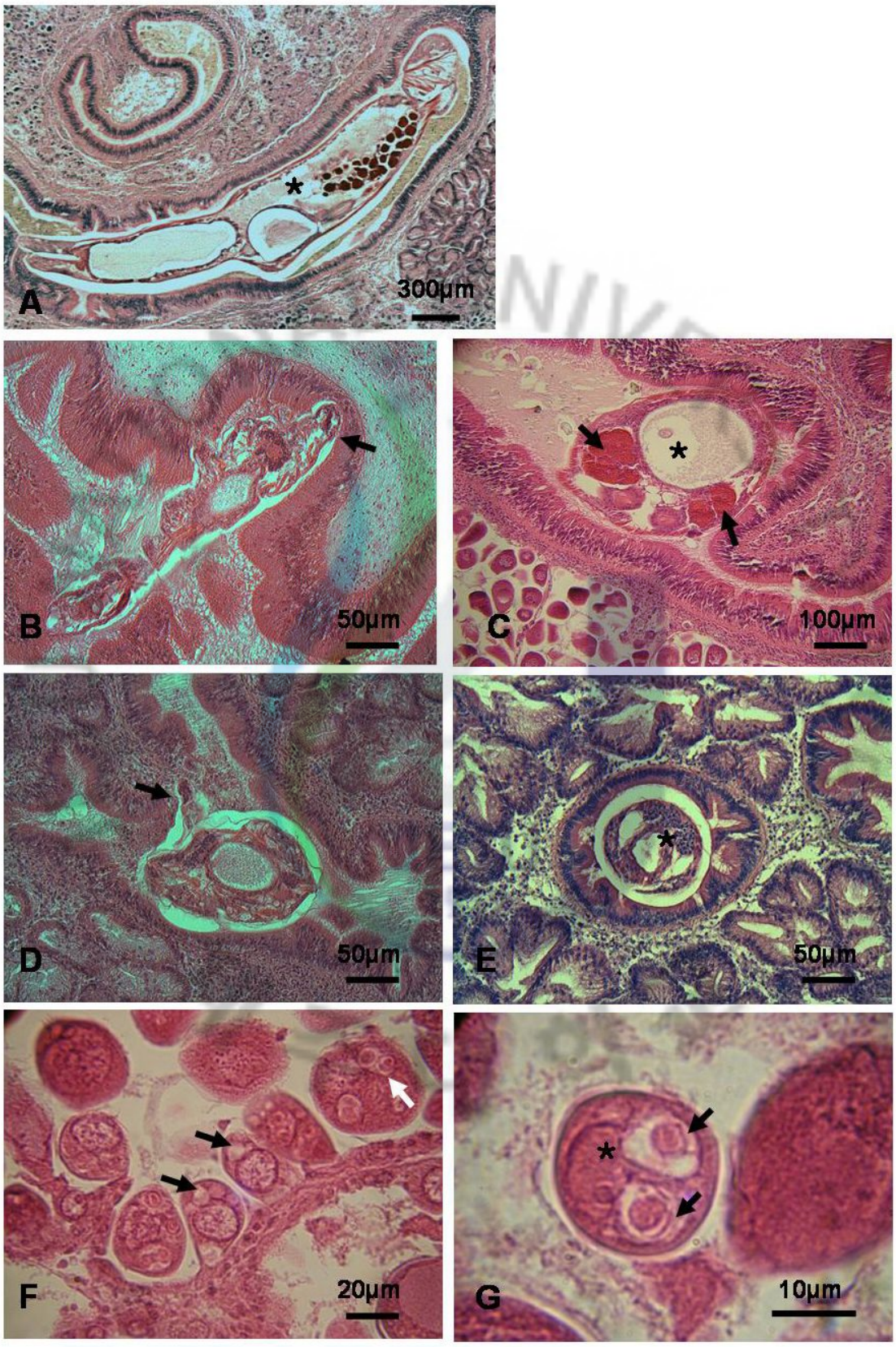

Figure 1-17

Figure 1-17 Histological section of Manila clams infected with parasite (H&E). 1-17A: Copepod (*) in intestinal lumen. 1-17B: The appendage of copepod (arrow) attached to the digestive tract epithelium. 1-17C: The copepod (*) contained with egg sacs (arrows). 1-17D: The appendage of copepod protruded from lumen to subepithelial connective tissue (arrow). 1-17E: Segmented of copepod (*) obstructed in secondary duct. 1-17F: Marteilioides in oocyte cytoplasm was in early stage (black arrows) and sporulation stage (white arrow). 1-17G: The sporulation stage of Marteilioides (arrows) in oocyte pressed the nucleus (*) to peripheral.

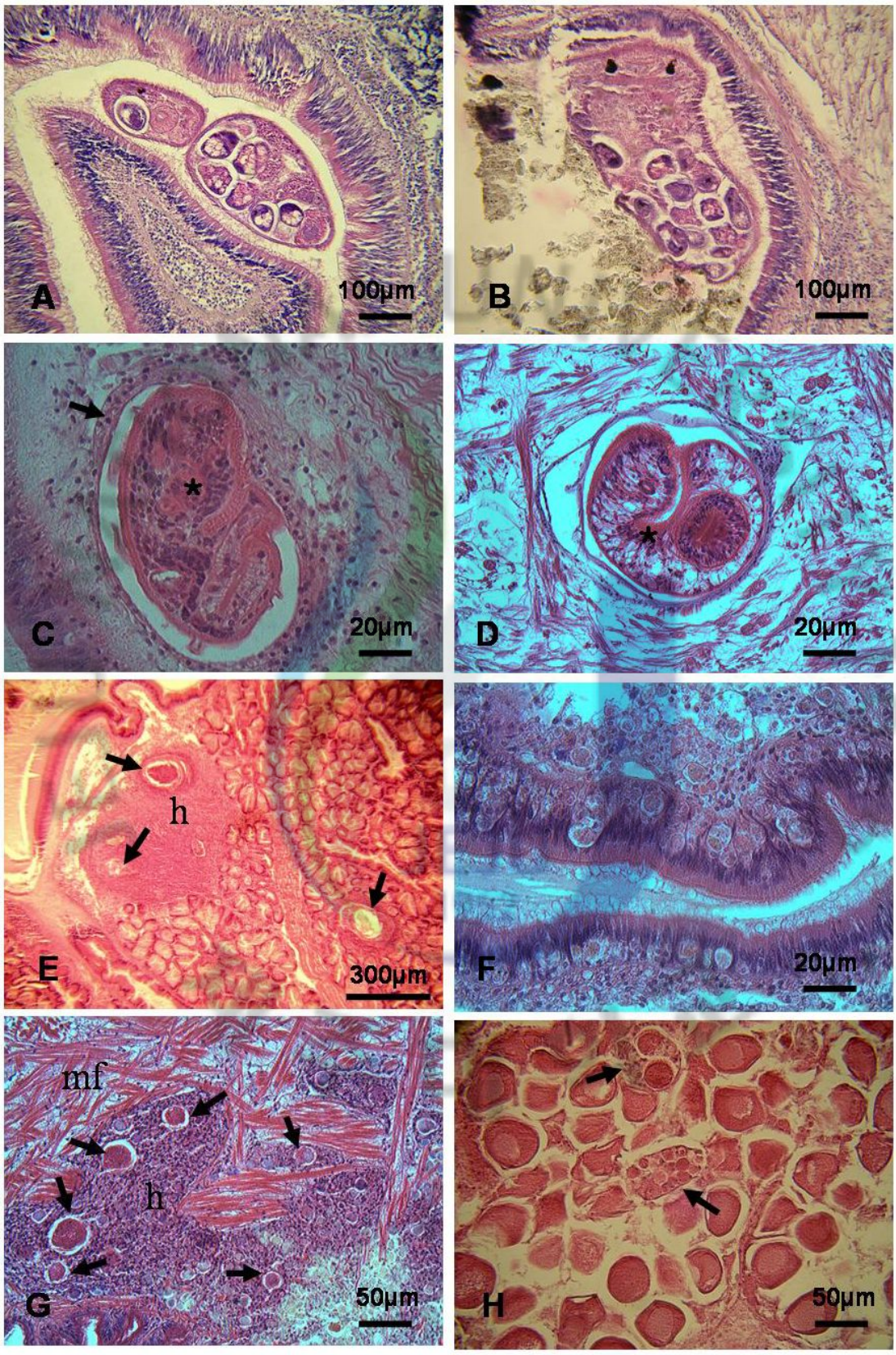

Figure 1-18

Figure 1-18 Histological section of Manila clams infected with parasite. 1-18A and 1-18B: Turbellaria in digestive tract. The body wall was covered by cilia. Turbellaria contained with anterior mouth, eye spots and embryo inside. 1-18C: Metacestode (*) in digestive tract connective tissue was surrounded by the capsule (arrow) of host origin. 1-18D: Metacestode (*) between muscle fiber. 1-18E: The unidentified eosinphilic parasites (arrows) with progressive hemocyte (h) accumulation. 1-18F: The irregular shape intracytoplasmic inclusion in intestinal epithelium. 1-18G: The round unidentified eosinophilic foreign bodies (arrows) infiltrated between foot muscle fiber with hemocyte infiltration. 1-18H: The intracytoplasmic eosinophilic inclusion bodies (arrows) in oocyte.

The unidentified encysted parasite

The encysted parasite was found in connective tissue or take part in the muscle fiber. Most of them died and was absorbed as can see in an eosinophilic ground substance with was accumulated with a large number of hemocyte. In this case, the type of parasite was not capable to predicted and classified as unidentified encysted parasite. The unidentified encysted parasite was found in low prevalence and cannot be observed in Jeju and east coast sampling sites (Fig 18E and table 1-8).

Other unidentified parasites

The other parasites which cannot be identified were found in only 1 or 2 Manila clam /samples. Some of them were described in Figure 1-18F-H.

4. Digestive gland condition

The digestive gland atrophy average semi-quantitative score of each sampling site was shown in table 1-11 and figure 1-19. The highest score of digestive gland atrophy in April-May was observed in Weri at the score 2.03 while in November was observed in Geogumdo at the score 2.08. Among all sampling sites, the sampling site from Jejudo except Sungsun, the Manila clams were in the lowest atrophy score. The different level of digestive gland atrophy was shown in figure 1-6.

The other pathologic condition of digestive gland was demonstrated as % prevalence (table 1-11). Necrosis of digestive tubule showed increased eosinophilia in the area affected. The digestive cells membrane cannot observed as necrotic cells are unable to maintain integrity and their contents leak out. The nucleus shrank and increase basophilia. The architecture of the tubular of digestive gland stilled observed but the cells clumped together and surrounded by

hemocyte. Localized necrosis showed extensive focal area of clumping digestive gland tubule. The area affected was limited in 1-2 lobules and normal part of the gland can be observed (Fig. 1-5A). While generalized necrosis showed severe diffuse eosinophilia in whole tissue (Fig. 1-5B). The necrosis of digestive gland was observed in low prevalence (Table 1-11). The normal digestive gland showed the well arrangement of the tubule which each tubule was attached each others (Fig. 1-5A). The hemocyte infiltration which not associated with parasite in digestive gland showed the increase number of hemocyte in connective tissue between the tubule (Fig. 1-5C). The vacuolization of digestive gland cells showed the small vacuoles in basement of cells (Fig. 1-5D). The density of the cell infiltration was varied. The prevalence of localized and generalized hemocyte infiltration was described in Table 1-11. The erosion of digestive gland cell into the tubule lumen (Fig. 1-5E) was found in most sampling site but the prevalence was not high. In case of the tubule was not attached each other but no sign of cell infiltration between tubule, the whole tubule together with the lumen size was decrease (Fig. 1-5F) and termed as digestive gland tubule shrinkage.

The prevalence of lipofuscin in digestive gland was shown in table 1-11. From H&E stained section, the small golden brown granules were distributed in connective tissue and the digestive gland cells (Fig. 1-5G). Lipofuscin was confirmed using Schmorl reaction, and the lipofuscin granules were stained with dark blue color (Fig. 1-5H).

Table 1-11 The average of digestive gland atrophy score and prevalence (%) of the digestive gland pathological lesions of Manila clams collected from 23 sampling sites in Korea

Sampling site	Sampling period	Atrophy score	Necrosis		Hemocyte infiltration		Shrinkage of tubule	Cell erosion	Vacuolization	Lipofuscin
			Localized	Generalized	Localized	Generalized				
1. Naeri	Apr 07	1.44	2.56	0	0	2.56	0	0	0	2.50
	Nov 07	0.98	5.00	0	5.00	5.00	0	0	0	7.50
2. Seonjae	Apr 07	1.23	0	0	0	2.50	2.50	2.50	0	0
	Nov 07	1.10	0	0	0	10.00	0	2.50	0	0
3. Weri	Apr 07	2.03	0	0	0	5.13	2.56	0	0	0
	Nov 07	0.68	2.63	0	0	5.26	10.53	0	0	7.50
4. Jonghyun	Apr 07	1.48	0	0	3.03	0	12.12	0	0	0
	Nov 07	1.38	0	0	0	2.50	0	5.00	0	0
5. Sungum	Apr 07	1.38	0	0	0	0	7.69	2.56	0	0
	Nov 07	1.54	0	0	0	15.38	2.56	0	0	0
6. Bakmiri	Apr 07	1.00	5.13	0	2.56	28.21	10.26	10.26	0	0
	Nov 07	1.66	7.89	0	0	2.63	0	7.89	0	2.50
7. Padori	Apr 07	0.84	6.45	0	0	0	0	3.23	0	0
	Nov 07	0.69	0	0	0	2.56	0	0	0	10.00
8. Hwangdo	Apr 07	1.00	2.50	0	0	30.00	0	2.50	0	0
	Nov 07	1.21	0	0	0	2.56	0	0	0	0
9. Boryung	Apr 07	0.83	0	0	0	2.50	0	17.50	12.50	0
	Nov 07	1.00	0	0	0	27.50	7.50	0	0	2.50
10. Gochang	Apr 07	1.00	0	0	0	2.63	0	2.63	0	0
	Nov 07	1.38	0	0	0	5.00	0	2.50	0	0
11. Mooahn	Apr 07	1.60	15.00	0	30.00	0	12.50	7.50	2.50	0
	Nov 07	1.75	2.78	0	0	22.22	2.78	0	0	0
12. Gangjin	Apr 07	0.89	10.81	0	5.41	18.92	2.70	0	0	0
	Nov 07	1.11	0	0	2.63	23.68	5.26	0	2.63	0
13.Geogumdo	Apr 07	1.50	6.25	6.25	6.25	12.50	21.88	25.00	0	0
	Nov 07	2.08	0	0	0	2.50	2.50	17.50	0	0
14. Sachon	Apr 07	0.91	6.06	0	0	0	3.03	0	0	5.00
	Nov 07	1.93	0	0	0	5.00	20.00	5.00	0	7.50
15. Tongyong	Apr 07	0.62	0	0	0	28.21	2.56	0	0	2.50
	Nov 07	1.44	0	2.56	0	10.26	15.38	2.56	0	2.50

Table 1-11 (continued) The average of digestive gland atrophy score and prevalence (%) of the digestive gland pathological lesions of Manila clams collected from 23 sampling sites in Korea

Sampling site	Sampling period	Atrophy score	Necrosis		Hemocyte infiltration		Shrinkage of tubule	Cell erosion	Vacuolization	Lipofuscin
			Localized	Generalized	Localized	Generalized				
16. Geojae	Apr 07	0.95	5.41	0	0	21.62	5.41	5.41	2.70	20.50
	Nov 07	1.68	10.53	0	2.63	57.89	10.53	7.89	0	2.50
17. Masan	May 07	1.44	0	0	0	5.13	2.56	7.69	5.13	2.50
	Nov 07	1.72	0	5.13	0	5.13	10.26	0	0	0
18. Weido	May 07	0.32	0	0	0	0	0	0	0	2.50
	Nov 07	0.85	0	0	0	0	0	0	0	7.50
19. Sungsan	May 07	1.18	0	0	0	10.00	12.50	5.00	2.50	0
	Nov 07	0.68	2.50	0	0	5.00	0	0	0	0
20. Seogwipo	May 07	0.28	0	0	0	0	0	0	0	1.00
	Nov 07	0.78	0	0	0	0	0	0	0	0
21. Gumnung	May 07	0.20	2.50	0	2.50	0	0	2.50	2.50	10.00
	Nov 07	0.48	0	0	0	0	0	0	0	15.00
22. Ulsan	Apr 07	1.00	0	0	0	2.50	5.00	0	0	2.50
	Nov 07	1.10	0	0	2.56	0	12.82	5.13	0	2.50
23. Pohung	Apr 07	0.82	0	0	0	0	0	2.63	0	1.00
	Nov 07	1.13	0	0	0	0	0	2.50	0	2.50

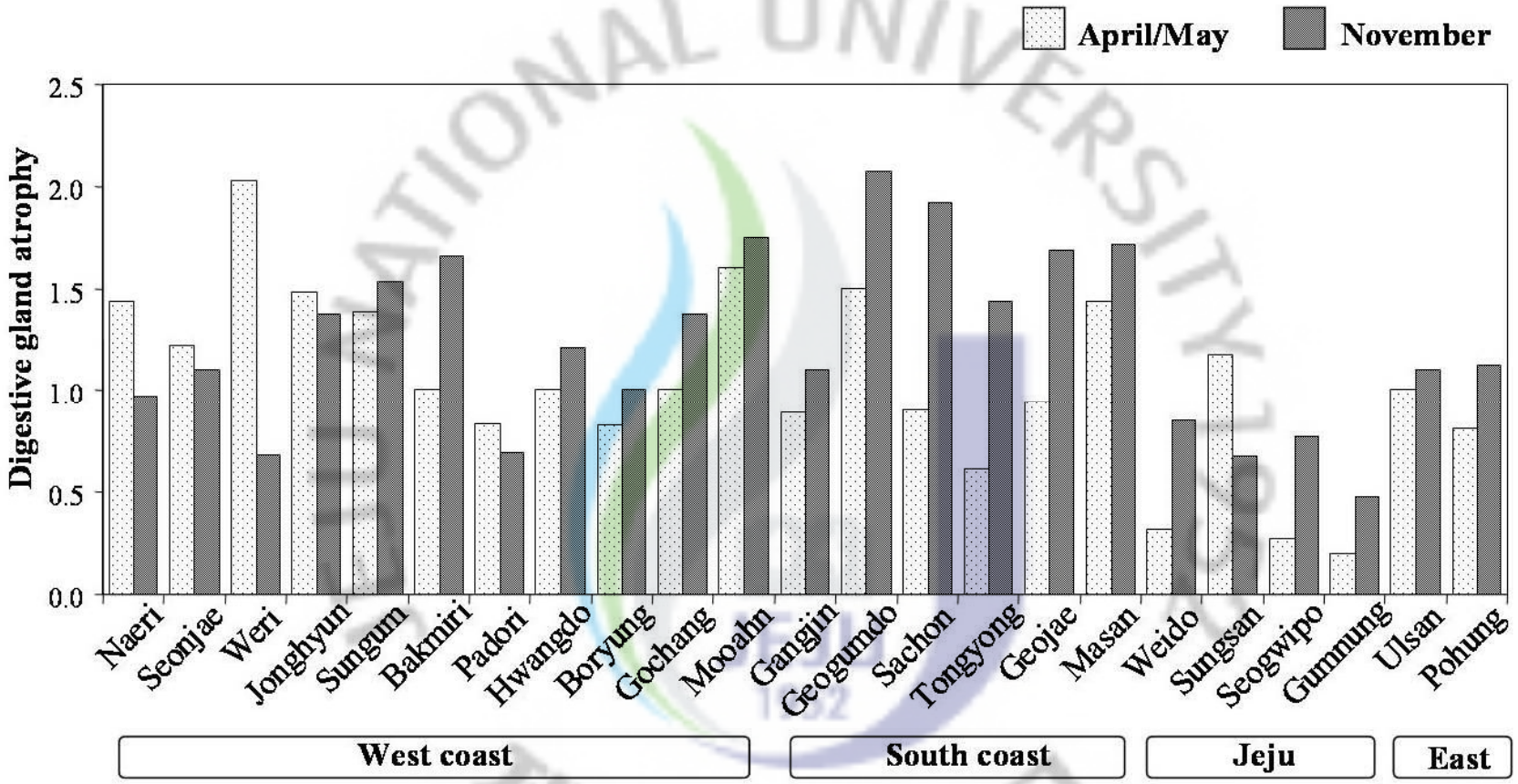

Figure 1-19 The average of digestive gland atrophy score of Manila clam *Ruditapes philippinarum* collected in April or May and November from 23 clam beds

Discussion

1. Condition index, gametogenesis and digestive gland atrophy

Numerous studies have reported that condition index of marine bivalves varies seasonally with gonad maturation and subsequent spawning (Park and Choi, 2004; Park et al., 2006a; Kang et al., 2007; Joaquim et al., 2008). The decreasing condition index observed in November corresponded with the increasing of spent clam after spawning season in summer while the clams in April were in developing stage. Among the clam beds, the condition index observed in November was not obviously different. Whereas the clams from west coast clam beds exhibited the higher condition idex in April than the clams from south and east coast. The estimation of chlorophyll-a from satellite ocean data indicated that the average chlorophyll-a in April was higher in the Yellow Sea (west coast) than in the East China Sea (south coast) while the wide range was observed in the East sea (Rebstock and Kang, 2003). The plenty of food availability may be an advantage of the Manila clam beds in west coast to be good locations for clam culture.

The digestive tubule cells were columnar epithelium but can change to simple cuboidal or squamous epithelium depending on the feeding phase (Winstead, 1995). Kim and Powell (2004) indicated that the clams with low condition were also the clam with high scores of digestive gland atrophy. The score of digestive gland atrophy in individual clams in this study did not negatively correlate with the condition index. However in most of sampling sites, the digestive gland atrophy was higher in November compare with in April. From the annual variation of chlorophyll-a concentration observed by Yang et al. (2008), Phytoplankton blooms in Gyeonggi Bay, Yellow Sea (1997-1999) occurred notably in the spring and late summer. While in November, the chlorophyll a level sustained at the baseline. The variation of

chlorophyll-a in south coast was in the similar trend. Refered to MOMAF (2001), Ngo et al. (2006) mentioned the seasonal variation in chlorophyll-a concentration from Gosung Bay, southern coast of Korea during January-December 2000 that the maximum value was observed in April and the minimum values were detected from October to December. Since the chlorophyll a represented the food availability in the sea, the reduction of phytoplankton in the sea in November may be the caused of higher digestive gland atrophy in this period. Cartier et al. (2004) suggested that the digestive gland index (calculated base on digestive gland weight) seems to be sensitive to gametogenesis and to energy storage cycle while effects of the environmental parameters (temperature and phytoplankton) were detected but did not explain entirely the digestive gland index variations. The increase of digestive gland atrophy score in November may be explained that it was the normal annual cycle which related with the seasonal food availability and possibly gemetogenesis.

2. Parasite infestation

Parasites observed in Manila clams in Korea can be classified based on the pathogenicity to host: (1) the parasite that can cause disorders in Manila clams and (2) the parasite which present without any associated pathology. According to histological examination, *Perkinsus*, cercaria, metacercaria, rickettsia, larval cestode Marteilioides and copepod infestation were possible a causative of harmful impacts to the host however depend on the number of parasite and the severity of host reaction. While pathologic lesion and host response was not observed in the clam infested with turbellaria and gregarine.

The sampling site in west coast and south coast showed obviously higher parasite species richness and prevalence. The tidal flat developed in west coast and south coast of Korea was the main area of Manila clam productions while Jeju Island and East coast sampling

sites were natural clam beds. The high density of clam beds may be one of the factor promotes the parasite infection. The condition index of west coast sampling site was higher than other region even though the parasite infestation was also high. The results suggested that the location of clam bedding is the greatly important factor affects the clam production than the non existence of parasite.

From the recent status of parasite infection in Manila clam landing in Korea, it was obvious that even the heavy infection of certain parasite and pathologic condition did not defeat the clam condition in our study regardless of the good physical condition. Therefore, instead of concerning about clam condition, the high parasitic infection area should be under surveillance for the sudden death induced by external stress condition such as pollution and environmental change.

2.1 *Perkinsus* sp.

Due to the occurrence of *Perkinsus* which was observed in most of sampling sites and the high prevalence of *Perkinsus* in many sampling sites, it was suggested that this protozoan was the main parasite of Manila clam landing in Korea. However the condition index of Manila clam in this study did not clearly show the correlation with *Perkinsus* infection both intensity and prevalence and the mortality was not capable to evaluate. The mortalities of bivalve causes by *Perkinsus* have been report in *Ruditapes philippinarum* from Korea (Park et al., 1999; Park and Choi, 2001). *Perkinsus* has been reported as a causative agent for growth reduction and reproductive retardation (Park et al., 2006a). The early infections of *Perkinsus* could not be readily detected in histology preparations but the examination of physical alterations in tissue sections provides important details of the effects of parasite on the host (McLaughlin and Faisal, 1999). Although it could not determine the growth and reproductive

output of the Manila clam however, microscopic observation supported that heavy infection effect the normal function of important organs of Manila clams especially gill, digestive gland and also retarded the growth of gonad follicle. The granulomatous hemocytic aggregates frequently seen at the gill bases may adversely affect the filter-feeding mechanism through hindering the related gill movement (Lee et al., 2001). High level of infection affects spawning frequency and reduces egg production (Park et al., 2006a). The histopathological characteristic of *Perkinsus* infection of our study was similar as reported in previous. The distribution of the trophozoites is attributable to their association with connective tissue and to their asscociation with blood vessels (Monte et al., 1995). The similar eosinophillic substances involved with *Perkinsus* infection was mentioned by Lee et al. (2001). Montes et al. (1995) stated that the eosinophilic substances originate from hemocytes and mainly consist of a defense molecule, and named polypeptide 225. Similar to the study of Park and Choi (2001) that *Perkinsus* was not detected among the clams collected from beaches located on the east coast in East sea and some sampling site along the coast of Jeju island. In contrast, the prevalence and infection intensity were much higher in the west and south coast sampling sites. The high density of clams at commercial clam beds and the type of bottom which were mostly mud or silty mud were thought to be associated with the higher prevalence of *Perkinsus*. The low infection intensity and prevalence of *Perkinsus* of Manila clam from Jeju and east coast were also mentioned in previous studies (Ngo and Choi, 2004; Park and Choi, 2001; Choi and Park, 2001). As *Perkinsus* sp. is infectious and can be transmitted from infected to uninfected oysters (Chu, 1996), the low infection could be associated with a much lower density of clams than that reported from commercial clam beds in west coast (Ngo and Choi, 2004). Practically, to minimize of Manila clam density in commercial beds was difficult. The Manila clams cultured in high endemic area especially Hwangdo, Boryung, Mooahn and Geogumdo which

had high weight prevalence and infection intensity should be bewared of suddenly mortality induced by environmental change or other stress. Because the physical condition of heavily infected clams may not properly function and induce the clams susceptible to stress than normal animal.

2.2 Cercaria trematode

The main pathogenicity of the gonad trematode cercaria in Manila clams was gonad castration. Similar results of gonad castration were also discussed in other bivalved infested with cercariae such as *Eurhomalea lenticularis* (Valderrama et al., 2004), *Tapes decussate* (Abdallah et al., 2009) and *Amiantis purpulata* (Cremonte et al., 2001). Valderrama et al. (2004) stated that the parasitic castration of *Eurhomalea lenticularis* was not only due to mechanical effects but also from an alteration of the kinetics of gametogenesis. The gonad acini appeared to be compressed by sporocysts which caused a reduction in their size and contained fewer numbers of germ cells. The mortality of infested clams was not able to evaluate in this study, however Abdallah et al. (2009) suggested that the high number of sporocyst caused death of the mollusk which the death of parasitized mollusks occurred before the total emergence of the cercaria. The temporal variation of cercaria infection was not in the same trend in every sampling site. The sampling sites from north-west coast showed the increasing prevalence in November while most of sampling sites from south-west and south coast, the prevalence of cercaria were higher in April. The environmental factors such as temperature and salinity might involve in the distribution of trematode. Abdallah et al. (2009) mentioned that the high summer temperature can limit the longevity of the free larval stage (miracidia) and consequently reduce the rate of infestation of mollusks and observed a clear correlation between the increase in salinity and the increase in the rate of parasitism. Cremonte et al. (2001)

concluded the maximum of monorchiid cercaria infection in purple clam in southwest Atlantic Ocean was in autumn. The intensity of infestation was not correlated with the prevalence but seem to relate with the growth stage of parasite. The growth stage of cercaria was recorded divided into germ ball, sporocyst and cercaria. As the parasite grew, more severity of gonad tissue destruction was observed. In spite of the severe pathology associated with cercaria infestations, there is usually little host response to the parasite (Lauckner, 1983). In agreement with Kim and Powell (2007), there was little or no aggregation of host hemocytes around healthy sporocysts however hemocytes were occasionally observed around dead or degenerating parasites.

2.3 Metacercaria

The metacercaria observed in mantle cavity was thought to be the digenean in the Family Gymnophallidae which used Manila clam as the secondary intermediate host. Most metacercaria in this Family used bivalves as first intermediate hosts and, with rare exceptions, charadriiform and anseriform birds as definitive hosts (Cremonte et al., 2008). Due to the complex life cycle and the requirement of intermediate and final host, the variation of metacercaria prevalence in different sampling site and the higher prevalence of metacercaria found in November maybe related to the availability of the host especially the bird migration in winter.

The metacercaria located in mantle cavity was reported in various species of bivalve in Korea. In 1989, Yu et al. (1993) reported the flukes belonging to the family Gymnophallidae from Manila clam purchased from fish market in Seoul. After Metacercariae were collected and experimental infection to mice was conducted, the adult worms were collected from the intestinal content and observed under microscope. The adult worms were morphologically

identified to *Parvatrema timondavidi*. The other Gymnophallid fluke was found from the surf clam *Mactra veneriformis* collected from a tidal flat located in Sochon-gun, Chungchongnam-do, Korea and identified as *Parvatrema chaii* (Sohn et al., 2007).

Due to the similar of gross appearance, location and host species, the metacercariae found in this present study was possible to be *Parvatrema timondavidi.* Base on the sequence of internal transcribed spacer (ITS) regions of ribosomal RNA gene of metacercaria and sporocysts in Manila clams, Yanagida et al. (2009) revealed that *P. timondavidi* was the same species as *P. duboisi*. Yanagida et al. (2009) indicated that *Parvatrema duboisi* has a two-host life cycle using Manila clams as the first and second intermediate host. The fork-tailed cercariae found in Manila clams of our study was similar to the one reported as *P. duboisi* (*T. timondavidi*). We doubted that both metacercariae located in mantle cavity and fork-tailed cercaria in gonad found in our study was the same species. However it was not possible to identify the cercaria by morphology and the genetic information of the parasite was required to answer this hypothesis. The only pathogenicity related to metacercaria was the mantle hyperplasia. Metacercariae do not normally cause parasitic castration and are generally considered to cause little if any detriment to their host (Lauckner, 1983). Even in the animal contained with high number of metacercariae seemed to be in a good state of health (da Silva et al., 2009). The effects produced by metacercariae the following maybe diagnosed: general debilitation, shell gapping, shell deformities, chemical erosion of shells, production of pearls and chalky concretions, hypertrophy or atrophy of affected host tissues, alterations of host behavior favoring detection and predation by final host, increase in oxygen uptake and metabolic rate, changes in host response to environmental stress, autotomy of body parts (siphons) and in heavy infestations, host death (Lauckner, 1983).

Various kinds of marine bivalves were reported to carry the metacercarial stage of

gymnophallid trematodes in Korea, among those, *Gymnophalloides seoi* is the only specie infecting humans (Lee et al., 1996). Even though the metacercariae of *G. seoi* have never been found in any molluscan species except oysters. There is a possibility that other marine bivalves serve as the second intermediate host and maybe sources of small intestine flukes of human *G. seoi* infection (Lee and Chai, 2001). Concerning to food safety, the identification of trematode in Manila clam should be performed to confirm if the trematode in Manila clam was the same species that infected human or not.

2.4 Marteilioides

Marteilioides chungmuensis, a pathogen Paramyxea, has been reported in Pacific oyster *Crassostrea gigas* (Park and Chun, 1989), Manila clam *Ruditapes philippinarum* (Lee et al., 2001; Itoh et al., 2005) and Iwagaki oyster *Crassostrea nippona* (Itoh et al., 2004b). From past 20 years, the occurrence of *M. chungmuensis* in Korea was mainly reported in *C. gigas*. The pasasite induces the formation of visible tumor-like gonad nodule, resulting in a loss of marketability (Park and Chun, 1989; Itoh et al., 2003a; Ngo et al., 2003). Therefore Marteilioides is considered one of the most serious problems for oyster production in Korea (Park et al., 2003). Lee et al. (2001) reported the first time in Marteilioides infestation from the Manila clams of the Hadong coast and Namhae coast over the southern coasts in Korea. The Manila clams infected with marteilioides in the present study also only the clams from southern coast sampling sites (Gangjin, Geogumdo, Sachon, Tongyong and Geojae) which located in the oyster farming area. Therefore Marteilioides infection in Manila clams was suspected to be an accidental infection that the pathogen transmitted from the Pacific oyster. Unlike the infection in oyster, the tumor-like lesion in Manila clam caused by Marteilioides was not observed. However other impacts of the parasites to Manila clam such as prolonged

spawning activity (Tun et al., 2008a), mass mortality (Park, 2005) and low fertilization rate (Park, 2005) which reported in Pacific Oyster should be evaluated.

2.5 Rickettsia-like organism

The fluctuation of rickettsia-like organism (RLO) infection in Manila clam seems not to relate with sampling site location. Most of infected Manila clam contained with small number of RLO and the pathogenicity to host was not obvious. The amorphous basophilic inclusions (ABI's) containing either rickettsiae, chlamydiae or mycoplasmas seem to be ubiquitous for these mollusks regardless of species, location, environment factors and depth (Lauckner, 1983). Similar to our study, infections appear to evoke little host response or pathological effect. Colonies are mostly found inside the cytoplasm of the digestive tubule epithelial cells without signs of host response (Cremonte et al., 2005). Rickettsia or Chlamydiae-like organisms in the cytoplasm of epithelial cells of the digestive tubules was observed in the venerid clam *Pitar rostrata* from Uruguay (Cremonte et al., 2005). Prokaryotic inclusions normally were observed within the epithelial cells of the digestive system. In some mytilids, prokaryotic inclusions were associated with the gill or renal tissues. Inclusions found in the digestive tract were usually roundish and those in the gill and kidney rather amorphous in shape. No apparent pathological effects or host responses to prokaryotic infection were detected (Kim and Powell, 2007). However Rickettsia was reported to be associated with disease in *Tellina tenuis* by Buchanan (1978). The shells of affected individuals were markedly thinner and chalky in nature, and their digestive gland was pale yellow compared with the usual dark brown colour. A high proportion of digestive gland cells were necrotic. Their cytoplasm contained membrane bound inclusion bodies which in turn contained viral particles. Heavy and extensive cytophatological lesions of rickettsial organisms

in the oyster *Crassostrea ariakensis* were present in the epithelial cells of the gills, digestive gland, mantle and digestive tube. The parasitized cells were greatly hypertrophied and eventually ruptured (Wu and Pan, 2000). Presently, the RLO was not a main problem of manila calm culture in Korea but the careful monitoring should be concern as the increasing of prevalence and intensity of infection can lead to health problem of Manila clam.

2.6 Gregarine-like organism

Gregarine apicomplexans are large single-celled parasites that inhabit the intestines and other extracellular spaces of nearly every group of invertebrates, particularly annelids and insects (Leander, 2007). The wide spread presence of gregarines in animal and little attention by the scientific community, most of gregarines remain unknown (Leander, 2007). While gregarine-like organism in this study was not classified as an important pathogen in Manila clam, the well known gregarine Nematopsis has been reported to be a pathogenic to *Cerastoderme edule* (Azevedo and Cachola, 1992), *Arcuatula arcuatula*, *Anadara granosa*, *Perna viridis* and *Paphia undulate* (Tuntiwaranurak et al., 2004). Nematopsis destroys the gill (Azevedo and Cachola, 1992; Carballal et al., 2001; Tuntiwaranurak et al., 2004) and induced a slight and focal hemocytic response (Carballal et al., 2001) and was reported to be a cause of high mortality in Cerestoderme edule in Portugal (Azevedo and Cachola, 1992). The pear-shaped gregarine-like organism found in digestive tract epithelium of Manila clams from Korea has the same characteristic with the one reported in *Tapes philippinarum* from British Columbia (Bower et al., 1992) and in the cockle *Cerestoderma edule* from Spain (Carballal et al., 2001). The unidentified gregarine in intestine was not a cause of clam mortality and only induced a mild and focal hemocytic resonse (Carballal et al., 2001) or distention of the intestinal circular muscle in the heavily infected clam (Bower et al., 1992).

2.7 Turbellaria

The flat worms, turbellarian are carnivorous predators or scavengers, feeding on nearly any available animal matter. More than 100 species of turbellarians are known to be symbiotic with other invertebrates. Some of these are simply commensals that derive some protectionfrom their associations, showing only physical modifications for temporary attachment. Others, however, feed upon their hosts, causing various degrees of damage and displaying true physiological dependency on the relationship (Brusca and Brusca, 2003). While Turbellaria are predominantly free-living predators or scavengers, members of the orders Rhabdocoela and Alloeocoela associate more intimately with marine mollusks (Lauckner, 1983). From histological observation, we suggested that the turbellaria found in digestive tract of Manila clam was not parasitic but symbiotic or commensal. While the turbellaria, *Parvavortex cardii* was reported in the gut of *Abra tenuis*, *Scrobicularia plana* and *Tapes decussate* which the relation of Turbellaria and mortality was questionable (de Montaudouin et al., 2000). The turbellarian identified as *Paravortex cardii* was reported in intertidal cockles *Cerastoderma edule* from the northern Wadden Sea, Germany (Thieltges and Riese, 2006) and Ría de Arosa, Spain (Carballal et al., 2005). Different to the gut turbellarian, several authors have mentioned the presence of a turbellarian in the gill caused pathological effects on the gill of mussel. The turbellarian *Urastoma cyprine* inhabited the mantle cavity, between the lamellae of the demibranch of the mussel. The affected area exhibited disarrangement of the gill filaments, the blood sinuses were engrossed and hemocyte infiltration was found surrounding the area where the parasite was placed (Robledo et al., 1994; Cáceres-Martínez et al., 1998). Aside from negative effects on eastern oyster *C. virginica*, the visible presence of worms on the gills could contribute to a drop in demand by the lucrative half-shell market (Bataller and Boghen, 2000). Although recorded pathological effects in the studies were not related to a negative

effect on the CI, the possibility of an increase in the negative effect if mussel culture develops and the mussel population size increases must not be overlooked (Thieltges and Reise, 2006).

2.8 Larval cestode

The term 'metacestode' used by many authors to describe stages found in invertebrate (intermediate) hosts, applied to any larval form between the egg and adult or to larval in the general or collective sense (Lauckner, 1983). Cestode infection did not seem to significantly damage the oyster. The information of cestode cysts, characterized by encapsulation of larval cestodes by layers of connective tissue fibers, was observed routinely. Encapsulated larval cestodes often appeared to be disintegrating and in the process of resorption by the host. (Kim and Powell, 2007). The capsule of host origin which surrounded the metacestode was similar to those described by Lauckner (1983), Cremonte et al. (2005) and Park et al. (2008). The metacestode infected Manila clam from Japan also exhibited massive hemocyte infiltration around the encysted parasites, indicating that metacestode infections impact the host defense system (Park et al., 2008). Hine and Thorne (2000) suggested that the greater number of metacestodes in wild rather than hatchery-reared oysters probably reflects proximity of wild oysters to definitive hosts. The tapeworm was well known and frequently harmful parasites of man and animals. However, none of the larval or postlarval tapeworms occurring in marine bivalves are known to infest humans (Lauckner, 1983).

2.9 Copepod

The affects of copepod infestation observed by several authors were explained in a single way that it may cause damage of focal area but does not kill the host. Davey and Gee (1988) reviewed that the copepod *Mytilicola intestinalis* caused local metaplastic changes in the

epithelium of the intestine and rectum. Normal ciliated columnar cells were reduced to cuboidal nonciliated types. A large parasite or several smaller ones apparently produced some erosion of gut epithelial cells, but this erosion and the metaplastic changes were so localized that repair would be rapid. The effects of the copepod was presented on blue mussel physiology includes reduced total flesh weight; reduced relative proportions of ash, lipid and protein in the flesh; reduced relative weight of the digestive gland compared to total flesh weight; increased protein digestion; and increased filtration and respiration rates (Davey and Gee, 1988). The copepod *Pseudomyicola spinosa* infested the blue mussel *Mytilus galloprovincialis* showed the low condition infected and some histological disorders such as obstruction of the intestine, digestive epithelia rupture and encapsulations around digestive tissues. However no evidence of host mortality associated with a high number of copepods (Olivas-Valdez and Cáceres-Martínez, 2002). The copepod causes some degree of damage, but apparently, it does not kill its host, maintaining the relationship copepod/host in equilibrium. However heavily infected mussels showed the lowest CI supporting the fact that the tissue lesions caused by the copepod may affect the condition of the host (Olivas-Valdez and Cáceres-Martínez, 2002). Since the copepod in Manila clam did not showed the negative effects to the CI and the prevalence was low, to date the copepod may not be the serious problem of Manila clam culture.

3. Digestive gland condition

The digestive gland is a compound, tubular gland. In addition to its digestive role, the digestive gland stores glycogen and lipids (Boucaud- Camou and Henry, 2003). Such abnormal histology of digestive gland tubules can be used as an indicator of the general physiological condition of bivalves (Eble and Scro, 1996). Histological analysis of Manila clam revealed

different digestive gland conditions in individual clam from different sampling sites. Beside of the pathogenicity due to infectious agent which was already explained, non infectious abnormalities of digestive gland were identified in this part. Although the etiological organism was not observed, the possibility that uncertain condition of digestive gland may or may not related with the infectious microorganisms was not ruled out. Several studies proposed that unspecific stress, disease, environment pollution or non feeding condition caused epithelial atrophy and large circular lumen (Winstead, 1995; Eble and Scro, 1996). Since the digestive epithelium is known to be important in digestion of foods, and detoxification, it could be considered that the atrophic changes of the epithelium lead to the weakness of clams and consequently massive mortality (Kim and Powell, 2004). In our study, the progressive digestive gland atrophy was observed in only few samples. The prevalence of other digestive gland disorders was also low and some were thought to be related with normal cycle of digestive gland degeneration except the intensive generalized necrosis which seems to affect the normal function of the organ. The erosion of digestive tubule epithelium present in lumen was explained by Couch (1985) as a necrotic material which might occur at any time during the atrophic process but often in final event. The oysters suffering from this condition also had systemic hemocytosis and vesicular connective tissue edema, indicating a state of physiological stress. The shedding of digestives cell resulted in a breakdown of the epithelium's structural integrity. In a few instances the degeneration and loss of digestive cells lead to digestive tubule necrosis (Syasina et al., 1997). Digestive gland atrophy was not the only parameter reported as stress indicator. The occurrence of nonspecific inflammatory responses is probably indicative stress, unrecognized injury, or the presence of submicroscopic agents. It has been noted that systemic hemocytosis is often associated with starvation and spawning-induced stress as well as chemically-induced stress (Couch, 1985). Large vacuole containing semi-fluid

material presented in digestive tubules was thought to be the remains of lysed digestive and/or secretory cells suggested an extensive process of tubule autophagy and regeneration (Johnson et al., 1996). Syasina et al. (1997) observed the poor digestive diverticular condition of *Mizuhopecten yessoensis* located in the heavily polluted area and suggested that morphological changes, such as vacuolization of digestive cells, mass shedding of digestive and basophilic cells and the occurrence of atrophic and necrotic tubules, suggest the possibility of chronic toxicity. Usheva et al. (2006) mentioned that the increase of vacuolization of cells of the digestive gland is one of the most usual phenomena recorded in bivalve under the effect of xenobiotics of organic and inorganic natures. This study provides the information of lipofuscin accumulation from Manila clam inhabited in 23 clam beds of Korea. As review by Koukouzika et al. (2009), lipofuscin deposition is also regarded as indicative of contaminant exposure and revealing a general response to pollution. In our study the prevalence was low and no obvious extremely lipofuscin deposited in clam from any sampling site. Koukouzika et al. (2009) observed the elevated of lipofuscin in winter and extremely accumulate in spring, suggested that the fluctuation of lipofuscin may be also depend on the normal seasonal variation. To consider Manila clam as a biomarker for pollution, the complete year study of lipofuscin accumulation and also the other digestive gland pathological lesions should be performed.

Part II

Comparative studies on the reproduction and physiological condition of *Marteilioides chungmuensis* infected oyster, *Crassostrea gigas* from Tongyong, Korea

Abstract

We investigated the comparative study on the reproductive output and physiology of *Marteilioides chungmuensis* infected Pacific oyster, *Crassostrea gigas* from Tongyong province, south coast of Korea during December 2007. The infected oyster showed the multiple yellowish nodules on the oyster body. The average tissue wet weight of uninfected and infected oyster was not significant different. The parasites were found both in early and advance stage in oocyte. Hemocyte accumulation could be observed in some samples. All infected oysters were in late development or ripe stage while uninfected oyster were in resting or spent stage. The percentage of infected eggs ranged from 7.45-82.99%. The egg produced from infected oyster were undersized (24.17±5.59 μm) and ELISA results revealed the low number of egg productions (average GSI=7.52±5.50). More oysters from infected group had severe digestive tubule atrophy. But the digestive gland atrophy indexes showed not big differences between uninfected and infected oyster. Biochemical compositions of uninfected oyster and infected oyster were significantly different ($p<0.05$). The protein in infected oyster was significant higher than uninfected oyster ($p<0.05$) due to the oocytes which remain only in the infected oysters while the carbohydrate and lipid level of Marteilioides infected oyster were significant lower than uninfected oyster ($p<0.05$). These results may be caused by environment stresses and lead mortality. Our study indicated that *M. chungmuensis* infection in oyster decrease the total production output and may effect to the oyster health in long term.

Introduction

Marteilioides chungmuensis, a pathogen Paramyxea, has been reported in Pacific oyster *Crassostrea gigas* (Park and Chun, 1989), Manila clam *Ruditapes philippinarum* (Lee et al., 2001; Itoh et al., 2005) and Iwagaki oyster *Crassostrea nippona* (Itoh et al., 2004b). Among the few host species, Pacific oyster is known to be susceptible to infect with *M. chungmuensis*. As the oocytes of female oysters were the site of infection, Marteilioides cause ovary enlargement and deformations that was rejected from the market resulting in economic losses to oyster farmers in Korea (Ngo et al., 2003) and Japan (Itoh et al., 2002). Marteilioides infection can also cause spawning failure by delaying spawning and demaging ripe oyster oocytes (Park et al., 1999; Park et al., 2003; Tun et al., 2008b; Ngo et al., 2003). Therefore the impact of *M. chungmuensis* infection was not only effect to the appearance of oyster but seem to effect to the overall oyster reproduction output.

Park and Chun (1989) observed the development of *M. chungmuensis* by electron microscope and reported the stage of parasite consisted of primary cell, secondary cell and tertially cell which differentiated into a sporont. Itoh et al. (2004) succeeded in using parasite-specific DNA probes and electron microscope to detect early development stages of parasite in epithelial tissue of the gills, mantle and labial palps. The occurrence of *M. chungmuensis* in oyster cultured in Korea was observed in developing and fully mature eggs of oysters in late June to January. High infection level was appeared in late June, August and November when oysters were spawning (Ngo et al., 2003). Similarly, the study of Tun et al. (2007) described the prevalence of *M. chungmuensis* from Japan increased from July to September, when oysters were in spawning period and declined from October to April when oysters were in spent stage. Prevalence of infection was 70% or higher from August to October, then declined in November

was reported using nested PCR which is more sensitive method to detect the parasite infection than histology (Tun et al., 2008b).

From above, the studies in Marteilioides were well discussed in term of seasonal variation of prevalence (Tun et al., 2008b; Ngo et al., 2003), histopathology (Tun et al., 2008a), parasite development (Park et al., 1989; Itoh et al., 2004) and molecular diagnosis (Itoh et al., 2002; 2003). However the information in the quantity of egg production has not been evaluated. Several studies have been applied in the quantification of the reproductive effort using an enzyme-linked immunosorbent assay (ELISA) using the polyclonal antibodies (Choi et al., 1993; Kang et al., 2003; Park and Choi, 2004; Park et al., 2005; Long et al., 2008). We investigated the effect of *M. chungmuensis* infection on the reproductive output of infected oyster by the ELISA assessment. To clearly understand the affects of *M. chungmuensis* to the oyster reproduction, we used usual histology method for observed histopathology of *M. chungmuensis* in pacific oyster, measured egg diameter with image analysis and evaluated gametogenesis development.

The effects of Marteilioides on the oyster related to physiologic parameter in term of biochemical composition changes were discussed by Park et al. (2003). The variation of energy storage in bivalve related to reproductive cycles (Li et al., 2006), site variation (Kang et al., 2000) and culture method (Ngo et al., 2006). Abnormal histology of digestive gland tubules can be used as an indicator of the general physiological condition of bivalves (Eble and Scro, 1996). In this study, we compared the biochemical composition and digestive gland atrophy to carry out the differences in physiological states of infected and uninfected oyster.

Materials and methods

1. Sampling efforts

The fresh Pacific oyster meat without shell from Tongyong was sent to laboratory in December 2007. Tongyong province is located on the south coast of Korea (Fig. 2-1), where several semi-enclosed bays and islands are located. In this area, oysters are cultured intensively by suspending oyster ropes hanging from longlines. The oyster meat was then divided in to 2 groups; normal gross appearance oyster and oyster with tumor. The oyster was weighted and processed for histology and the remained tissue was lyophilized by freeze dryer. Freeze-dried oysters were homogenized using a mortar and pestle and keep in -20^{o}C for ELISA and biochemical composition assay.

2. Histology

A cross section of oyster body which contained gonad, digestive gland, gill and mantle was cut and fixed in Davidson's solution for 24 hours and preserved in 70% ethyl alcohol. The tissue was dehydrated and embedded in paraffin. Paraffin blocks were sectioned at 6 μm thickness and stained with Harris's haematoxylene and eosin Y. Histology slides were examined under a light microscope. Gonad development stage was categorized follow by Ngo et al. (2002). The egg diameter was measured from the histology slides using an Image analysis computer program. To compare the egg size produced from infected and uninfected oysters, the histology slides of oyster collected from Marteilioides uninfected area, Goseong Bay, South coast Korea were used as normal samples. Most of the normal oyster samples were in ripe stage. The percentage of infected eggs from each sample was calculated from the number of infected eggs divided by the total number of eggs observed from 2 medium magnification microscope

fields. The digestive gland atrophy was graded to scale 0–4 depended on the thinning of digestive tubule wall according to Kim et al. (2006).

3. Quantification of Marteilioides infected oyster eggs using Enzyme linked immunosorbent assay (ELISA)

Indirect ELISA was used to establish the quantity of the Marteilioides infected oyster. The freeze-dried oyster 20 mg were taken from each sample, dissolved in phosphate buffer containing 0.05% Triton X-100 (PBST) and homogenized with ultrasonifier. The homogenate was diluted to 500 fold to fit into the optimal antibody-antigen reaction. Each 100µl of homogenate including standard prepared by various concentrations of purified *C. gigas* eggs, negative control (indifferent oyster in PBST) and Marteilioides infected oysters were added into the polystyrene 96-well microplates. Two replicates were made for each sample and the microplates were incubated at 4^{o}C overnight. After incubation, the plate was washed 5 times with PBST, and 150µl of 1% bovine serum albumin was added to each well as a blocking agent. The plate was incubated for 1 hour in room temperature, washing with PBST and the rabbit anti-oyster egg IgG as primary antibody developed by Kang et al (2003) was added in each well and incubated for another 1 hour. After washing, 100 µl of goat anti-rabbit IgG alkaline phosphatase conjugate (1:100) was then added, incubated for 1 hour in room temperature and washed with PBST. Finally, 100 µl of p-nitrophenylphosphate (p-NPP) substrate dissolved in 0.1M glycine buffer was added as a coloring agent and the optical density of the end product was measured at 405nm using a microplate reader. The result was shown as % gonadosomatic index (%GSI).

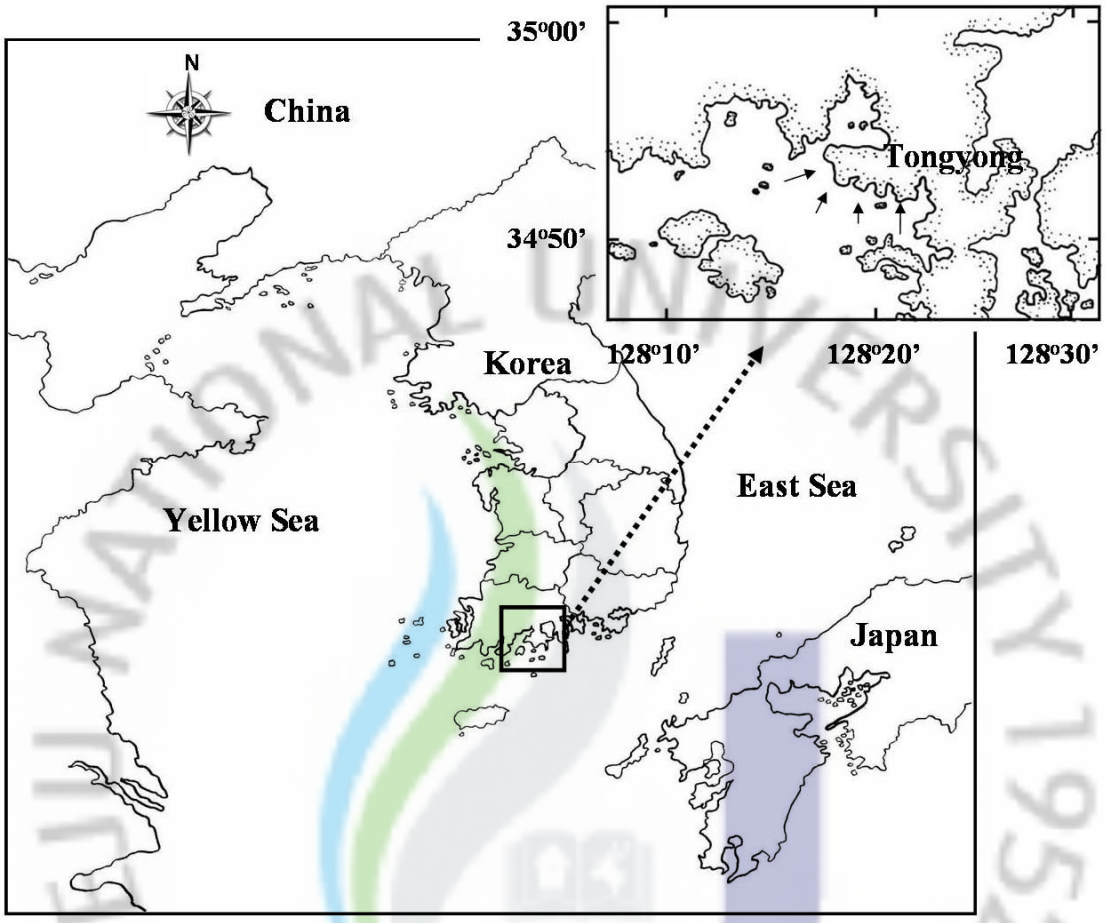

Fig. 2-1 Map shows sampling area

4. Biochemical composition analysis

Protein, lipid and carbohydrate contents were determined in freeze-dried remained oyster tissue after histology section. Protein content was determined using the method of Lowry et al. (1951) after alkaline hydrolysis with 0.1M NaOH at 37°C for 2 hours using bovine serum albumin as standard. Carbohydrates were determined as glucose by the phenol-sulfuric method (Dubois et al., 1956) using dextrose anhydrase as standard. Total lipids were extracted using a mixture of chloroform and methanol (Bligh and Dyer, 1959) and measured gravimetrically. The estimation of the total protein, carbohydrate and lipid was based on dried tissue weight of each individual oyster and expressed as mg/g tissue dried weight.

Results

1. Gross and appearance of the oysters

The Pacific oysters were divided into uninfected and Marteilioides infected group by Marteilioides pathognomonic gross lesions. The average tissue wet weight was not significant different by the uninfected oyster was 6.94±2.53 g and infected oyster was 7.25±1.78 g. The external appearance of infected oyster showed the multiple spherical or oval firm yellowish smooth-surface nodules on the oyster body (Fig. 2-2). The nodule size was varied from 50 mm to 1.5 mm.

2. Histology

From the light microscope observations (Fig. 2-3), parasites were found only in the oocytes therefore all infected oysters were female. *M. chungmuensis* invaded oocytes cytoplasm and sometime pressed oocyte nucleus to the edge of the cell. The infected oocytes were both

immature oocytes which attached to the gonad follicle wall and mature oocyte which free in follicle lumen. The small eosinophilic cytoplasmic inclusion bodies in oocyte were described as unsporulation form of *M. chungmuensis*. This form of parasite was the early stage of *M. chungmuensis* that found in immature oocytes. The sporulation stage which contained 1-2 large spherical (10 μm) eosinophilic sporonts in one parasite was mainly found in mature free oocyte. 1-2 parasites could be observed in one oocyte. This stage was called advanced stage. Hemocyte infiltration in follicle was found in some samples. The varied degree of hemocyte infiltration was observed in gonad follicle and surrounded the oocytes.

The number of oysters in each reproductive stage was demonstrated in table 1. Most of uninfected oyster (60%) was in resting stage which the gonad was inactive, and sex was unable to distinguish. Follicles were small and had wide interfollicle space. The early development of spermatogenesis was observed 28% of uninfected oyster. This stage characterized by the developing of spermatogonia in the follicles. The few numbers of male oysters was in spent stage (10%). The follicle shrank and few sperm remained in follicle. The only one uninfected female oyster was found in spent stage which follicles shrank markly and contained few oocytes.

The gonad of the infected oyster contained with high number of immature and mature oocyte. The Marteilioides infected oyster were in ripe (76%) or late development stage (24%). The ripe stage determined by the full packing of oocytes in follicles. Most of oocyte populations were free mature oocyte which had polygonal configuration. Late development stage was characterized by the high number of oocytes in follicle. Many developing oocytes which varied in size on the follicle wall were observed.

From Marteilioides infected oyster, the eggs in gonad were not all infected by the parasite. The percentage of infected eggs ranged from 7.45-82.99%. The average of infection was at 30.48±20.95%

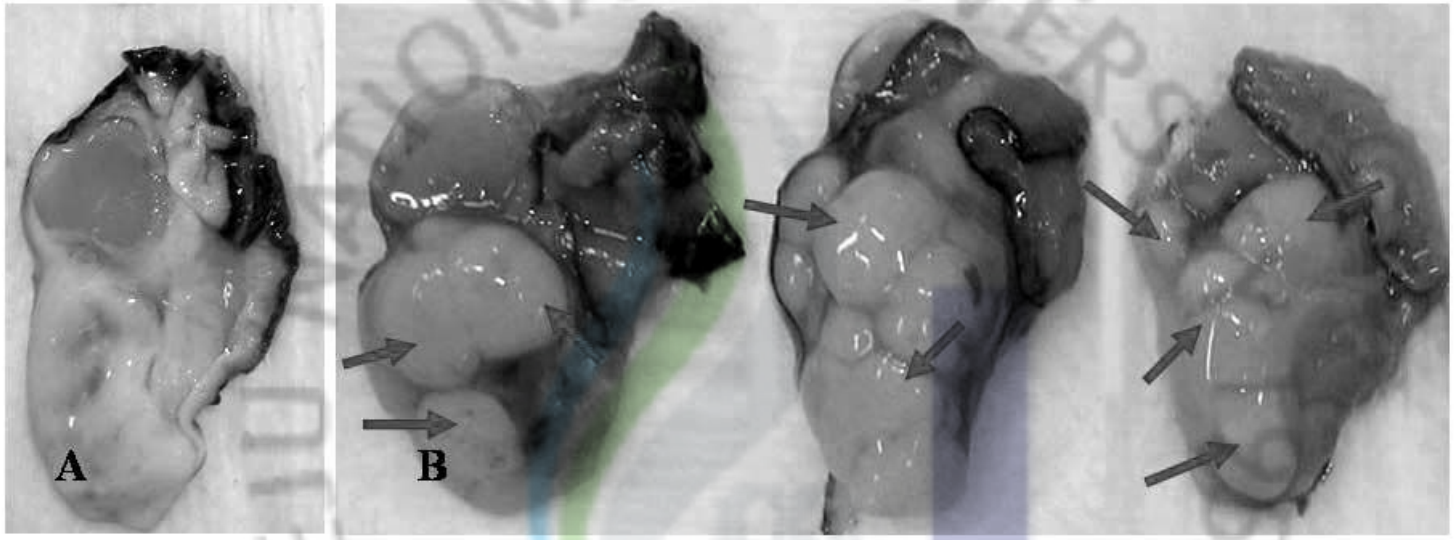

Figure 2-2 The Pacific oyster *Crassostrea gigas*. A: Normal oyster. B: *M. chungmuensis* infeted oyster shows the multiple nodules on the body (arrows).

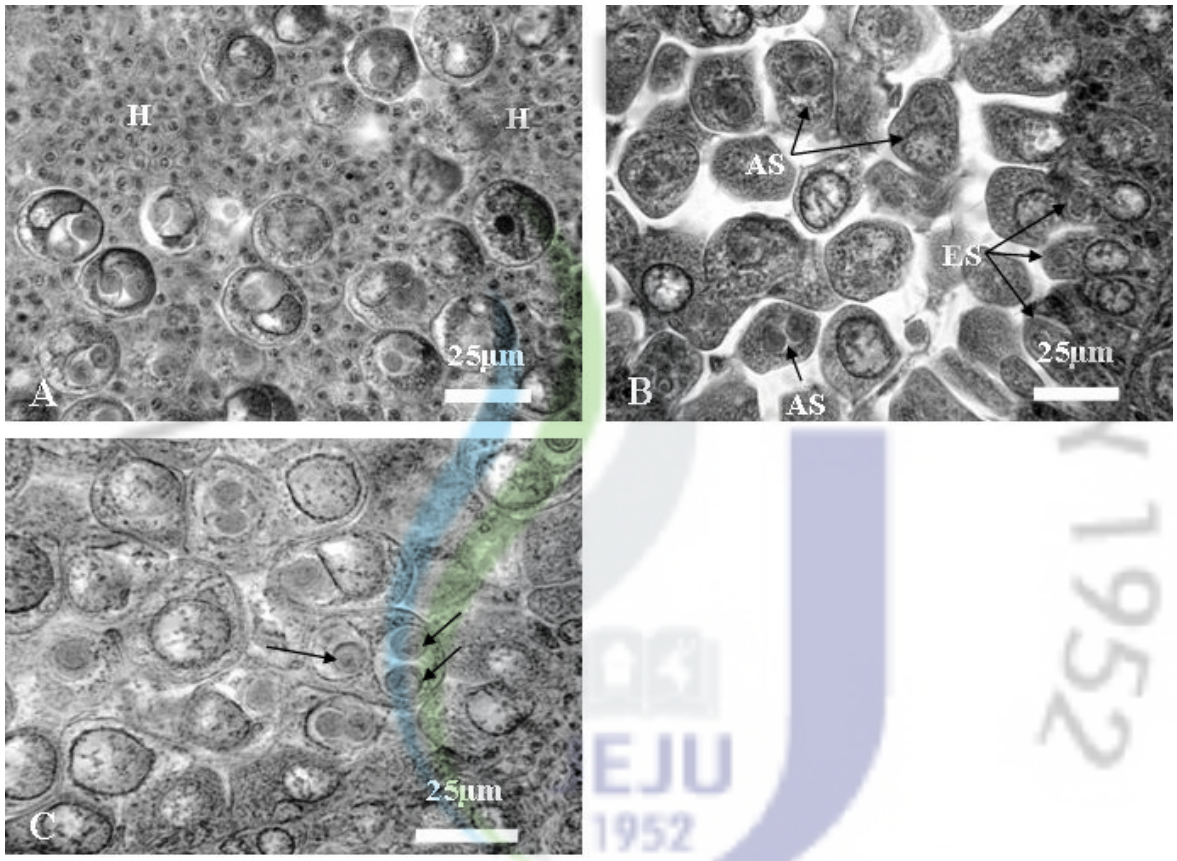

Figure 2-3 Histological section of *M. chungmuensis* infected Pacific oyster. A: The gonad follicles contain with *M. chungmuensis* infected eggs (arrows). Hemocyte accumulation can be observed (H). The parasite invaded oocyte cytoplasm and pressed oocyte nucleus to the edge of the cells. B: The sporulation stage or advanced stage (AS) are mainly found in mature free oocytes. The early stage (ES) of parasite mostly infected the oocyte attached to the follicle wall. C: The sporulation stage of 1 primary cell contains with 1-2 sporont(s) (arrows).

Table 2-1 The number and percentage of oysters at each reproductive stage.

Reproductive stage	Uninfected oyster		Infected oyster	
	N	%	N	%
Resting	59	59.60	0	0
Early developing	28	28.28	0	0
Late developing	0	0	23	23.96
Ripe	0	0	73	76.04
Spawning	1	1.01	0	0
Spent	11	11.11	0	0
Total	99	100.00	96	100.00

Table 2-2 The average egg diameter (μm) and standard deviation (SD) of normal oysters from uninfected area and *M. chungmuensis* infected oyster.

Egg diameter (μm)	Normal oyster	Infected oyster (parasite-free egg)	Infected oyster (infected egg)
Mean	39.63	24.37	23.92
SD	5.65	6.12	4.81
Range	23.76-66.41	9.85-50.02	8.78-40.01
N	650	593	464

3. Egg size

The egg size from infected oysters was divided to parasite-contained egg and parasite-free eggs. The egg size produced from normal oyster and Marteilioides infected oyster was compare by Histological section. The average diameter of normal oyster eggs was 39.63±5.65 µm and the infected oyster eggs were 24.17±5.59 µm (Table 2-2). The infected oyster eggs were significantly ($p<0.05$) smaller than normal oyster from Goseong. From the infected oyster, the egg diameter was also measure separately from infected egg and parasite-free egg. The diameter of infected egg and parasite-free egg was not significant different with the sizes were 23.92+4.81 and 24.37+6.12 µm.

4. Digestive gland atrophy index

The digestive gland atrophy index in uninfected oyster and infected oyster were 1.34 and 1.76. The number of oysters that found in each score was shown in table 2-3. Normal digestive tubule (score 0) in Pacific oyster showed typical columnar epithelia and lumen nearly occluded while abnormal digestive tubule (score 4) showed the progressive digestive gland epithelium destruction and wide lumen (Fig. 2-4).

5. ELISA

Reproductive effort of the female *C. gigas* was assessed in this study using the rabbit anti-oyster egg IgG in ELISA. ELISA was performed in only Marteilioides infected group (N=96) due to lack of egg in gonad of uninfected group. Average gonadosomatic index (GSI) of Marteilioides infected oyster was 7.52±5.50 %. The minimum and maximum value of GSI was 0.53 and 30.37%. Table 2-4 summarized the GSI in *C. gigas* reported from other studies.

Table 2-3 The number and percentage of oyster in different semi-quantitative score of digestive gland atrophy (DGA).

DGA	Uninfected oyster		Infected oyster	
	N	%	N	%
Score 0	25	25	6	6
Score 1	33	33	41	43
Score 2	25	25	19	20
Score 3	14	14	19	20
Score 4	2	2	10	10
Total	99	100	95	100

Table 2-4 Gonadosomatic index (GSI) in *C. gigas* reported from other studies

Study area	Sampling period	% GSI	Reference
Tongyong, South coast, Korea	December, 2007	7.52±5.50	Present study
Gosung Bay, South coast, Korea	May, 2000 (developing) June, 2000 (ripe) July, 2000 (spawn)	26.0 42.3 14.5	Kang et al. (2003)
Gosung Bay, South coast, Korea	April, 2000 (developing) June, 2000 (ripe) (surface, bottom of a long-line suspended culture)	16.4, 15.6 49.5, 41.0	Ngo et al. (2006)
Normandy, France	August, 2005 (full maturity)	59.6	Royer et al. (2008)

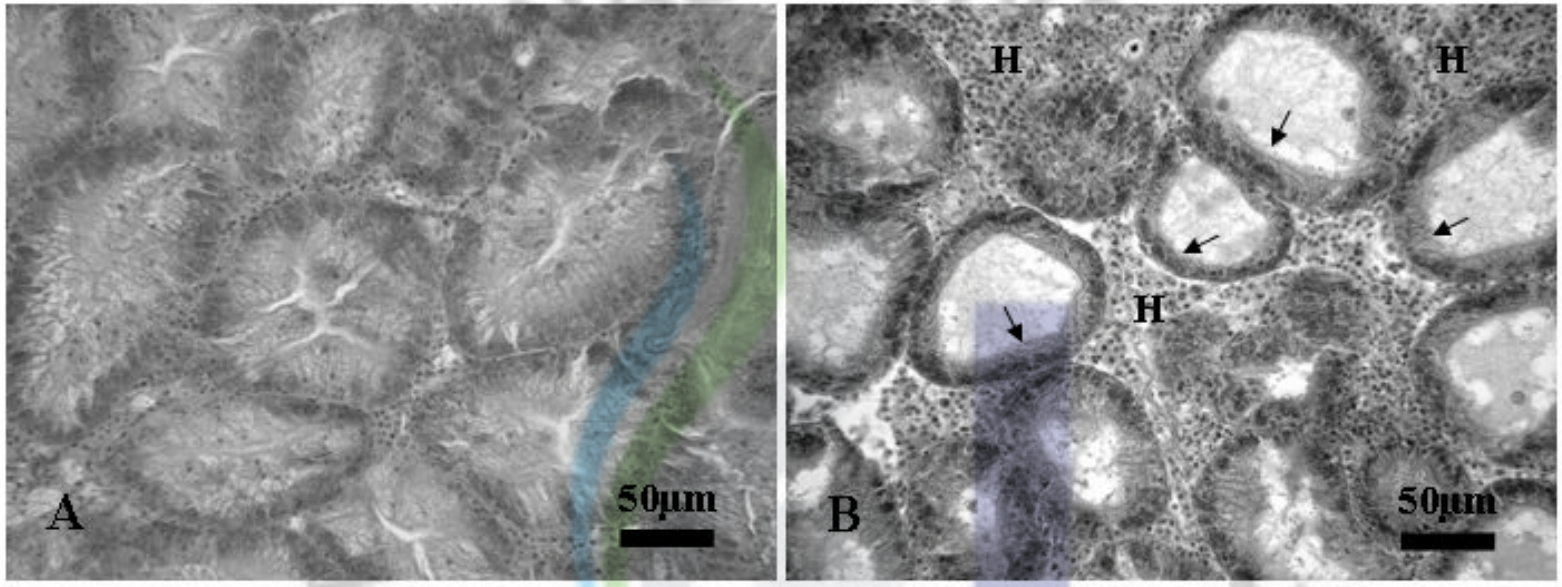

Figure 2-4 Digestive tubules of *C. gigas*. A: Normal digestive tubule, score at 0. B: Atrophy digestive tubules, score at 4. The epithelium cells (arrow) were extremely thin and became cuboidal. The intertubular connective tissue was filled with hemocyte accumulation (H).

6. Biochemical composition

Table 2-5 demonstrated biochemical composition of uninfected and *M. chungmuensis* infected oyster. Protein was the main composition in oyster tissue followed by carbohydrate and lipid. Protein concentration of infected oyster (403.84±29.94 mg/g) was significant higher than uninfected oyster at $p<0.05$. In contrast, carbohydrate and lipid level of Marteilioides infected oyster were significant lower than uninfected oyster. Carbohydrate concentrations were 369.76±50.97 and 108.68±44.41 mg/g in uninfected oyster and infected oyster. Lipid concentrations were 94.39±17.90 and 85.93±18.30 mg/g in uninfected oyster and infected oyster.

Table 2-5 Biochemical composition (mg/g tissue) of uninfected oyster and *M. chungmuensis* infected oyster

Biochemical composition parameter (mg/g tissue)	Uninfected oyster		Infected oyster	
	Mean	SD	Mean	SD
Protein	343.40	23.55	403.84*	29.94
Carbohydrate	269.76*	50.97	108.68	44.41
Lipid	94.39**	17.90	85.93	18.30
N	50		50	

Significant: *$p<0.001$, **$p<0.05$

Discussion

1. Characteristics of *M. chungmuensis*

From past 20 years, the occurrence of *M. chungmuensis* in *C. gigas* was mainly reported from the south coast of Korea. Park and Chun (1989) reported for *M. chungmuensis* for the first time in oyster from Hansan-Geoje Bay with the prevalence 5.3-6.7%. In 2003, Park et al. revealed the prevalence from 13.3 to 57.1% in 1996 and from 28.6 to 61.5% in 1997 from Jinhae Bay, south coast of Korea. Ngo et al. (2003) reported the occurrence of *M.chungmuensis* in year 2000 from Gosung Bay. In this study, our samples also obtained from culture area in south coast which is enzootic area for *M. chungmuensis*. Histology observation revealed the characteristics of *M. chungmuensis* was identical to the previous studies in Korea and Japan (Park and Chung, 1989; Itoh, 2002; Ngo et al., 2003; Tun et al., 2008b). The separation of infected or uninfected oyster grouped was performed by gross appearance only. Every infected oyster was female however it was possible that some of male or indifferent oysters might contain with parasite but did not show any abnormal lesions. Male oysters could be invades by *M. chungmuensis* but parasite had no ability to grow or sporulate (Itoh et al., 2004) therefore *M. chungmuensis* did not cause any pathological lesion on gross appearance of oysters. Normal annual gametogenesis of *C. gigas* cultured in south coast of Korea indicated the active development during April-May and July-August while in December oysters were in indifferent and spent stage (Ngo et al., 2002). In case of infected oysters, the ripe eggs remained in the gonad until sampling period in December while uninfected oysters were in reproductively inactive. Tun et al. (2007) observed 5-month extension of the reproductive period in infected female oyster in *M. chungmuensis* epizootic area. Gametogenesis in oyster is synchronized such that the egg and sperm are released concurrently help to benefit the maximum number of zygote

(Thompson et al., 1996). From histology the infection caused unsynchronized gametogenesis in male and female oysters resulting in the releasing of eggs from infected oyster into water column was not concurrent with the male spawning period. Therefore the high number of eggs both infected and not infected by Marteilioides was not release to the water column during the sperm was ready to fertilize and cause to the decreasing of oyster spat production. We reported the percentage of infected eggs by determine the % infected eggs from microscopic examination of histology slide. The underestimation of intensity of infection might be concerned because the early stage of *M. chungmuensis* was not able to detect.

Ngo et al. (2003) observed large accumulations of hemocytes within or around the follicle wall in heavily infected oysters. It was interesting that no phagocytosis of infected oocytes in oysters collected in winter while uninfected oocytes in normal oyster was found to be phagocytized which introduced the hypothesis that *M. chungmuensis* inhibited phagocytic activity of hemocyte in order to survive in the eggs. In our study, hemocyte accumulations were found in large number of samples which collected in winter time. The distribution of hemocyte infiltration was varied in number of cells and tissue area involved. The presence or level of hemocyte infiltration in infected gonad seemed not to relate with the intensity of infection (% infected oocyte) in this study.

2. Quantification of reproductive effort

The impacts of *M. chungmuensis* on oysters directly resulted in failure of female oyster reproduction. Our study revealed the undersized mature eggs of infected oysters compared with normal oysters in varied sampling period and decreasing of egg production was suspected by ELISA assessment. ELISA indicated that the level of mean GSI in infected oyster was much lower than reported in other studies during active gametogenesis period but equivalent to

spawning (September-October) or spent (December-January) oysters in Ngo et al. (2006) and Kang et al. (2003) observations (Table 3). The histology of infected oyster was classified to be late development or ripe stage from number of oocytes in follicles, follicle size, oocyte shape and stage of oocyte which normally exhibited high GSI. The low GSI was possibly due to some part of gonad was degenerated and substituted by connective tissue. Therefore gonad of infected oyster was not entirely utilized to produce gamete. As individual oyster had different degree of gonad tissue inflammation, GSI was widely ranged from 0.53 to 30.37%. Park et al. (1999) determined the effect of *M. chungmuensis* on the development and growth of oyster larvae produced from parasite infected oysters and suggested that both infected and uninfected eggs from an infected oyster had a low fertilization rate and developed much slower than the uninfected oyster.

3. Digestive gland atrophy

The digestive tubule cells were columnar epithelium but can change to simple cuboidal or squamous depending on the feeding phase (Winstead, 1995). Several studies proposed that unspecific stress, disease, environment pollution or non feeding condition caused epithelial atrophy and large circular lumen (Winstead, 1995; Eble and Scro, 1996). Weis et al. (1995) observed a higher prevalence of more severe metaplasia of the digestive gland atrophy of oysters living on high level of copper and arsenic. The increase of digestive gland epithelium destruction was reported in the mussel *Crenomytilus grayanus* with trematodes invasion (Uscheva and Frolova, 2006). From all 95 infected oysters, only 10 oysters showed progressive digestive gland epithelium destruction (score 4). It is likely that *M. chungmuensis* infection was not directly effects the digestive physiology of the oyster. However, base on the result that the digestive gland of *M. chungmuensis* infected oyster showed higher mean score of digestive

gland atrophy than uninfected oyster, it was possible that the infection could induce stress in oyster and digestive gland atrophy was a side effect of a non specific response to stressful conditions.

4. Biochemical composition

Biochemical composition in *C. gigas* in this study showed the concentration of protein was highest followed by carbohydrate and lipid. Berthelin et al. (2000) suggested that proteins are the major component of *C. gigas* throughout the year while glycogen and lipids remain at a low level. The evaluation of biochemical composition of oyster oocytes showed that the predominant of dry egg constituent was protein (40-50%) (Lee and Hefferman, 1991; Massapina et al., 1999; Kang et al., 2003). The significant higher protein concentration in Marteilioides infected oyster maybe due to protein concentration in oocytes which remained only in infected oyster. The carbohydrate of infected oyster was lower than uninfected oyster more than 50%. The annual changes of carbohydrate in oyster showed high value of glycogen before gametogenesis and dropped during ripeness because the oysters used glycogen in oocyte production (Park et al., 2003). Then the carbohydrate recovered again after spawning (Ngo et al., 2006; Dridi et al., 2007). In winter, whereas the gonad tubule size reduced, storage cells were increase and resulted in the elevation of glycogen storage (Berthelin, 2000). Oppositely, the infected oysters sampled in December seemed to fail in the carbohydrate storage. As Dridi et al. (2007) mentioned that carbohydrate content and especially glycogen storage was considered as bioindicators of environment status and reflected further environment stress, the continuously decrease in glycogen level of infected oyster may lead to nutritional stress and cause of mortality (Tun et al.,2008b; Park et al., 2003). The oyster eggs contained with relatively high total lipid about 20% (Kang et al., 2003; Massipina et al., 1999). The pattern of lipid

accumulation appeared to relate with gametogenesis development and fell after spawning as a result of egg releasing (Thompson et al., 1996; Kang et al., 2000; Dridi et al., 2007). Even though the infected oysters were in active gametogenesis, we found that the total lipid concentration was lower than uninfected oyster. As lipids play an important rule in membrane constituents and reserve energy in embryonic development (Dridi et al., 2007), it was hypothesized that the unsatisfied development of embryo observed by Park et al. (1999) was the result from the scarce amount or unqualified of lipid composition in the oyster eggs.

Part III

Report on the occurrence of *Marteilioides chungmuensis* (Paramyxea) in Pacific Oyster (*Crassostrea gigas*), Suminoe Oyster (*Crassostrea ariakensis*) and Manila Clam (*Ruditapes philippinarum*) in Korean waters

Abstract

Marteilioides chungmuensis is a protozoan parasite recognized as a significant pathogen of the Pacific oyster. The diagnosis of this parasite was routinely examined base on the characteristic of parasite under microscope. During routine monitoring in 2007, histological observations in Pacific oyster *Crassostrea gigas*, Suminoe oyster *C. ariakensis* and Manila clam *Ruditpes philippanarum*, revealed the parasite *Marteilioides chungmuensis* in cytoplasm of oocytes which characterized by the sporulation stage contained 1-2 sporonts in primary cells. To confirmative identified this protozoa, the PCR assay was performed to amplified overlapped fragments of 18s rDNA gene sequence of *M. chungmuensis*. PCR products were sequenced and the phylogenetic affinity of the sequences was determined. The *M. chungmuensis* isolated from Pacific oyster, Suminoe oyster and Manila clam in this study were indentity and also with the ones previously reported in *C. gigas* from Japan and Korea. Herein we reported for the first time of the occurrence of this parasite in Suminoe oyster cultured in Korea.

Introduction

Marteilioides is a paramyxean parasite which is an important group of protists infecting various groups of marine vertebrates (Berthe et al., 2004). However, Marteilioides itself has been reported as a causative agent of ovarian tumor only in Pacific oyster and the occurrence of this protozoan in other bivalve species was rare (table 3-1). Differentiating, detecting parasites and diagnosing parasitic infections have been performed by numerous methods, ranging from the more steadfast morphological and biological techniques to using state-of-the-art biochemical, immunological and molecular assays (Zarlenga and Higgins, 2001). The diagnosis of the Paramyxean parasites are usually easily and only the simple method such as stained tissue imprints and histology have been often used for this parasite group detections (Berthe et al., 2004). However the limited of detection Marteilioides by standard histological techniques or gross observation in early infection may lead to mis-diagnosed (Itoh et al., 2003b). Therefore the full sequence of small subunit ribosomal RNA of Marteilioides was reported (Itoh et al., 2003b) and the development of In situ hybridization (ISH) for parasite detection was established (Itoh et al., 2003a). ISH can visualized parasite development in the host tissue and determine the parasite's location. So this method provided a good tool to clarify the parasite's development. Itoh et al. (2003b) was successful in developing of PCR detection techniques which served high sensitivity and specificity.

The detection and identification of parasites in aquaculture has concentrated on the ribosomal RNA (rRNA) gene array, the ribosomal DNA or rDNA. These genes are ideal targets for diagnosis because they occur in tandem array along the chromosome and this multi-copy arrangement provides multiple targets for primer or probe binding (Cunningham, 2002). The rRNA genes are useful targets for diagnosis tests because there are many copies in the

genome, which can help to ensure good sensitivity, and they offer a mosaic of conserved and variable regions which allow analyses at various levels of resolution (Berthe et al., 2004). The complete sequence of SSU rDNA is useful in the development of molecular probes for the parasite and can help to establish a molecular phylogenetic position for this genus. To date, the diagnosis of Marteilioides in Korea was focused on gross examination and histological observation. The information of rRNA gene sequence of Marteiliodes isolated from *C. gigas* in Korea was reported in GenBank accession number DQ063583 by Park and Choi (2005, unpublished). The sequence of 1197 base pairs indicated that the *Marteiliodes chungmuensis* found in *Crassostrea gigas* in Korea is the same species with the one reported in Japan.

The objective of this study was to report the presence of *M. chungmuensis* in Pacific oyster, Suminoe oyster and Manila clam. Since using molecular diagnosis may fail to detect a particular strain of a pathogen, Berthe et al. (2004) suggested that as many strains as possible from a wide geographic range should be sequenced. Therefore this study reported the small subunit rDNA sequence of *Marteilioides chungmuensis* isolated from the pacific oyster *C. gigas*, Suminoe oyster *C. ariakensis* and Manila clam *Ruditapes philippinarum* collected from different location in Korea.

Table 3-1 Host species of *Marteilioides chungmuensis*

Host	location	reference
Pacific oyster *Crassostrea gigas*	Jinhae bay, Korea	Park et al. (2003)
Pacific oyster *Crassostrea gigas*	Gosung bay, Korea	Ngo et al. (2003)
Pacific oyster *Crassostrea gigas*	Pukman bay, Tongyong, Korea	Jeong et al. (2005)
Pacific oyster *Crassostrea gigas*	Ushimado, Okayama prefecture, Japan	Itoh et al. (2002)
Spiny oyster *Saccostrea echinata*	Darwin harbour, Australia	Hine and Thorne (2000)
Iwagaki oyster *Crassostrea nippona*	Okayama prefecture, Japan	Itoh et al. (2004)
Manila clam *Ruditapes philippinarum*	Kyongnam province, Korea	Lee et al. (2001)
Manila clam *Ruditapes philippinarum*	Oita prefecture, Japan	Itoh et al. (2005)

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Materials and Methods

1. Sample afford

The Pacific oyster *Crassostrea gigas* meat from Tongyong (Fig. 3-1) suspected infection with Marteilioides was sent to the lab and processed for the study of parasite impact to physiological condition of the oyster (see in Part II). The oysters showed the tumor on soft body was selected and the needle biopsy was performed to confirm Marteilioides infection. The infected oysters which microscopically observed the parasite in the egg were then slightly cut on the nodule to allow the eggs release from the tissue. The oyster eggs were washed with phosphate buffer saline (PBS; 0.15M NaCl, 0.01M NaH_2PO_4, pH 7.5) for removing tissue debris (Fig. 3-2). The eggs were preserved in absolute ethanol and stored at -70°C.

The Suminoe oyster *Crassostrea ariakensis* and were collected for routine monitoring. Forty or thirty Suminoe oysters were collected monthly from an estuarine area of the Seomjin River on the southern coast of Korea from January to July 2007 (Fig. 3-1). The only one Marteilioides infected oyster was detected from histological slide.

The National survey of Manila clam *Ruditapes philippinarum* was performed in April and November 2007 (see in Part I). The infected clams were found in south coast sampling sites. The middle part of oysters and clams tissue were removed and processed for histological slides. The remained tissue was lyophilized, grinded and stored as freeze-dried power at -70°C.

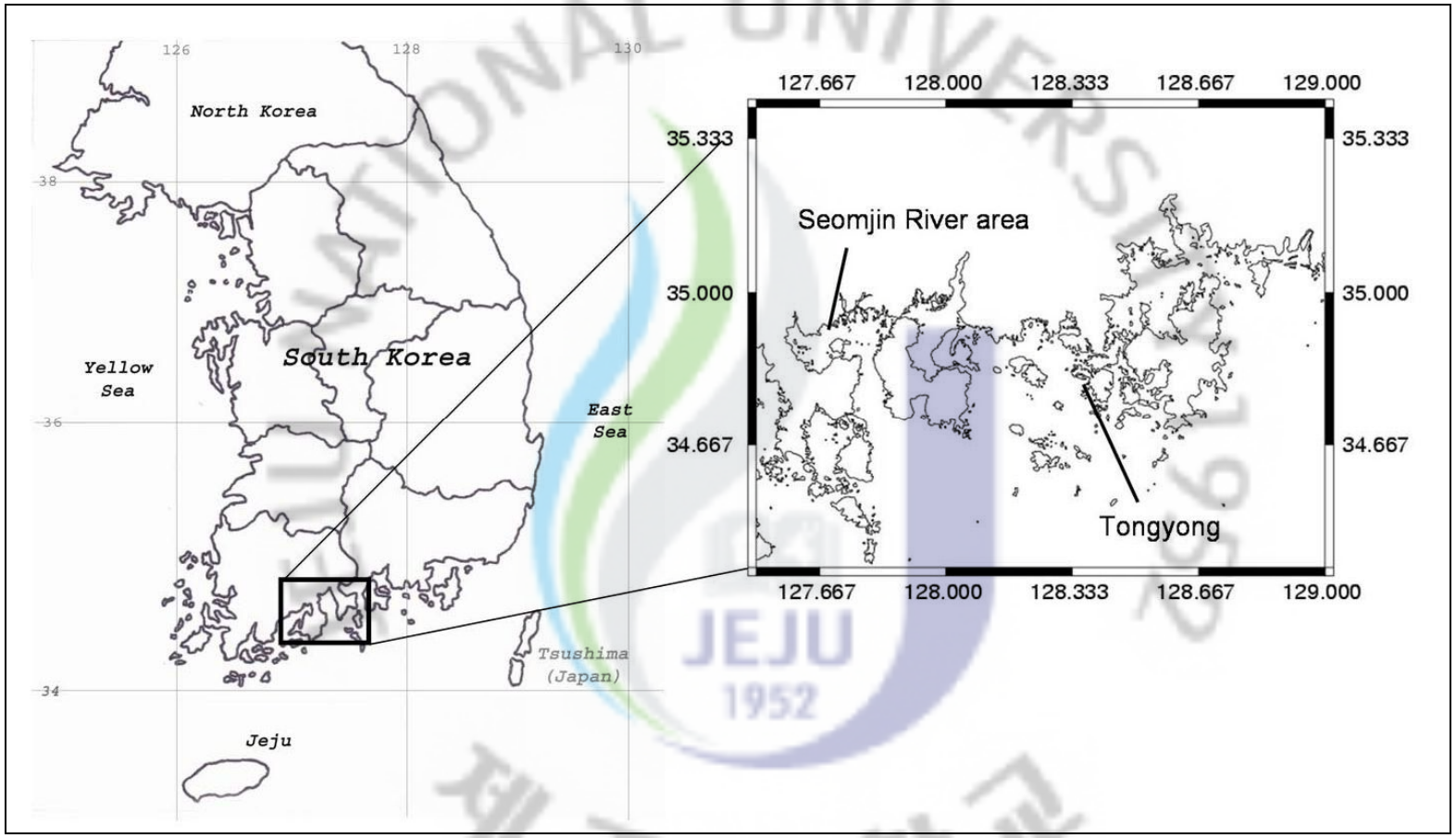

Figure 3-1 Map shows the location where the samples were obtained.

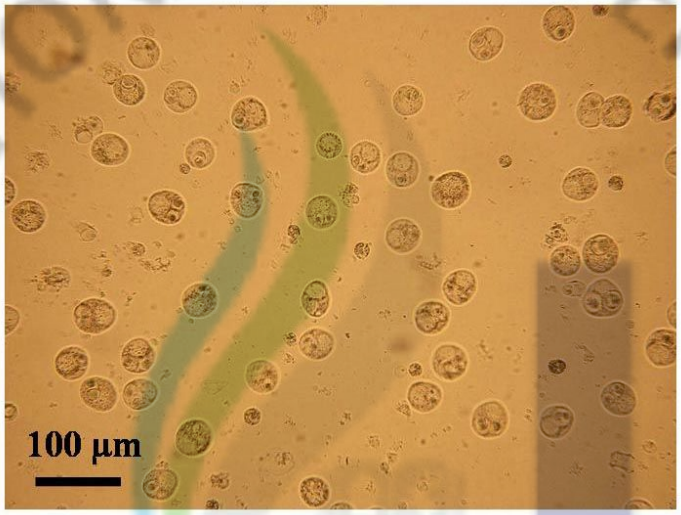

Figure 3-2 The pacific oyster eggs contained with *Marteilioides chungmuensis* collected from infected oyster.

2. Histological preparation

The cross sections, approximately 5 mm thick which contained visceral organ, gill and mantle were taken from each sample and were fixed in Davidson's solution for 24 hours and preserved in 70% ethyl alcohol. The tissue was then embedded in paraffin and sectioned at 5 μm thickness. The tissue were placed on the glass slide and deparaffinized with xylene. Then the tissue was rehydrated with ethanol series and stained with Hematoxylin & Eosin, dehydrated with ethanol series and mounting.

3. DNA amplification, cloning and sequencing

The total DNAs of *M. chungmuensis* infected in pacific oyster were extracted from the oyster eggs preserved in absolute alcohol at -70°C while the parasite of Suminoe oyster and Manila clam DNA was extracted from the freeze-dried whole clam tissue using DNeasy® tissue kit (Qiagen) following the manufacturer's tissue protocol. The concentration of purified DNAs was measured using a spectrophotometer and then the DNA was used as a template for polymerase chain reaction (PCR) amplification.

The 18s rDNA of parasite was amplified by PCR using 2 pairs of primers; PD-18S-F and OPR-2; OPF-2 and PD-18S-R (Table 3-2, Fig. 3-3). PCR reactions were carried out in 50 μl volume according to the standard conditions of Takara Ex Taq (Takara Shuzo). The thermal cycle protocol was follow: Preheating 94°C for 5 min and 30 cycles of denaturation 94 °C for 5 min, annealing 54 °C for 30 sec and extension 72 °C for 2 min, and a further elongation 72°C for 5 min. Amplified product was analyzed using electrophoresis on 1% agarose gel. The gel was cut and DNA was purified by silica-based membrane bound spin column using Power

Gel Extraction Kit (DyneBioinc, Korea). Purified DNA was ligated with pGEM®-T Easy Vectors (Promega) followed the manufacturer's protocol and transform to *Escherichia coli* DH5 alpha. The recombinant plasmid DNA was isoloated based on the modified alkaline lysis method using AccuPrep® Plasmid Extraction Kit (Bioneer). Sequencing reaction was carried out with Automatic genetic analyzer (Applied Biosystems 3130xL). To determine the sequence of 18S rDNA, the oligonucleotide universal primer for sequencing M13 forward and reverse primer was used with the internal primer OPF-3 and 530f (Table 3-2, Fig. 3-3).

4. Data analysis

BLAST searching and pairwise alignment program calculated the similarity of the determined nucleotide sequences of Marteilioides. The sequences were aligned using CLUSTAL W program (Thompson et al., 1994). Phylogenetic analyses were conducted in MEGA 4.0 (Tamura et al., 2007). The evolutionary history was inferred using neighbor-joining method (Saitou and Nei, 1987). Confidence estimated was obtained on the basis of bootstrap generation of 1,000 replicates (Felsenstein, 1985). The numbers of base differences per site from analysis between sequences were conducted in MEGA 4.0 (Tamura et al., 2007).

Table 3-2 The primers used for PCR reaction and DNA sequencing.

Primer	Direction	Sequence	Tm	reference
PD-18S-F	forward	5′- AAC CTG GTT GAT CCT GCC AT-3′	55.4	Itoh et al. (2003a)
OPR-2	reverse	5′-GAC CTT CCG ATT ATC CGC CC-3′	59.1	Itoh et al. (2003b)
OPF-2	forward	5′-CCG CGT TTA CAC CTG TGA CC-3′	57.4	Itoh et al. (2003b)
PD-18S-R	reverse	5′-GAT CCT TCT GCA GGT TCA CCT-3′	54.4	Itoh et al. (2003a)
OPF-3	forward	5′-GGC TGA ATA CCT CTG CC-3′	46.9	Itoh et al. (2003b)
530f	forward	5′- GTG CCA GCM GCC GCG G-3′	64.2	Vossbrinck et al. (1993)

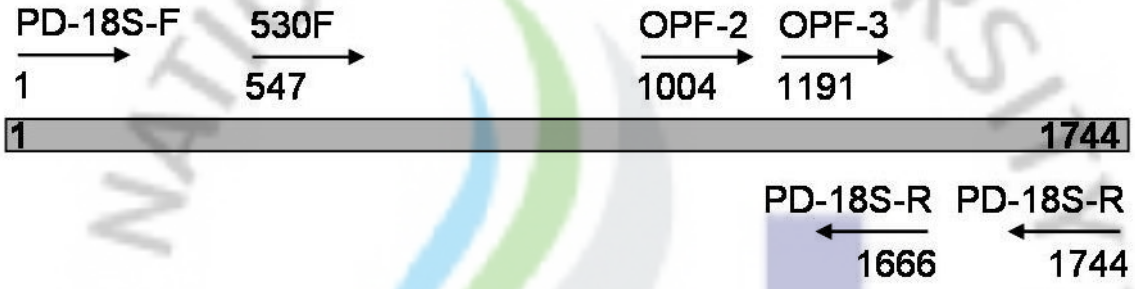

Figure 3-3 Primer annealing sites used in DNA amplification and sequencing the 18S rDNA of *Marteilioides chungmuensis* base on the sequence reported by Itoh et al. (2003b) accession number AB110795.

Results

1. Histological morphology

The various stages of *M. chungmuensis* were observed in Pacific oyster eggs (Fig. 3-4A). The sporulation occurred in oocyte characterized by 1-2 sporonts in the plasmodium. The early stage of parasite were found in early developinging eggs which attached to the follicle wall.

Out of all the Suminoe oyster tissue observed from January to July 2007, only one sample was suspected to be infected with Marteilioides. The infected Suminoe oyster was in early development stage of gametogenesis. The small oval shape oocytes were attached with follicle walls. The follicle size was small. Most of the parasite found in Suminoe oyster was in early stage. The unstained round shape inclusions located in oocyte cytoplasm (Fig. 3-4B). The sporulation was seldom observed in this sample as the parasites were still in early stage.

The prevalence of *Marteilioides* in Manila clam in April and November 2007 was mentioned in Part I. The parasites observed in Manila clam eggs were both early and mature stage (Fig. 3-4C). The parasites was easily detected especially the sporulation stage due to the large eosinophilic sporonts which detached from the primary cells. The mature parasites showed 1-2 sporonts in plasmodium. In one individual egg, 1-3 parasites can be observed.

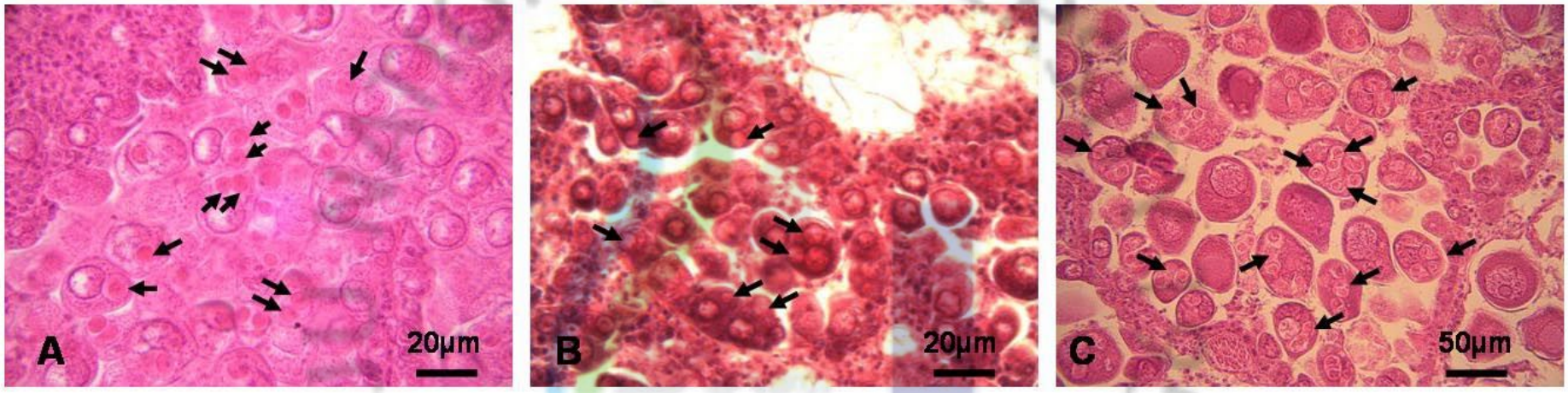

Figure 3-4 Histological pictures of *Marteilioides chungmuensis* infection in *Crassostrea gigas* (3-4A), *C. ariakensis* (3-4B) and *Ruditapes philippinarum* (3-4C). The arrows indicated the parasites in egg cytoplasm.

2. Molecular analysis

The PCR using PD-18S-F/OPR-2 primer pair and OPF-2/PD-18S-R for the *M. chungmuensis* 18s rDNA yielded the expected 1675 bp and 741 bp product (Fig. 3-5). The thicker DNA bands were observed in Lane 1 which was the sample from oyster eggs while the faint bands were observed in the DNA extracted from Suminoe oyster and Manila clam tissue.

The overlapped nucleotide sequences obtained from amplified PCR products were successfully sequenced and finally the 1716, 1734 and 1734 bp of 18s rDNA gene of *M. chungmuensis* originated from *C. gigas* (Fig. 3-6), *C. ariakensis* (Fig. 3-7) and *R. philippinarum* (Fig. 3-8) were established and deposited in GenBank database (Accession no. GU132547, GU132548 and GU132549). GenBank Blast search showed closet identity of our 3 sequences with *M. chungmuensis* isolated from Pacific oyster *C. gigas* in Japan (AB110795) with identities value of 99%, 99% and 98% in the samples from *C. gigas*, *C. ariakensis* and *R. philippinarum* respectively.

To assess the genetic affinity of each parasite sequence, the 18s rDNA sequence of *M. chungmuensis* and closed species were used in a comparative neighbour joining analysis. The GenBank accession numbers of the 11 sequences previously deposited in GenBank and the 3 sequences reported in this study were shown in table 3-3. Final 18s rDNA gene sequence alignment used in the phylogenetic analysis consisted of 669 bp including gaps and missing data. The neighbour-joining method showed the taxonomic position of *M. chingmuensis* infecting Pacific oyster and Suminoe oysters were identical to *M. chungmuensis* previously reported in Japan (Itoh et al., 2003a) and Korea (Park and Choi, unpublished). The *M. chungmuensis* isolated from Manila clam was identified as other strain supported by bootstrap value of 100. *Marteilia refringens* was found to be nested within the same clade with Marteilioides which this clade belong to Paraxyxea (Fig. 3-9). The estimates of evolutionary

divergence between sequences and the nucleotide similarity were demonstrates in table 3-4.

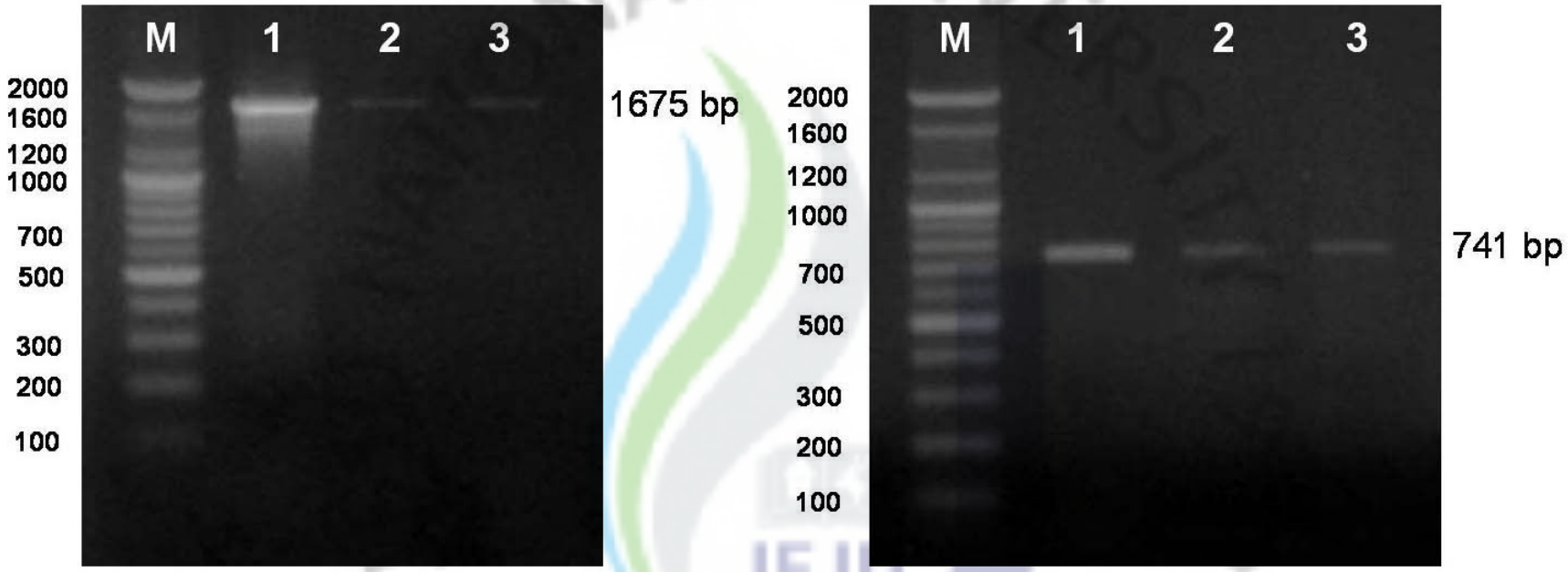

Figure 3-5 A photograph of an agarose gel stained with ethidium bromide and illuminated with UV irradiation showing PCR product from the primer PD-18S-F/OPR-2 (1675 bp) (left) and primer OPF-2/PD-18S-R (741bp) (right). Lane 1: *M. chungmuensis* obtained from *C. gigas*; Lane 2: from *C. ariakensis*; Lane 3: from *R. philippinarum*; M: 100 bp DNA marker.

```
   1  aacctggttg atcctgccat tcctgtgaac tagcgttctg gactaagcca tgcgagtgta
  61  agctcaagac ctgcatcagg tcgaagccgc agacggctcc gtacaagata gttaatccaa
 121  ggctcgatcc aattgcggac atctctggga aactagagcc attacgtgca aactaatgcc
 181  aggttcgcct ggtgcagtta tcggtctgga acgtgccggc ttgccggtgt tcaggtgact
 241  ctggataacc tcgcttaccg cgagtaccac tcgacggtgt gagccacgaa gctatcaccc
 301  atcaattagg cggtacggta gtggcgtacc gcgatcgtca cgggtacggg gaatcagggt
 361  tcgattccgg agagcatgcc tgagagaagg cagacgttgc tacggcatac agcaggcgcg
 421  caaatttgtc aatgttcgga tgaatgaacg agcaagaacg tatggaagga cgagacagtt
 481  ctgtcttcgt cacatccatg agggcagccc tcacagctgc ccgataacca ctggagggca
 541  agtctggtgc cagcagccgc ggtaattcca gctccgggag tttacgggat tattgctgag
 601  actaaaacgt ctgtagtcgg aaccaacggc ggagcgtgcg gtcggacccg ccgggcgaga
 661  tggccgctcc gccacgaccg ttcgttcgcc tcggcgtgcg tcggtccacg tccgtgaccc
 721  aaaccaggtg atcaaggcga gctcgtgctc gatggttttg catggaatcg tagaacgaaa
 781  cctgcgctcg gcgttccaag agcgcaggtg atgatcaacg ggagcggtcg gagacgagtg
 841  aactgcaggg cgaggggtaa aatctgatga tccttgcaag acagccgaaa gcgaaggcac
 901  tcgtctagag cgtttccgtc gataaaggac ggaagccacc gtagtcaaca ggattagata
 961  ccccggtagc ggtgtgcccc aaactatgcc gaccggcccg acgccgcgtt tacacctgtg
1021  accggtgttg ggtccgagga agctggagtg atttggcttc tgggagatta cggctgcaaa
1081  gctgaaactt aaaggaattg acggaagggc accatcaggt ctggattatg cggctcaatt
1141  ggattcaacg cccgacaccc caccaggaca ggagcgcccg atgaagcacc ggctgaatac
1201  ctctgccgat gggcgtgcgg gtggtgcatg gccgccgtag ttcgtgaggc gacttgtctg
1261  gttaattccg acaacgaacg taaccgcact gcccgaacca tggtgcgcgc cgtcgcggtg
1321  cgtcgccaaa ctcgggcaga caaatccgca acgagtctcg tctcggagcg gaaggaagct
1381  acggcgatgg caggtctgtg atgcccctag acgccctgga tcgcacgcgt aatacaatgc
1441  cggcccaagc gcgcaggttc gcccgagagg gtctacaaat cgcacgaaaa gccggcccag
1501  tcgggatcga ggactgcaac tctcctcgtg aacgcggaac acctcgtagt ggcgcgtcat
1561  taacgcgcca cgaatgagtc cctgtccttt gtacacaccg cccgtcgcta cagccaatta
1621  cgtgtcgtcg tcaagaggcc ggacggttcg ccctcgggcg gataatcgga aggtccctga
1681  acgtcggtac gaggagggtg taaaagtcgt aacaag
```

Figure 3-6 Partial sequence of 18S rDNA of *Marteilioides chungmuensis* isolated from Pacific oyster *Crassostrea gigas* (Accession number GU132547)

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   1  aacctggttg atcctgccat tcctgtgaac tagcgttctg gactaagcca tgcgagtgta
  61  agctcaagac ctgcatcagg tcgaagccgc aggcggctcc gtacaagata gttaatccaa
 121  ggctcgatcc aattgcggac atctctggga aactagagcc attacgtgca aactaatgcc
 181  aggttcgcct ggtgcagtta tcggtctgga acgtgccggc ttgccggtgt tcaggtgact
 241  ctggataacc tcgcttaccg cgagtaccac tcgacggtgt gagccacgaa gctatcaccc
 301  atcaattagg cggtacggta gtggcgtacc gcgatcgtca cgggtacggg gaatcagggt
 361  tcgattccgg agagcatgcc tgagagaagg cagacgttgc tacggcatac agcaggcgcg
 421  caaatttgtc aatgttcgga tgaatgaacg agcaagaacg tatggaagga cgagacagtt
 481  ctgtcttcgt cacatccatg agggcagccc tcacagctgc ccgataacca ctggagggca
 541  agtctggtgc cagcagccgc ggtaattcca gctccgggag tttacgggat tattgctgag
 601  actaaaacgt ctgtagtcgg aaccaacggc ggagcgtgcg gtcggacccg ccgggcgaga
 661  tggccgctcc gccacgaccg ttcgttcgcc tcggcgtgcg tcggtccacg tccgtgaccc
 721  aaaccaggtg atcaaggcga gctcgtgctc gatggttttg catggaatcg tagaacgaaa
 781  cctgcgctcg gcgttccaag agcgcaggtg atgatcaacg ggagcggtcg gagacgagtg
 841  aactgcaggg cgaggggtaa aatctgatga tccttgcaag acagccgaaa gcgaaggcac
 901  tcgtctagag cgtttccgtc gataaaggac ggaagccacc gtagtcaaca ggattagata
 961  ccccggtagc ggtgtgcccc aaactatgcc gaccggcccg acgccgcgtt tacacctgtg
1021  accggtgttg ggtccgagga agctggagtg atttggcttc tgggagatta cggctgcaaa
1081  gctgaaactt aaaggaattg acggaagggc accatcaggt ctggattatg cggctcaatt
1141  ggattcaacg cccgacaccc caccaggaca ggagcgcccg atgaagcacc ggctgaatac
1201  ctctgccgat gggcgtgcgg gtggtgcatg gccgccgtag ttcgtgaggc gacttgtctg
1261  gttaattccg acaacgaacg taaccgcact gcccgaacca tggtgcgcgc cgtcgcggtg
1321  cgtcgccaaa ctcgggcaga caaatccgca acgagtctcg tctcggagcg gaaggaagct
1381  acggcgatgg caggtctgtg atgcccctag acgccctgga tcgcacgcgt aatacaatgc
1441  cggcccaagc gcgcaggttc gcccgagagg gtctacaaat cgcacgaaaa gccggcccag
1501  tcgggatcga ggactgcaac tctcctcgtg aacgcggaac acctcgtagt ggcgcgtcat
1561  taacgcgcca cgaatgagtc cctgtccttt gtacacaccg cccgtcgcta cagccaatta
1621  cgtgtcgtcg tcaagaggcc ggacggttcg ccctcgggcg gataatcgga aggtccctga
1681  acgtcggtac gaggagggtg taaaagtcgt aacaaggtat ttgtaggtga acctg
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Figure 3-7 Partial sequence of 18S rDNA of *Marteilioides chungmuensis* isolated from Suminoe oyster *Crassostrea ariakensis* (Accession number GU132548).

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   1  aacctggttg atcctgccat tcctgtgaac tagcgttctg gactaagcca tgcgagtgta
  61  agctcaagac ctgtaccagg tcgaagccgc agacggctcc gtacaagata gttaatccaa
 121  ggctcgatcc aattgcggat atctctggga aactagagcc attacgtgca aactaatgcc
 181  aggttctcct ggtgcagtta tcggtctgga acgtgccggc ttgccggtgt tcaggtgact
 241  ctggataacc tcgcttaccg cgagtccaac tcgacggtgt gagccacgaa gctatcaccc
 301  atcaattagg cggtacggta gtggcgtacc gcgatcgtca cgggtacggg gaatcagggt
 361  tcgattccgg agagcatgcc tgagagaagg cagacgttgc tacggcatac agcaggcgcg
 421  caaatttgtc aatgttcgga tgaatgaacg agcaagaacg tatggaagga cgagacagta
 481  ctgtcttcgt cacatccatg agggcagccc tcacagctgc ccgataacca ctggagggca
 541  agtctggtgc cagcagccgc ggtaattcca gctccgggag ttcacgggat tattgctgag
 601  actaaaacgt ctgtagtcgg aaccaacggc agcgcgtgcg atcggaccca ccgggcgagg
 661  tcgccgcagt gccacgaccg tccgttcgcc tcggcgtgcg tcggtccacg tccgtgaccc
 721  aaaccaagtg atcaaggcga gctcgtgctc gatggttttg catggaatcg tagaacgaaa
 781  cctgcgctcg gcgttccaag agcgcaggtg atgatcaacg ggagcggtcg gagacgagtg
 841  aactgcaggg cgaggggtaa aatctgatga tccttgcaag acagccgaaa gcgaaggcac
 901  tcgtctagag cgtttccgtc gataaaggac ggaagccacc gtagtcaaca ggattagata
 961  ccccggtagc ggtgtgcccc aaactatgcc gaccggcccg acgccgcgtt tacgcatgtg
1021  accggtgttg ggtccgagga agctggagtg atttggcttc tgggagatta cggctgcaaa
1081  gctgaaactt aaaggaattg acggaagggc accatcaggt ctggattatg cggctcaatt
1141  ggattcaacg cccgacaccc caccaggaca ggagcgcccg atgaagcacc ggccgaatac
1201  ctctgccgat gggcgtgcgg gtggtgcatg gccgccgtag ttcgtgaggc gacttgtctg
1261  gttaattccg acaacgaacg taaccgcact gcccgaacta tggtgcgcgc ccgcgggtgc
1321  gtcgccaaac tcgggcagac aaatccgcaa cgagtctcgt ctcggagcgg aaggaagcta
1381  cggcgatggc aggtctgtga tgcccctaga cgccctggat cgcacgcgta atacaatgcc
1441  ggcccaagcg cgcaagttcg cccgagaggg tctacaaatc gcacgaaaag ccggcccagt
1501  cgggatcgag gattgcaact ttcctcgtga acgcggaaca cctcgtagtg gcgcgtcatt
1561  aacgcgccac gaatgagtcc ctgtcctttg tacacaccgc ccgtcgctac agccaattac
1621  gtgtcgtcgt caagaggccg gacggttcgc cttcgggtgg ataatcggaa ggtccctgaa
1681  cgtcggtacg aggagggtgt aaaagtcgta acaaggtatt tgtaggtgaa cctgc
```

Figure 3-8 Partial sequence of 18S rDNA of *Marteilioides chungmuensis* isolated from Manila clam *Ruditapes philippinarum* (Accession number GU132549).

Table 3-3 Identification of analyzed 18S rDNA sequences

Species	Accession number	Source
Haplosporidium montforti	DQ219484	*Haliotis tuberculata*
Haplosporidium edule	DQ458793	*Penaeus vannamei*
Haplosporidium nelsoni	AB080597	*Crassostrea gigas*
Haplosporidium lusitanicum	AY449713	
Haplosporidium costale	U20858	oyster
Urosporidium crescens	U47852	
Marteilia refringens	AJ250699	*Mytilus edulis* and *Ostrea edulis*
Marteilioides chungmuensis	AB110795	*Crassostrea gigas* (reported in Japan)
Marteilioides chungmuensis	DQ063583	*Crassostrea gigas* (reported in Korea)
Marteilioides chungmuensis	GU132547	*Crassostrea gigas* (present study)
Marteilioides chungmuensis	GU132548	*Crassostrea ariakensis* (present study)
Marteilioides chungmuensis	GU132549	*Ruditapes philippinarum* (present study)
Allogromia sp.	Z69607	
Peneroplis planustus	FM877697	

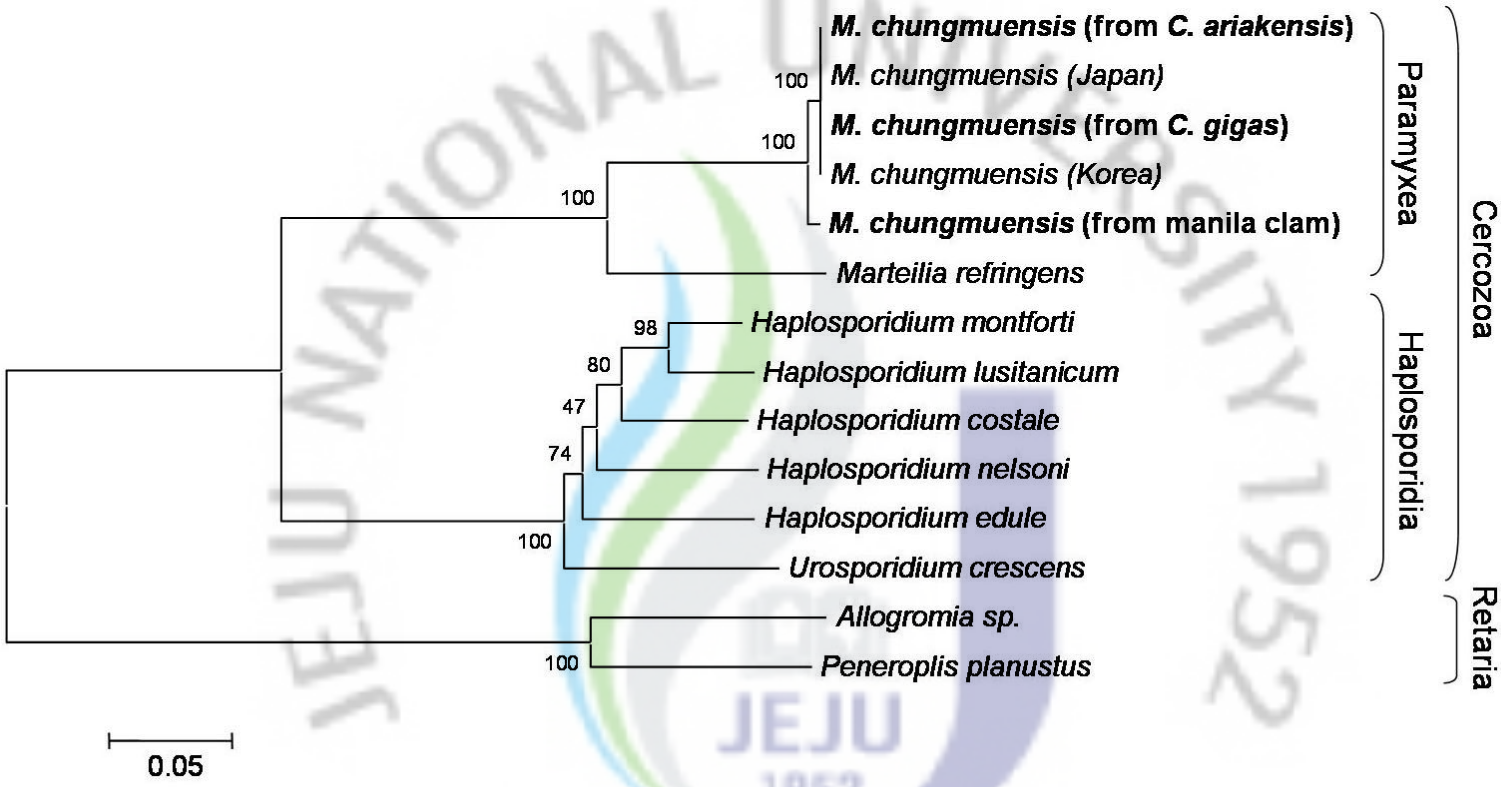

Figure 3-9 The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.35466407 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 669 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

		Nucleotide similarity													
		H.mon	*H.edu*	*H.nel*	*H.lusi*	*H.cos*	*U.cres*	*M.refr*	M._Jap	M._Kor	M._gig	M._ari	M._clam	*Allo sp*	*P.pla*
Evolutionary divergence	*H.mon*		86.8	86.3	93.4	88.8	82.2	58.9	59.8	59.8	59.8	59.8	59.6	36.4	36.4
	H.edu	0.132		84.9	86.3	87.4	83.2	58.5	58.7	58.7	58.7	58.7	58.4	34.2	36.6
	H.nel	0.137	0.151		87.3	87.6	83.8	57.3	58.6	58.6	58.6	58.6	58.2	35.8	36.9
	H.lusi	0.066	0.137	0.127		90.5	83.1	57.6	58.5	58.5	58.5	58.5	58.2	35.3	34.9
	H.cos	0.112	0.126	0.124	0.095		83.9	58.6	58.7	58.7	58.7	58.7	58.6	34.8	34.9
	U.cres	0.178	0.168	0.162	0.169	0.161		56.1	57.6	57.6	57.6	57.6	58.0	35.3	36.0
	M.refr	0.411	0.415	0.427	0.424	0.414	0.439		82.2	82.2	82.2	82.2	82.2	33.8	33.8
	M._Jap	0.402	0.413	0.414	0.415	0.413	0.424	0.178		100.0	100.0	100.0	99.0	33.1	33.5
	M._Kor	0.402	0.413	0.414	0.415	0.413	0.424	0.178	0.000		100.0	100.0	99.0	33.1	33.5
	M._gig	0.402	0.413	0.414	0.415	0.413	0.424	0.178	0.000	0.000		100.0	99.0	33.1	33.5
	M._ari	0.402	0.413	0.414	0.415	0.413	0.424	0.178	0.000	0.000	0.000		99.0	33.1	33.5
	M._clam	0.404	0.416	0.418	0.418	0.414	0.420	0.178	0.010	0.010	0.010	0.010		33.3	33.7
	Allo sp	0.636	0.658	0.642	0.647	0.652	0.647	0.662	0.669	0.669	0.669	0.669	0.667		81.4
	P.pla	0.636	0.634	0.631	0.637	0.651	0.640	0.662	0.665	0.665	0.665	0.665	0.663	0.186	

Table 3-4 Estimates of Evolutionary Divergence between Sequences. The number of base differences per site from analysis between sequences is shown in left-lower part and nucleotide similarity is shown in right-upper part. All results are based on the pairwise analysis of 14 sequences. Analyses were conducted in MEGA4. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 669 positions in the final dataset. *H.mon=Haplosporidium montforti, H.edu=H.edule, H.nel=H.nelsoni, H.lusi=H.lusitanicum, H.cos=H.costale, U.cres=Urosporidium creascens, M. refr=Marteilia refringens,* M._Japan=*Marteilioides chungmuensis* reported in Japan, M._Kor=*M. chungmuensis* reported in Korea, M._gig; M._ari; M._clam= *M. chungmuensis* isolated from Pacific oyster; Suminoe oyster and Manila clam in this study. *Allo sp= Allogromia sp*, *P.pla=Peneroplis planustus*

Discussion

The *M. chungmuensis* is an important parasite of Pacific oyster because it causes pathological affect to the host. The infection rate of *M. chungmuensis* in Korea has increased every year and the parasite occurs throughout the yeat (Park, 2005). In contrast, the low prevalence of marteilioidosis in other species was mentioned (Hine and Thorne, 2000; Lee et al., 2001; Itoh et al., 2004; Itoh et al., 2005) including Suminoe oyster and Manila clam in this study.

Oysters are considered to be the most important molluscan shellfish in Korean aquaculture. In Korea, the Pacific oyster is widely cultured along the southern coast where a number of small, shallow bays are protected by numerous islands (Choi, 2008) thus the occurrence of *M. chungmuensis* was mainly reported from south coast area. The histological characteristic of parasite in our samples supported that the animal may infect with *M. chungmuensis*. The 18s rDNA sequence of parasite confirmed the *M. chungmuensis* isolated from three bivalves species are identical or very closed strain. Therefore it is possible that the protozoan *M. chungmuensis* which normally infected Pacific oyster in endemic area of southern coast was transmitted to Suminoe oyster and Manila clam. However those bivalves may be not a natural host of parasite, due to the high prevalence and severe pathological affects were not observed. Furthermore, the habitat of Suminoe oyster was in estuaries which different from Pacific oyster and may effect the growth and survival of the parasites. The Suminoe oyster has been considered a potential aquaculture species in Korea. The wild populations have been identified in two major estuaries, the Seomjin and Kawha River estuaries (Yoon et al., 2008). To increase output of the Suminoe oyster culture, improving the quality and production of seed, the studies in reproductive biology of this oyster has been done (Kim, 2009; Yoon et al., 2008). To date there was no report about the parasite infection in the Suminoe oyster cultured

in Korea. Our studies first reported the *M. chungmuensis* in Suminoe oyster from Seomjin River. Even though the prevalence was low and the impact of parasite to this oyster was not clear, we suggested not overlooking the infection of this parasite since it may affect the reproduction of the oysters especially when the intensive culture established.

The ultrastructure of the parasite which contained with two sporonts in plasmodium with each sporont containing one spore was described (Itoh et al., 2005). As the feature of spore was identical to those *M. chungmuensis* reported in Pacific oyster, Itoh et al. (2005) suggested that it is possible that Marteilioides found in Manila clam was the same species. Lee et al. (2001) reported the *Marteilioides* sp. infected in Manila clam and the diagnosis was based on histological studies. The DNA sequencing of the parasite isolated from Manila clam in this study indicates the high identity with *M. chungmuensis* previously reported in Korea and also with those isolated from Pacific oyster and Suminoe oyster. *M. chungmuensis* was also reported in *C. nippona* in Japan (Itoh et al., 2004). The result supported that this parasite may has wide host range, able to infect other bivalve species and not have strict host specificity especially in the oysters (Itoh et al., 2004).

While *M. chungmuensis* caused tumor-like nodule in the gonad of Pacific oyster resulting in the rejection from the market, the Manila clam infected with this parasite did not show any gross lesion. Itoh et al. (2005) revealed that the parasite may not have a negative impact on clams however it is possible that Marteilioides infection may also reduce fecundity of the clam and hence cause a resource reduction and further investigation is required.

rRNA is present in all extant species and presumably dates back to the earliest forms of life. It thus reflects the evolutionary history of life itself, and can be used to establish the evolutionary relationships between all species on earth (Smit et al., 2007). The information in 18s rRNA sequence is useful to classified this parasite group because the protozoan Cercozoa is

the only eukaryote phylum established primarily as a result of molecular phylogenetic discoveries (Cavalier-Smith and Chao, 2003a). According to Cavalier-Smith and Chao (2003a), the molecular data indicated that Haplosporidia and Paramyxea are an order Ascetosporea in Phylum Cercozoa. The result of phylogenetic analysis supported that Marteilioides was grouped with the Paramyxean which is same with *Marteilia refringens*. The *M. chungmuensis* isolated from Manila clam was dimorphism separated from those isolates from oyster which may related with the parasite biology such as life cycle, host reaction and vilurence.

Part IV

Induction of *Perkinsus olseni* hypnospore in Manila clam *Ruditapes philippinarum* without Fluid Thioglycollate Medium

Abstract

The *Perkinsus* culture using fluid thioglycollate medium (FTM) has been extensively adapted for perkinsosis diagnosis. The hypnospores develop in FTM are spherical, uninucleated cells, characterized by their large defined vacuole and a peripheral ring of cytoplasm which stain blue-clack with Lugol's iodide. There has been reported of this *Perkinsus* stage in *Perkinsus marinus* but not available for *P. olseni* and the studies of *Perkinsus* hypnospore were mainly focused on the hypnospore from FTM culture. In this study the existence of hypnospore stage of *P. olseni* was evaluated in Manila clam *Ruditapes philippinarum* under different condition. The live Manila clam from known high infection area (Sonchae and Hwangdo) was used in this study. The hypnospore formation was confirmed by 2M NaOH treatment and Lugol's iodide solution staining. We successful detected the small hypnospore in live clam (4.29±0.55 μm) (no incubation) and in clam tissue incubated in conical tube, petridish and the whole clam (with shell) wrapped with parafilm. The highest growth of hypnospore was found in wrapped clam which is imitated the phenomenon of diseased clam that the infected clam may burrow in the sediment and died within the shell. The hypnospore produced from this method was increase up to 70 μm after 6 days incubation at 25°C while low temperature (4°C) was highly affected the enlargement of hypnospore. The anaerobic condition and decomposed death tissue of the clam seems to be the factor that promoted the hypnospore growth. This study indicates that hypnospore can survive in low temperature and under unfavorable condition (low nutrient, aerobic condition) and seawater was not the essential factor for hypnospore growth. The result supported that hypnospore is the key stage of *Perkinsus* to prolong the life span in the environment.

Introduction

The protistan parasites of the genus *Perkinsus* affects a wide variety of mollusks around the world such as *Perkinsus marinus* in the eastern oyster *Crassostrea virginica* (Mackin et al., 1950; Hewatt and Andrews, 1954), *Perkinsus olseni* in the abalone *Haliotis rubra* (Lester and Davis, 1981), pearl oyster *Pinctada maxima* (Norton et al., 1993), Manila clam *Ruditapes philippinarum* (Choi and Park, 1997; Hamaguchi et al., 1998), Venus clam *Protothaca jedoensis* (Park et al., 2006b), Undulated surf clam *Paphia undulate* (Leethochavalit et al., 2004), *P. atlanticus* in Carpet shell clam *Tapes decussates* (Azevedo, 1989), *P. qugwadi* in Japanese scallop *Patinopecten yessoensis* (Bower et al., 1992) and *P. honshuensis* in Manila clam *R. philippinarum* (Dungan and Reece, 2006). *Perkinsus* has been reported as a causative agent of mass mortalities (Andrew and Hewatt, 1957; Azevedo, 1989; Andrews, 1996; Choi and Park, 1997). Therefore, among molluscan diseases, *Perkinsus* has resulted in the most severe economic losses and researchers from around the world have focused considerable effort on studying the *Perkinsus* parasites (Villalba et al., 2004).

The small, nearly spherical sporelike bodies of *P. marinus* are the forms most commonly observed in oyster, normally range from 2-20 μm. The most distinctive feature is a very large, partially eccentric vacuole occupying the greater portion of the cell (Ray and Chandler, 1954). The reproduction of *P. marinus* in the host oyster was binary fission which immature thalli divide progressively into 2, 4, 8 or more cells, the cytoplasm appearing to cleave as nuclear division proceeds. The nuclear production in the immature thallus to form a multinucleate stage without corresponding cytoplasmic cleavage was also observed. When liberated in the sea water, stages in the oyster begin a saprophytic reproductive cycle which differs in some respects from that in the oyster. The growth results in laying down a definite

wall which is cellulose. The sporangia produce aplanospores which may develop flagella and become zoospores in seawater (Mackin and Boswell, 1956).

Andrews (1996), according to Mackin (1962) mentioned that transmission of *Perkinsus* is direct from infected dying oysters to other hosts of the species. All stages appear to be infective or become so when the host dies and prezoosporangia are released into marine waters. Placing uninfected oyster in the same container with infected oysters, the uninfected ultimately become infected. Chu (1996) compared the infectivity of the two life stage, meront and prezoosporangia to figure out the principle stage for transmitting disease in the field. Results indicated that meronts are probably the primary transmission agent in nature due to the higher infection prevalence and intensity than oysters inoculated with prezoosporangia.

Ray (1952) discovered a simple technique for detecting the organism in oyster tissue. The method consists of placing pieces of tissue in a fluid thioglycollate medium (FTM), which has been fortified with antibiotics to suppress bacterial growth. When the *Perkinsus* cells were placed in nutrient media containing yeast extract and dextrose, enlargement of cell took place very rapidly. Cells beginning at about 10 microns may reach 70 microns in size in two to three days incubation. Such enlarged cells have thickened walls which turn blue when treated with Lugol's solution (Ray, 1954). The hypnospores are spherical, uninucleated cells, characterized by their large defined vacuole and a peripheral ring of cytoplasm (Perkin and Menzel, 1967).

Without FTM, the enlarge form of *P. marinus* reaching a maximum diameter of 4 μm and possessing a thick, cyst-like wall have been observed in dying oyster and even live oyster which similar to the one which develop under cultural condition (Ray and Chandler, 1954). Mackin (1961) described the hypnospore which characterized by a thickened wall, increase size, and increases relative size of the vacuole with its contained nutrient material, rarely seen in oyster tissues, they probably develop in seawater under certain unknown conditions and serve as

overwintering spores. Chu (1996) stated that the prezoosporangia (hypnospores) which developed after placed the meronts in FTM was sometimes observed in moribund and dead oyster tissues. In culture condition, after incubation in FTM, the hypnospore usually produce zoospores after transfer to seawater in 4-5 days. However it is unclear whether prezoosporangia released in sea water from moribund and deceased oyster would sporulate in nature.

In Korea, the Manila clam populations from commercial clam beds showed high levels of *Perkinsus* infection and exhibited severe pathological symptoms and high infection intensity. Consequently, Park and Choi (2001) suggested that Perkinsosis may responsible for the annual mass mortality of clams and possibly long-term decline in harvests (Park et al., 1999; Park and Choi, 2001). The *Perkinsus* of Manila clam landing in Korea was identified as *P. olseni* (Choi et al., 2005). To date, there is no reported of the natural existence of *P. olseni* hypnospore in dead clam tissue. Furthermore, the studies of hypnospore stage of *Perkinsus* were mainly focused in the FTM cultured hypnospore, such as morphology and sporulation (Perkins and Menzel, 1966), effect of temperature and salinity (Chu and Greene, 1989) and hypnospore enlargement in different tissue (McLaughlin and Faisal, 1999).

The environmental condition was clearly a factor control of *Perkinsus* infection (Andrew and Hewatt, 1957). Temperature appears to be the most important environmental factor affecting the large scale geographic distribution of *P. marinus*. The highest parasite prevalences and intensities are observed following maximal summer temperature. Infection intensity declines as temperatures decline in winter, however the parasite persists at temperatures as low as 0-5 ^{o}C (Burreson and Ragone Calvo, 1996). In this study we attempt to ascertain the existence of the hypnospore stage of *P. olseni* in lived or deceased Manila clam induced in different condition and temperature. The experiment was applied to imitate the

natural phenomenon of moribund clam in clam bed to find out the condition which promoted the growth of *Perkinsus* hypnospore in clam tissue.

Materials and Methods

1. The formation of hypnospores in clam tissue without FTM

To obtaine *Perkinsus* hypnospores from Manila clams tissues, Manila clams were collected from Sonchae (commercial clam bed located in west coast which is high *Perkinsus* infection area). The clams were divided to 4 groups (10 clams each). 20 clams were opened and the whole body tissue were chopped, pooled and seeded in ten 15 ml-conical tubes. Five samples in conical tube were incubated without any addition (Group 1) and the other five tubes were added with 5 ml sterile seawater each (Group 2). Group 1 and 2 was performed to find out wheather seawater was the essential factor for hypnospore formation. In group 3, 10 whole live Manila clams were put in plastic chamber, sea water was supplied and the chamber was sealed. The experimental condition of this group was imitated the situation when the moribund clam slowly died in the sea. In group 4, 10 whole live clams were individual wrapped with parafilm and put in plastic chamber. The wrapped Manila clams were created under the idea that diseased clam may bury under the bottom sediments and died within the closed shell due to the pressure of the sediments. Clams in group 4 were assumed to be in an anaerobic condition or lower oxygen condition compare with group 3. Every sample was kept in dark condition at room temperature for 6 days. The hypnospore formation was detected following the method of Choi et al. (1989). Briefly, the clam tissue were treated with NaOH 2M for 30 minutes (2 times) to digest the remaining clam tissue and washed with phosphate buffer saline (PBS; 0.15M NaCl, 0.01M NaH_2PO_4, pH 7.5) by centrifuge at 3000 rpm for 5 minute. The pellets (hypnospore) from each group were pooled, resuspended in PBS and confirm by Lugol's iodide staining. The size of hypnospore was measured by image analysis.

2. Comparative study of the growth and number of hypnospore cultured in FTM and incubated in anaerobic induced condition

From the result of experiment 1, the hypnospores were possible and easily induced without using FTM especially in the wrapped clam. The FTM provided high nutrient and anaerobic condition which are ideal for hypnospore growth. Therefore all *Perkinsus* including different life stages are retrieved from the host and become hypnospore (Fisher and Oliver, 1996). In this experiment, the size and number of hypnospore induced from the wrapped clam was compared with FTM culture by using the same individual clams.

Ten Manila clams from Sonchae (commercial clam bed located in west coast which is high *Perkinsus* infection area) were opened and longitudinal dissected into 2 parts and the weight of each part were measured. One part of clam tissue was seeded in 10 ml Fluid thioglycollate medium (FTM) (Fluka) fortified with antibiotic at final concentration; chloramphenicol (Oxoid) 0.16 mg/ml and nystatin (Oxoid) 160 unit/ml to suppress the bacterial and fungi growth (Ray, 1954). The other part was put back in the shell and wrapped with parafilm to promote anaerobic condition. After incubation for 1 week in dark at room temperature, hypnospore were observed after 2M NaOH digestion (Choi et al., 1989) and stained with Logol's iodide 2%. *Perkinsus* hypnospores which are spherical and stained with blue colour were counted under light microscope using hemocytometer. The photo of hypnospore was taken and the hypnospore diameter was measured from the digitized images using Image Pro® image analysis software. The size and number of the hypnospores from individual clams were compared.

3. Enlargement of hypnospore in different conditions and temperatures

The similar size of Manila clams from Hwangdo (commercial clam bed located in west

coast which is high *Perkinsus* infection area) was used for the experiment. The clams (total 480 clams) were divided to 4 groups (120 clams each). Since the gill was found to be the major site of *Perkinsus* infection (Azevedo, 1989; McLaughlin and Faisal, 1999), this experiment was conducted using gills. Total 480 clams were opened and the left and right gills were removed and individual divided into 2 parts. Each part of gill was weighted, one part was used for incubated at 4°C and the other part at 25°C (room temperature). In group 1; the gill was incubated in FTM at 4°C and 25°C. Group 2; the gill was directly put in conical tube and capped, incubated at 4°C and 25°C. This closed system (capped tube) was assumed to provide low oxygen condition. Group 3; the gill was place in Petri dish and incubated at 25 °C. This group was thought to be an aerobic condition as the petridish could allow air exposure. Group 4; the gill was individually wrapped in aluminum foil and incubated in closed chamber. The oxygen was absorbed with the simultaneous generation of carbon dioxide using AnaeroGen™ (Oxiod). The chambers which were anaerobic condition were then kept at 4°C and 25°C. The tissue preparation from every group was detected for the hypnospore every day until 6 day incubation. To digest the remaining clam tissue, the samples were incubated in 10 ml 2M NaOH for 30 min 2 times. The suspensions were then centrifuge at 3000 rpm and the pellets were washed in PBS. The hypnospores were count by hemocytometer under light microscope and measure the size using Image Pro® image analysis software.

4. The presence of hypnospore stage in live Manila clam

The Manila clams from Hwangdo (commercial clam bed located in west coast which is high *Perkinsus* infection area) were used in this study. Total 20 clams were open and the gilled was removed and directly digested with 5 ml 2M NaOH for 30 min. The suspension was centrifuge and NaOH was removed. The pellet was then washed with PBS by centrifuge at

3000 rpm for 5 minute. Finally the pellet was stained with 2% Lugol's iodide and observed under light microscope to determine the presence and abundance of hypnospore characterized by blue-black stained in clam tissue.

To confirm that only hypnospore can survive in 2M NaOH and the cell wall stains with Lugol's solution, the trophozoite stage was cultured in DMEM according to Ordás and Figueras (1998). One ml of Manila clam hemolymph was seeded in 24-well polystyrene plates. One ml of sterile seawater prepared with antibiotic (4000 IU/ml penicillin/streptomycin) was added to each well. After incubation in 22°C, the seawater was removed and the pellet was resuspended with 1 ml of Dulbecco's Modified Eagle Medium (DMEM):Ham's F-12 (1:2) with 50 mM Hepes buffer, 3.5 mM sodium bicarbonate and 5% Fetal Bovine Serum (FBS) in 30 ppt sterile seawater. After 2 days incubation the trophozoite was collected and stained with Lugol's solution and treated with 2M NaOH.

Result

1. The formation of hypnospores in clam tissue without FTM

The variety size of spherical hypnospore characterized by blue-black staining after Lugol's iodide solution treatment was observed in every group. The average size of hypnospore in each group was shown in Table 4-1 and Figure 4-1. After 6 days incubation, the clam wrapped with parafilm (group 4) had the largest size of hypnospore and high density in number followed by the whole clams with seawater (group 3), the minced clam tissue in conical tube with seawater (group2) and the minced clam tissue in conical tube (group 1). The hypnospore from group 3 and 4 (whole clams with shell) were significant larger than group 1 and 2 (clam tissue).

2. Comparative study of the growth and number of hypnospore cultured in FTM and incubated in anaerobic induced condition

The size and number of hypnospore developed without culture medium were smaller than hypnospore in FTM in all clam samples. The hypnospore diameters were 11.8-137.0 μm and 6.8-70.29 μm in FTM-incubated and wrapped clam group respectively. Table 4-2 and figure 4-2 showed the hypnospore diameter in each samples. The number of *Perkinsus* hypnospore per mg tissue was 26-5216 cells and 15-3867 cells in FTM-incubated and wrapped clam group (Table 4-2 and Fig. 4-3). From the assumption that all *Perkinsus* cells in clam tissues become hypnospore in FTM, the result indicated that 15.82-53.40% of *Perkinsus* in clam tissue were possible to grow and develop into hypnospore. Figure 4-4 showed the hypnospore developed in FTM and in wrapped clam.

3. Enlargement of hypnospore in different conditions and temperatures

The hypnospore developed in FTM reached the maximum size within 5 days (54.7 μm) after incubate at temperature 25°C while the growth rate of hypnospore in others group (Table 4-3 and Fig. 4-5) was stable until 6 days incubation and the size of hypnospore was not greater than 10 μm. Among the experiment conditions, except the FTM assay, the hypnospore that grew in the conical tube has largest size followed by anaerobic chamber and the hypnospore developed in Petri dish. The clearly different of hypnospore growth was found in the different incubation temperature. At 4 °C the hypnospore diameter was much smaller than the one incubated in 25 °C in every group. The number of *Perkinsus* was not increase by incubation period (Table 4-4 and Fig. 4-6). The number of hypnospore detected from FTM 25 °C was higher than other groups.

4. The presence of hypnospore stage in live Manila clam

After digested the gill with 2M NaOH, the small hypnospore stained with light blue color after Lugol's solution treatment was observed (Fig. 4-7A). The average number of small hypnospore from 20 Manila clams was 466 cells/mg gill weight. The maximum and minimum numbers were 3087 and 4.1 cells/mg gill weight. The size of small hypnospore was found from 2.91-5.06 μm, the average size was 4.29±0.55 μm. While the hypnospore from live Manila clam was positively stained with Lugol's solution and survived in 2M NaOH, the trophozoite obtained from DMEM culture was not stained blue color by Lugol's solution but stained brown from the iodine (Fig. 4-7B) and after treated with 2M NaOH, all the cells were dissolved.

Table 4-1 The hypnospore size from the Manila clam incubated in different condition without FTM. **Clam tissue**; The clams were opened and the whole body tissue were chopped, pooled and seeded in 15 ml conical tubes without other addition. **Clam tissue with seawater**; whole body tissue were chopped, pooled and seeded in 15 ml conical tubes and add some sterile seawater. **Whole clam with sea water**; the Manila clams were put in plastic chamber, sea water was supplied and the chamber were sealed. **Wrapped clam**; the clams were wrapped with parafilm and put in sealed plastic chamber.

Group	N	Mean	SD	Maximum	Minimum
1. clam tissue	8	19.58	3.25	25.91	16.12
2. clam tissue with seawater	31	24.02	6.96	37.09	8.58
3. whole clam with seawater	25	30.88	11.95	63.76	9.56
4. wrapped clam	74	32.56	9.30	61.01	15.88

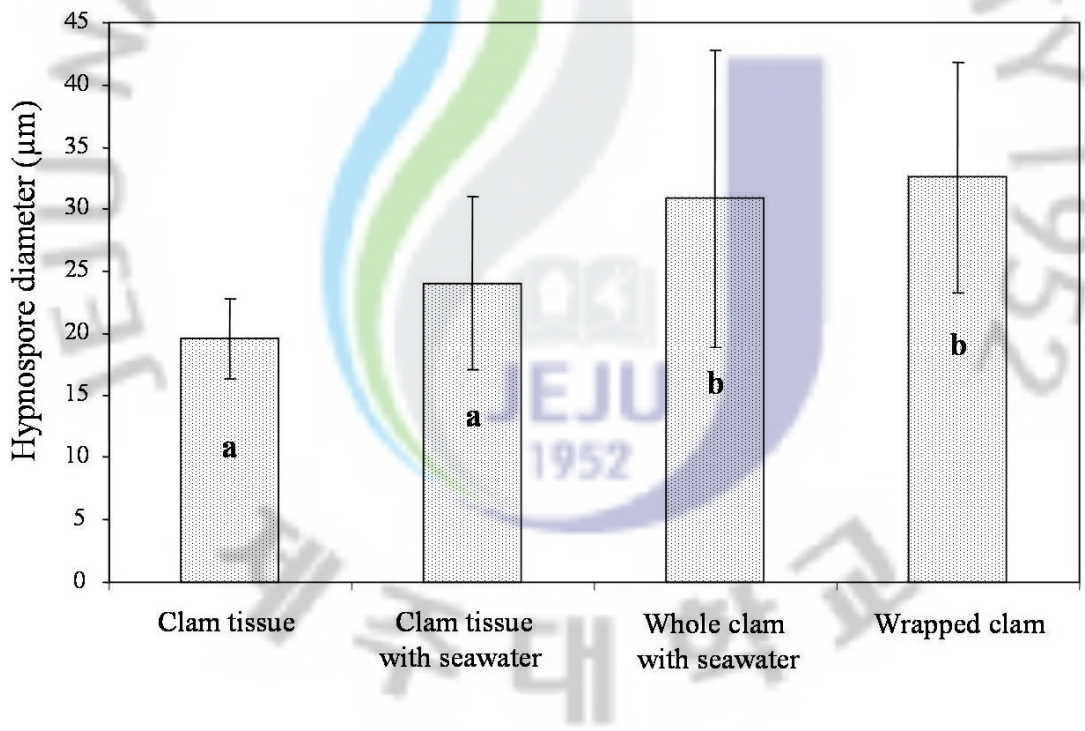

Figure 4-1 The size of hypnospore after incubation for 6 days at room temperature. **Clam tissue**; The clams were opened and the whole body tissue were chopped, pooled and seeded in 15 ml conical tubes without other addition. **Clam tissue with seawater**; whole body tissue were chopped, pooled and seeded in 15 ml conical tubes and add some sterile seawater. **Whole clam with sea water**; the Manila clams were put in plastic chamber, sea water was supplied and the chamber were sealed. **Wrapped clam**; the clams were wrapped with parafilm and put in sealed plastic chamber. The different letters on the bar indicated the significant different at p value<0.05.

Table 4-2 The size and number of hypnospore produced from Manila clam. The clam were divided into 2 parts; one part were incubated in FTM and the the other part were put in the shell and wrapped to create the anaerobic condition for 6 days at room temperature (25°C).

Clam	Hypnospore diameter (μm)										Number of hypnospore/mg tissue weight		
	FTM					Wrapped clam					FTM	Wrapped clam	% difference
	N	Mean	SD	Max	Min	N	Mean	SD	Max	Min			
1	76	39.88	14.46	88.37	11.84	80	23.66	10.82	66.63	7.53	1095	658	39.85
2	99	33.17	9.00	78.31	16.36	108	19.95	5.77	37.91	10.06	2833	1648	41.83
3	30	60.81	23.52	137.01	14.31	11	47.49	13.41	70.30	21.37	26	15	42.26
4	52	51.58	16.05	85.49	15.11	80	25.71	7.28	42.69	8.44	250	173	30.77
5	115	28.18	8.42	52.77	14.03	93	21.99	6.01	45.83	11.99	4782	3595	24.81
6	83	31.07	10.19	63.12	12.80	99	22.52	7.64	44.14	7.56	3972	1850	53.40
7	161	27.86	8.21	55.45	11.91	148	22.23	6.44	37.66	6.79	3199	1890	40.92
8	86	44.74	13.32	80.65	16.32	88	24.96	8.28	47.05	11.92	1137	957	15.82
9	107	34.44	8.56	70.29	17.62	138	20.44	6.33	42.68	6.55	5216	3867	25.85
10	81	45.13	12.70	86.61	23.07	81	27.40	10.68	59.32	7.66	284	233	17.82

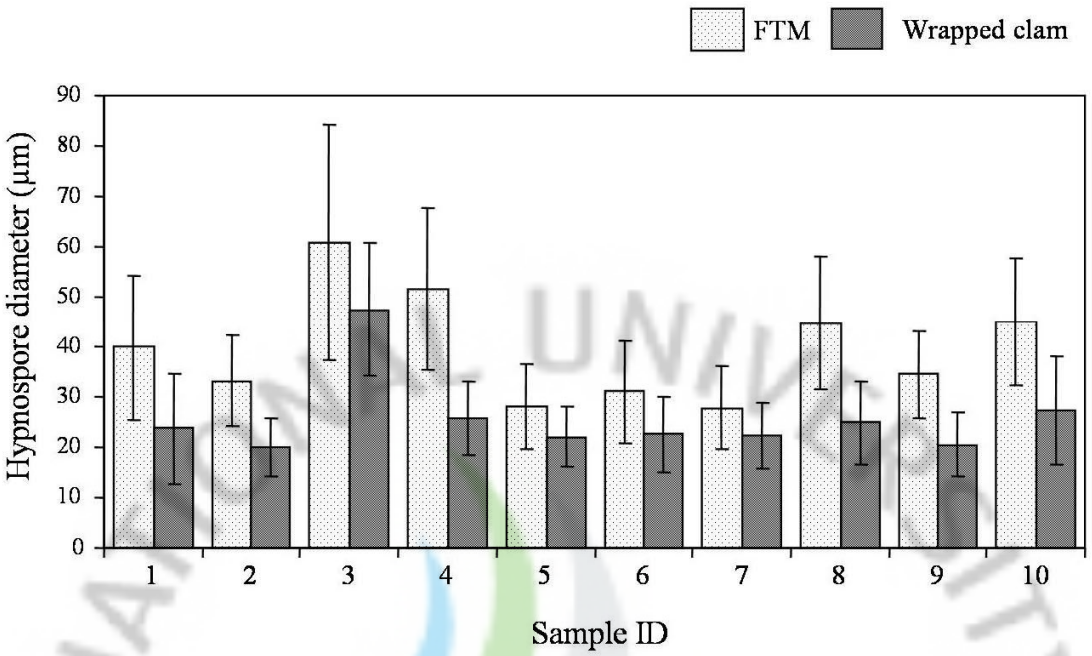

Figure 4-2 The size of hypnospore obtained from FTM assay and wrapped calm in 10 individual Manila clams

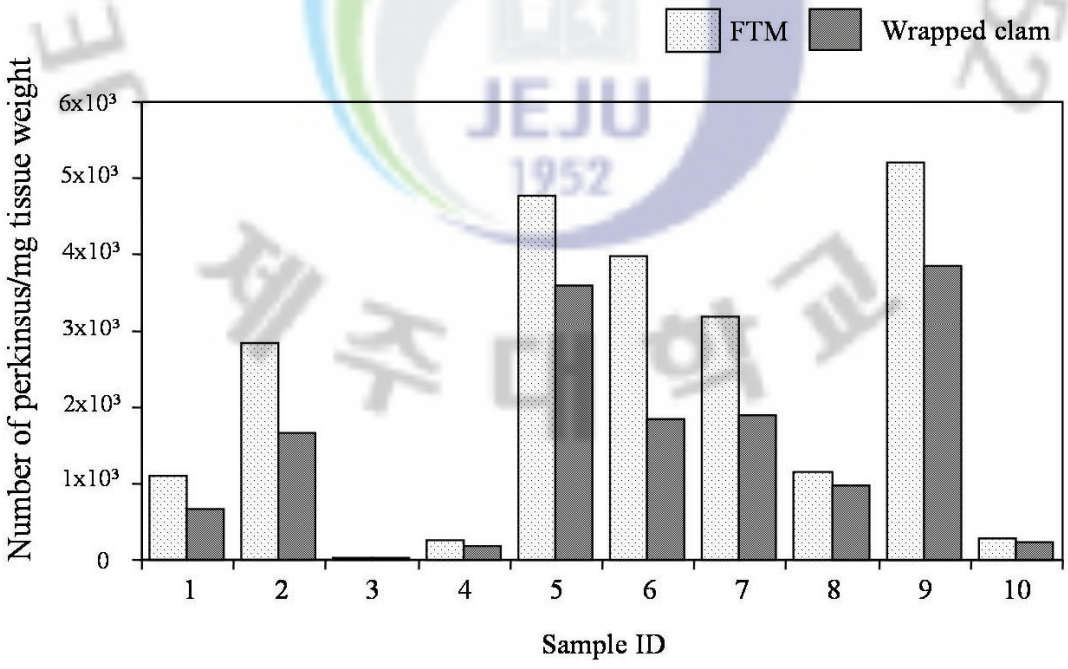

Figure 4-3 The number of hypnospore obtained from FTM assay and wrapped calm in 10 individual Manila clams

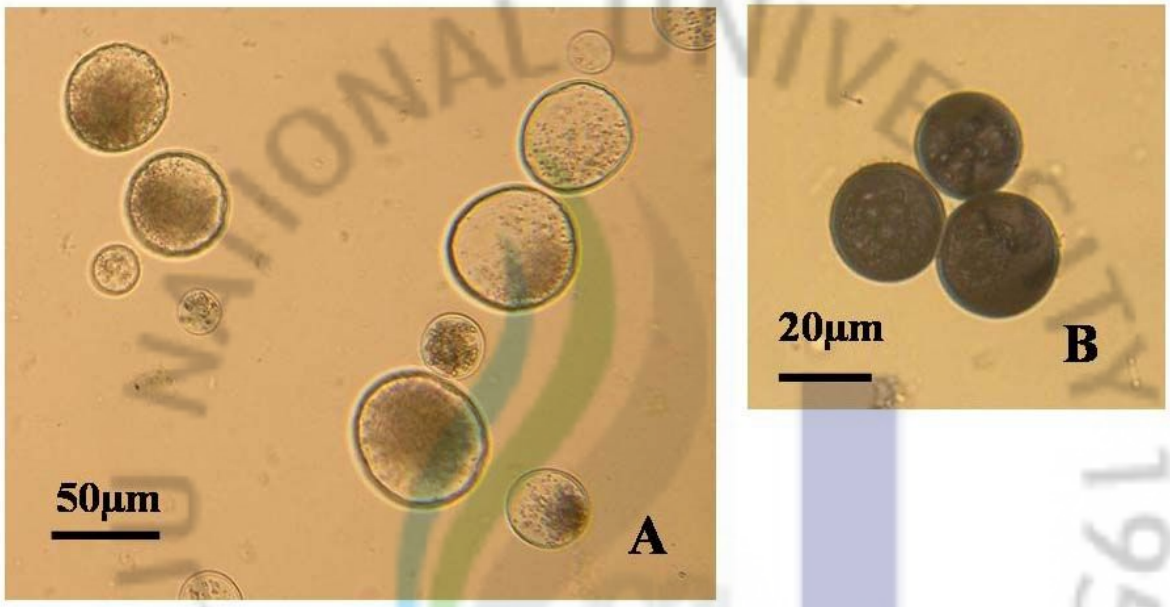

Figure 4-4 The hypnospore from clam tissue culture in FTM assay (not staining) (A) and the hypnospore developed in wrapped calm staining with Lugol's solution (noted the dark blue colour) (B) after incubate at 25°C for 6 days.

Table 4-3 The hypnospore size (μm) in different conditions and temperatures. The data showed the growth of hypnospore incubated in different conditions from 24 hr-6 days. **FTM 25°C**: the gill was incubated in FTM at 25°C. **FTM 4°C**: the gill was incubated in FTM at 4°C. **Tube 25°C**: the gill was directly put in conical tube and capped, incubated at 25°C. **Tube 4°C**: the gill was directly put in conical tube and capped, incubated at 4°C. **Petri dish 25°C**: the gill was place in Petri dish and incubated at 25 °C. **Anaerobic 25 °C**: the gill was individually wrapped in aluminum foil and incubated in anaerobic chamber at 25 °C. **Anaerobic 4 °C**: the gill was individually wrapped in aluminum foil and incubated in anaerobic chamber at 4°C.

	FTM						Tube						Petri dish			Anaerobic					
D	25°C			4°C			25°C			4°C			25°C			25°C			4°C		
ay	N	mean	SD	N	mean	SD	N	mean	SD	N	mean	SD	N	mean	SD	N	mean	SD	N	mean	SD
1	1145	21.71	3.21	818	9.24	1.70	439	9.98	1.69	63	5.53	0.23	666	5.78	0.65	662	6.33	1.20	482	4.44	0.37
2	1108	29.51	3.81	1090	7.69	1.05	695	9.04	1.90	611	5.34	0.74	597	6.05	0.72	610	9.32	2.36	469	4.77	0.60
3	1101	35.24	5.75	901	7.57	0.68	698	9.96	2.22	534	6.24	1.11	710	5.75	0.56	793	8.07	1.36	623	4.28	0.42
4	900	44.40	8.78	665	6.82	0.78	802	9.88	2.14	556	7.36	0.91	481	5.82	0.68	688	8.44	1.65	599	4.08	0.61
5	1197	54.70	7.53	549	8.20	0.75	474	8.80	1.01	373	6.94	0.81	760	6.23	0.79	771	9.40	1.66	664	4.23	0.66
6	1100	51.67	8.51	571	8.10	0.91	577	8.72	1.35	210	4.82	0.54	649	5.63	0.79	698	8.50	1.77	449	4.22	0.41

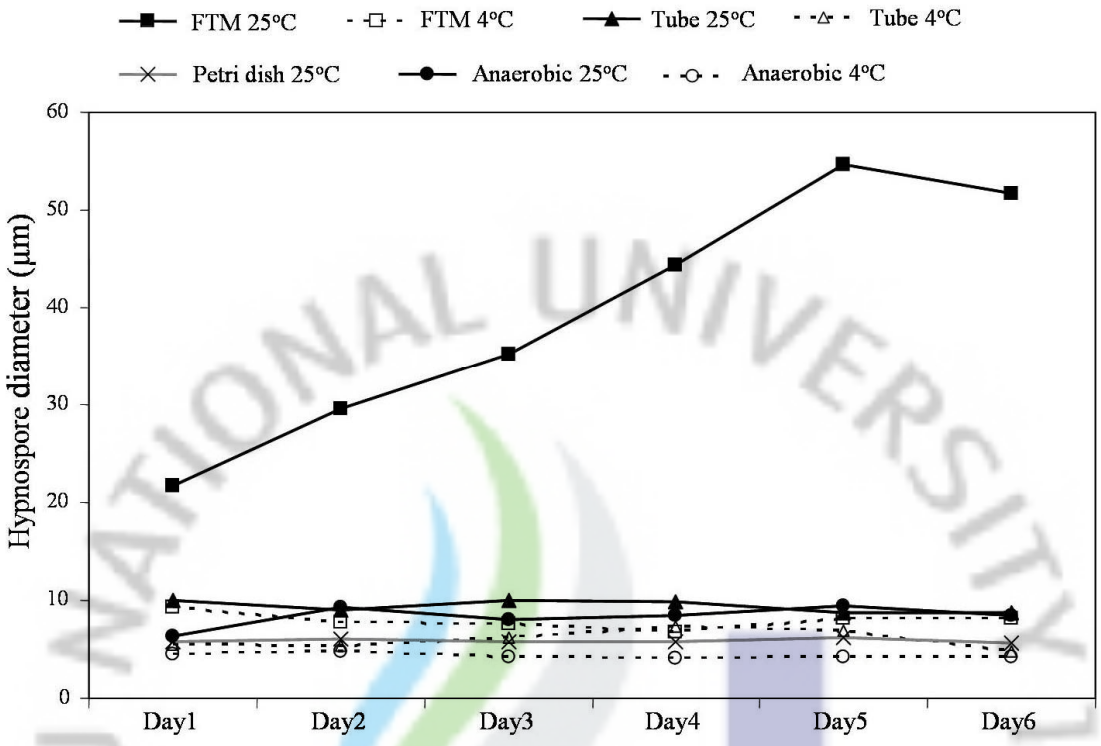

Figure 4-5 The growth of hypnospore incubated in different conditions from 24 hr-6 days incubation. **FTM 25°C**: the gill was incubated in FTM at 25°C. **FTM 4°C**: the gill was incubated in FTM at 4°C. **Tube 25°C**: the gill was directly put in conical tube and capped, incubated at 25°C. **Tube 4°C**: the gill was directly put in conical tube and capped, incubated at 4°C. **Petri dish 25°C**: the gill was place in Petri dish and incubated at 25 °C. **Anaerobic 25 °C**: the gill was individually wrapped in aluminum foil and incubated in anaerobic chamber at 25 °C. **Anaerobic 4 °C**: the gill was individually wrapped in aluminum foil and incubated in anaerobic chamber at 4°C.

Table 4-4 The number of hypnospore per mg of gill weight in different conditions and temperatures. The data showd the number of hypnospore incubated in different conditions from 24 hr-6 days. **FTM 25°C**: the gill was incubated in FTM at 25°C. **FTM 4°C**: the gill was incubated in FTM at 4°C. **Tube 25°C**: the gill was directly put in conical tube and capped, incubated at 25°C. **Tube 4°C**: the gill was directly put in conical tube and capped, incubated at 4°C. **Petri dish 25°C**: the gill was place in Petri dish and incubated at 25 °C. **Anaerobic 25 °C**: the gill was individually wrapped in aluminum foil and incubated in anaerobic chamber at 25 °C. **Anaerobic 4 °C**: the gill was individually wrapped in aluminum foil and incubated in anaerobic chamber at 4°C.

	FTM						Tube						Petri dish			Anaerobic					
Day	25°C			4°C			25°C			4°C			25°C			25°C			4°C		
	N	mean	SD	N	mean	SD	N	mean	SD	N	mean	SD	N	mean	SD	N	mean	SD	N	mean	SD
1	20	4070	3007	20	1616	1316	20	1016	779	20	75	188	20	3548	2274	20	2537	2812	20	2515	2390
2	20	4218	2304	20	3446	2562	20	2019	1167	20	1775	1529	20	2648	1846	20	2259	2596	20	2505	1869
3	20	3992	3079	20	2897	1592	20	1937	1590	20	1702	1402	20	3577	2857	20	2968	3929	20	3643	2755
4	20	5473	4540	20	2294	1830	20	1388	784	20	1859	1192	20	2369	1600	20	1851	1631	20	2650	3409
5	20	2784	1864	20	1645	1363	20	905	1032	20	1705	1846	20	2255	2146	20	2469	3060	20	2745	1498
6	20	4012	2568	20	2054	1729	20	1648	1696	20	641	666	20	2063	2092	20	3879	4635	20	2590	2180

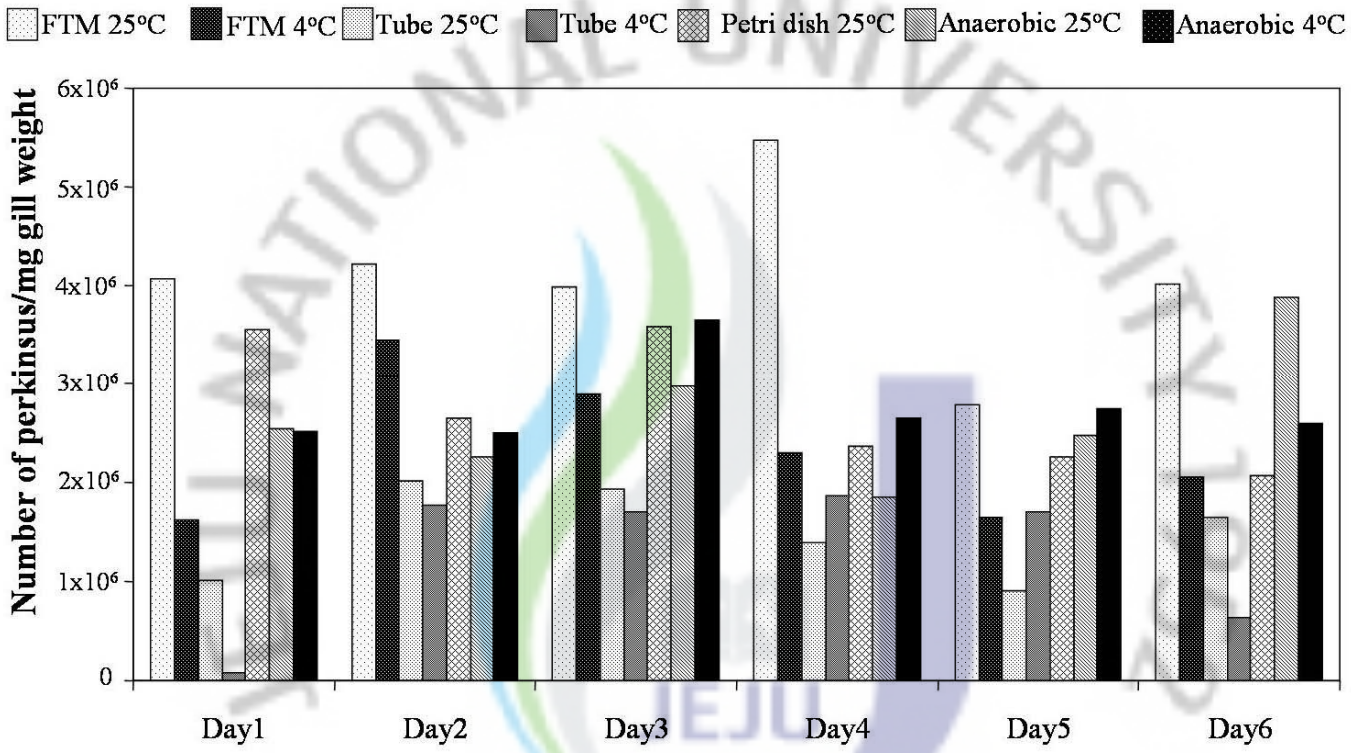

Figure 4-6 The number of hypnospore incubated in different conditions from 24 hr-6 days. **FTM 25°C**: the gill was incubated in FTM at 25°C. **FTM 4°C**: the gill was incubated in FTM at 4°C. **Tube 25°C**: the gill was directly put in conical tube and capped, incubated at 25°C. **Tube 4°C**: the gill was directly put in conical tube and capped, incubated at 4°C. **Petri dish 25°C**: the gill was place in Petri dish and incubated at 25 °C. **Anaerobic 25 °C**: the gill was individually wrapped in aluminum foil and incubated in anaerobic chamber at 25 °C. **Anaerobic 4 °C**: the gill was individually wrapped in aluminum foil and incubated in anaerobic chamber at 4°C.

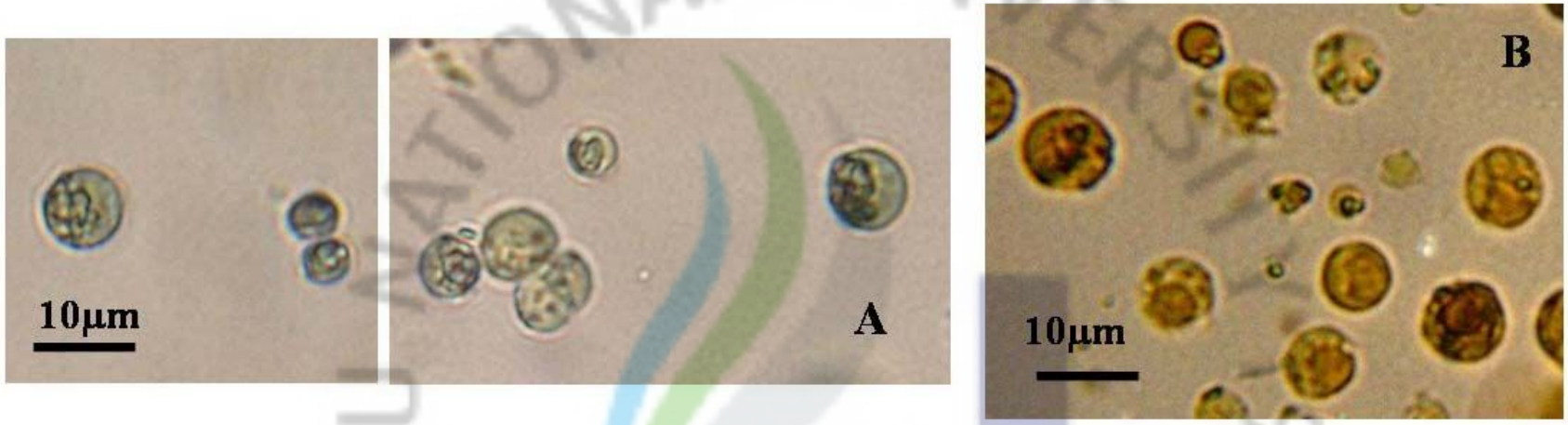

Figure 4-7 The hypnospore of *Perkinsus olseni* observed after directly digest Manila clam tissue with NaOH (no incubation) and stained with light blue color with Lugol's solution (A). The trophozoite cultured in DMEM for 2 days, after staining with Lugol's solution the dark blue color was not observed (B).

Discussion

The hypnospore developed in the clam tissue without nutrient supplement has a dominantly small size compare with the hypnospore cultured in FTM. To confirm that the spherical cell we observed was the real hypnospore stage of *Perkinsus*, we did the tissue staining by Lugol's solution. The hypnospore cell wall should be stained with blue color while the other uncultured trophozoite do not turn blue when treated with iodine solution (Mackin, 1962). The 2M NaOH digestion method also use together for double confirmation. Choi et al. (1989) developed a technique for extracting *P. marinus* hypnospores from infected oyster tissues cultured in FTM based on treating those tissues with 2M sodium hydroxide. This technique provides a preparation of hypnospores free of oyster tissue and other parasitic organisms such as *Nematopsis*. In our study, as the hypnospore developed without FTM was very tiny. The number of hypnospore presence in clam tissue was impossible to distinguish and the quantitative assessment was probably underestimated. The 2M NaOH method was favorable for illustrate the small hypnospore in clam tissue and recommended as a key step for natural hypnospore development detection.

The high nutrient especially dextrose and yeast extract and anaerobic condition were known to be the essential factor of hypnospore growth (Ray, 1966). For the first trial, we discovered that *Perkinsus* hypnospore can be induced without supplement of artificial medium. Only the clam tissue itself with or without seawater, the hypnospore was existed and capable to increase the size up to 63 μm in 6 days at room temperature. The hypnospore in both group of the whole clam incubation showed significant larger than those in the chopped tissue. The possible explanation was the chopped clam tissue may lead more air exposure to the *Perkinsus* cells thus the natural decomposed tissue in the clam shells provide more suitable condition of

hypnospore growing. The presence of sea water seems not to be a crucial factor of hypnospore development. Therefore in natural clam bed which is tidal flat, the ebb tide did not impact the vital of the parasites.

Mackin (1961) observed that after the host die, Dermocystidium cells (*P. marinus*) enlarge in the disintegrating tissues just as if they had been cultured in thioglycollate medium. Microscopic studies also indicated that the cytoplasm of the enlarged cells segmented to produce a number of small cells assumed to be spores. The hypnospore therefore seem to be a resistant spore or presporangium which undergoes development only after liberation from the host. After successfully induced the hypnospore formation without using the medium, we compared the growth and number of hypnospore produced in wrapped clam with the FTM assay using the same individual clams. The wrapped clam represented the phenomenon of the diseased clam in bottom sediment. The unhealthy clam which burrowed in sediment may slowly die with shell closed due to pressure of sediment and the weakness of adductor muscle. The decomposed tissue inside the shell generated anaerobic condition and promoted the growth of hypnospore. The result indicated that some hypnospore developed in wrapped clam can reach almost the same size with the ones developed in FTM. However the average diameter showed that the hypnospore developed in dead clams was smaller than from FTM. The hypnospore cultured in FTM did not increase by the times. There is evidence indicated that parasite numbers did not increase during incubation in FTM, even after several months since parasites did not multiply in culture (Fisher and Oliver, 1996). The number of hypnospore observed in decomposed clam tissue was also less than FTM. We postulated that as the FTM provided complete ideal condition for hypnospore development, all the *Perkinsus* cells in clam tissue turned to enlarged hypnospore. From the result, we concluded that in natural phenomenon of the dead clam, 15-50% of *Perkinsus* cells in clams body were died and the left

ones can survive and capable to grow in autolysis tissue. The presence of hypnospore stage which provides ability of *Perkinsus* to tolerance various environment condition is the important consideration for clam bed management to reduce the *Perkinsus* infection.

The comparative time series study of the growth of hypnospore was performed in experiment 3. In FTM, the hypnospore rapidly grew which within 1 day incubation at 25 ^{o}C, the diameter was larger than the hypnospore observed from non-FTM condition 2-3 times. While the size of hypnospore from FTM assay gradually increase day by day, the hypnospore from non-FTM condition seemed not to grow until 6 days. From the result, we can assume that the condition promoted in this experiment was not desirable for hypnospore development. In this experiment, we used gill tissue instead of whole clam tissue. The size of hypnospore produced in this study was remarkable smaller than in experiment 1 and 2, even tough the same anaerobic or low oxygen condition was provided (incubated in capped tube or anaerobic chamber). We observed that the decay of the gill tissue without clam body was slower than the whole clam. The bacteria and enzyme in digestive organ may be one of the fundamental factor facilitate the anaerobic condition and deterioration which is preferable for hypnospore growth.

Temperature was also an important impact to hypnospore growth. Villalba et al. (2005) mentioned that the temporal pattern of perkinsosis was significantly associated with the sea water temperature. The carpet shell clam in Galicia showed the annual spring peak of infection intensity occurred when seawater temperature was around 15 ^{o}C. In every group, the hypnospore incubated in 4 ^{o}C was smaller than in 25^{o}C. Interestingly, from the hypnospore size we conclude that the condition provided in this experiment was not suitable for the growth of hypnospore especially in low temperature; however the large number of hypnospores was still detected. This finding supported that the hypnospore is the strong stage

of *Perkinsus* which can survive even in inappropriate environment and is the important factor cause difficulty in disinfection the clam beds. According to the review by Villalba et al. (2005), transmission of *Perkinsus* maybe associated with host spawning, excretory activities, alternate host or vector activities, heterotrophic parasite proliferation, or periodic resuspension of parasite cells present in sediments, however, the primary mode of transmission occurs via the direct dissemination of parasite cells released from dead hosts.

Most of population of *Perkinsus* cell in clam tissue was trophozoite. We ensured the presence of small hypnospore or pre-hypnospore of *P. olseni* in live clam after directly digested by NaOH without incubation. Mackin (1961) suggested that the pre-hypnospore found in diseased oyster tissue are inactive, especially in cool periods. They appear to await liberation through death of the host or by diapedesis. However, warm period in winter may stimulate the pre-hypnospores to renewed reproductive activity.

For conclusion, the decomposed of *Perkinsus* infected Manila clam tissue promoted the desirable condition of hypnospore growth. The development of hypnospore was held during the low temperature period or inappropriate environment condition but not died. We did not estimate the maximum time the hypnospore can survive in sediment or in decayed clam tissue and practically, withdrawing all the Manila clams from clam beds until the parasite vanish is impossible. Therefore, because *Perkinsus* never can be eradicated from enzootic areas, management should focus on activities that will reduce the economic loss in the expectation of epizootic levels of infection (Krantz and Jodan, 1996). As the anaerobic condition is one of the factor effects the growth of hypnospore, frequently flipping the bottom sediment surface to increase oxygen exposure may be one of the method which can control the development of parasite.

Part V

First occurrence of the Paramyxean parasite *Marteilia* sp. in the Manila Clam (*Ruditapes philippinarum*) in Korea

Abstract

The paramyxean protozoan, *Marteilia* has been reported to be a causative agent of Marteiliosis or digestive gland disease in oyster and mussel. Histological examination of Manila clam (*Ruditapes philippinarum*) during routine monitoring in June 2009 revealed that the Manila clam collected from Tongyong (1 out of 40) and Goheung (1 out of 200) infected with *Marteilia* sp. The parasite located in digestive gland epithelium. The multiplication of tertiary and secondary cells occurred in primary cells formed a group of cell complex containing secondary and tertiary cells in plasmodium. The high intensity of parasite causes disruption of digestive tubule epithelium. The parasite and erosive epithelium were observed in lumen. *Marteilia* may impact the normal function of digestive gland. However the infected Manila clam in this study did not show remarkable decrease in condition index. Herein we first reported of *Marteilia* sp. in Manila clam in Korean waters.

The paramyxean protozoa of the genus *Marteilia refringens* has been reported as important protozoa that cause Aber disease which induced mortality of the oyster *Ostrea edulis* in France since 1968 (Figueras and Montes, 1988). Aber disease was named after the estuaries in Bretagne where mortalities were first reported. The other names of Marteiliosis were digestive gland disease and QX disease of Sydney rock oyster *Saccostrea glomerata* in Australia caused by *Marteilia sydneyi* (Berthe et al., 2004). The protozoan *Marteilia* is one of the important bivalve diseases found in various species of bivalve and wide geographical distribution. *Marteilia refringens* infected *Ostrea edulis*, *Tiostrea chilensis*, *Ostrea angasi*, *O. puelchana*, *Cardium edule*, *Mytilus edulis*, *M. galloprovincialis*, *C. gigas* and *C. virginica*. *Marteilia sydneyi* infects *Saccostrea cucullata* (Australia) and *M. christensemi* in *Scrobicularia plana* (France) (reviewed by Bondad-Reantaso et al., 2001). *Marteilia maurini* was detected in *M. edulis* and *M. galloprovincialis*. *Marteilia* sp. was observed in cockle *Cardium edule*, clams *Tapes rhomboids* and *Tapes pullastra* (reviewed by López and Darriba, 2006), razor clam, *Solen marginatus* in Spain (López-Flores et al., 2008) and Manila clam *Ruditapes philippinarum* in Japan (Itoh, 2005). The physiological impacts of Marteiliosis are emaciation and exhaustion of glycogen reserves, gross discoloration of the digestive gland, cessation of feeding and weakening. The digestive gland becomes brown to pale yellow in color. The mantle becomes translucent and shell growth ceases. The visceral mass loses its pigmentation and in heavily infected individuals appears shrunken and slimy. Mortality appears to be associated with sporulation of the parasite and disruption of the digestive tubule epithelia. (Bondad-Reantaso, 2001). The presence of the parasite reduces the condition index (Berthe et al, 2004). The high prevalence of *Marteilia* sp. in the flat oyster (*Ostrea edulis*) from the gulf of Thessaloniki, Greece along with high intensity of infection was concluded as a cause of the flat oyster extinction in that area (Virvilis and Angelidis, 2006). Negative association between

infection by *Marteilia refringens* and the development of mussel gonad was demonstrated by Villalba et al. (1993). The parasite in Genus *Marteilia* has not been reported in Korea. Only Marteiliodes which is also Paramyxean has been reported in Pacific oyster and Manila clam (See Part II and III).

The Key diagnostic features of the Phylum Paramyxea are the internal cleavage to produce cells within cells during sporulation (López and Darriba, 2006). The development stage of Marteilia (Grizel et al., 1974) was demonstrated in Figure 5-1. Continuous enlargement of the primary cell cytoplasm and, within it, increase in the number of daughter cells by serial endogenous budding and cell division, characterize the development of paramyxean (Berthe et al., 2004). In our study, Marteilia was found in histological sections during Manila clam monitoring program in June 2009 and October 2009.

The market-sized Manila clams were collected from the Tongyong and Goheung, the clam beds located in south coast of Korea. Total of 40 and 200 clams from Tongyong and Goheung were collected. The shell length was measured before the clam tissue was removed weighted, processed for histological slides and stained with Hematoxylin & Eosin. The condition index was calculated as Tissue weight divided by shell dried weight. The slides were observed by light microscope.

The characteristic of the Manila clams examined in this study was described in Table 5-1. Only one Manila clam from each sampling site was infected with *Marteilia* sp. The parasite located in digestive tubule epithelium. Both early and advanced stages of *Marteilia* sp. were observed. The development of secondary cell in primary cell was characterized by 3 to at least 5 basophilic secondary cells in primary cells (plasmodium). The plasmodium was round shape with approximate diameter 7.6-14.0 μm, with thin wall and fully contained with dividing secondary cell (Fig. 5-2). The second phase of sporulation (advanced stage) was characterized

by the production of tertiary cell in secondary cells. The plasmodium size increases to 11.9-17.4 μm. The secondary cells produce 2 tertiary cells inside. The halo space around the spore was remarkable and easily to distinguish between parasites and host cells. The various sizes of spore were observed. In both Manila clam samples, the high intensity of infection was noted. Most of digestive gland tubules in the section were infected by *Marteilia* up to more than 10 plasmodia in one tubule. The normal epithelium was not observed. The tubules were occluded by erosive destroyed epithelium cells and parasite plasmodium. The hemocyte infiltration associated with parasite infection was not discovered. Berthe et al. (2004) recognized that plasmodia within the epithelia of the stomach or digestive gland do not introduce any particular hemocytic reaction. However, the plasmodia located in the gill may be associated with a heavy infiltration of hemocytes in the surrounding area of the gill where the parasite was present.

The digestive gland or digestive diverticular is the organs function in absorption and intracellular digestion (Shaw and Battle, 1957). The high infections cause loss of condition as a consequence of reduced energy acquisition. Also the parasite may interfere directly with host feeding and absorption simply by its physical presence (Berthe et al., 2004). However, the infected Manila clam in our study did not show remarkable decrease in condition index (Table 5-1).

The different of parasite infectivity in different bivalve hosts was mentioned. Mussels seem to be more resistant to Marteilia than the oysters. Virvilis and Angelidis (2006) suggested that the mussels may act as a "reserved tank" of the parasite, from which the more susceptible oysters are affected. The low prevalence of *Marteilia* sp. in Manila clam observed in this study and the one reported in Japan (Itoh et al., 2005) presumed that the Manila clam is not a susceptible host for *Marteilia* infection. However, the high intensity of infection should

be considered. From the histopathological, the digestive gland of infected Manila clam was severely overwhelmed by the parasites and may not function probably. Owing to the few cases of *Marteilia* occurrence, we can not pointed out that the parasite was associated in morbidity or mortality in Manila clams as reported in oyster or mussel. The heavy infections of Marteilia coincide with periods of weak condition of the mussels. The highest percentage of heavy infections related with the post spawning periods (Villalba et al., 1993). To achieve more Marteilia positive Manila clam samples for studying the impact of this parasite, the repeat Manila clam sampling should be accomplished in spawning period during early of August (Kang et al., 2004).

Marteilia was believed to have a complex life cycle involving several hosts. There are some investigations supported that intermediate host may essential for *Marteilia* development. Audemard et al. (2004) supported that the calanoid copepod *Paracartia grani* was a host for *M. refringens*. The zooplankton from natural enzootic area was found infected with Marteilia (Carrasco et al., 2007). So far, we can not estimate how the parasite introduced to Korean water. From the uncertain parasite life cycle, it was possible that the parasite-infected intermediate host (plankton, copepod or other mollusk species) originated from endemic area was accidentally transported to Korea.

The recommended diagnosis methods of Marteilia were variable from simple direct detection method by wet mounts and digestive gland imprint to histology and transmission electron microscope (TEM). The molecular techniques were also developed for the parasite detection and identification. For polymerase chain reaction, PCR primers that target the ITS1 (internal transcribed spacer) region are able to amplify *M. refringens* and In situ hybridization (ISH) was developed using a probe target the SSU of rRNA gene of *M. refringens* but it was cross reacted with *M. sydneyi* and *Marteilioides chungmuensis* (Kleeman et al., 2002). López-

Flores et al. (2004) generated the *M. refringens* probe using IGS (intragenic spacer) which more specific. Sequencing is recommended as a final step for confirmatory diagnostic which target regions are the SSU rDNA, ITS1 and IGS. Among the diagnosis methods, histological techniques were mentioned as a gold standard which may conduct with TEM or ISH (OIE, 2009).

Based on the characteristic of parasite in histological section, we reported the first time of the *Marteilia* sp. in Manila clam from Korea. In this study we can not determine the species of Marteilia. According to Itoh et al. (2005), Marteilia infections have not been reported from clam species in Marteilia refringens enzootic areas, suggesting that Marteilia in the clam may be a new species in this genus. The molecular techniques were required to complete the parasite identification in species level.

Table 5-1 Characteristics of the examined Manila clams

Location	N	Sampling period	Tissue wet weight (g)	Shell length (mm)	Condition index
Tongyong	40	June 2009	1.16±0.32	29.4±2.3	0.47±0.09
Goheung	200	October 2009	1.64±0.41	35.5±2.1	0.37±0.08
***Marteilia* infected Manila clam**					
Tongyong	1	June 2009	0.77	25.5	0.48
Goheung	1	October 2009	0.99	33.3	0.37

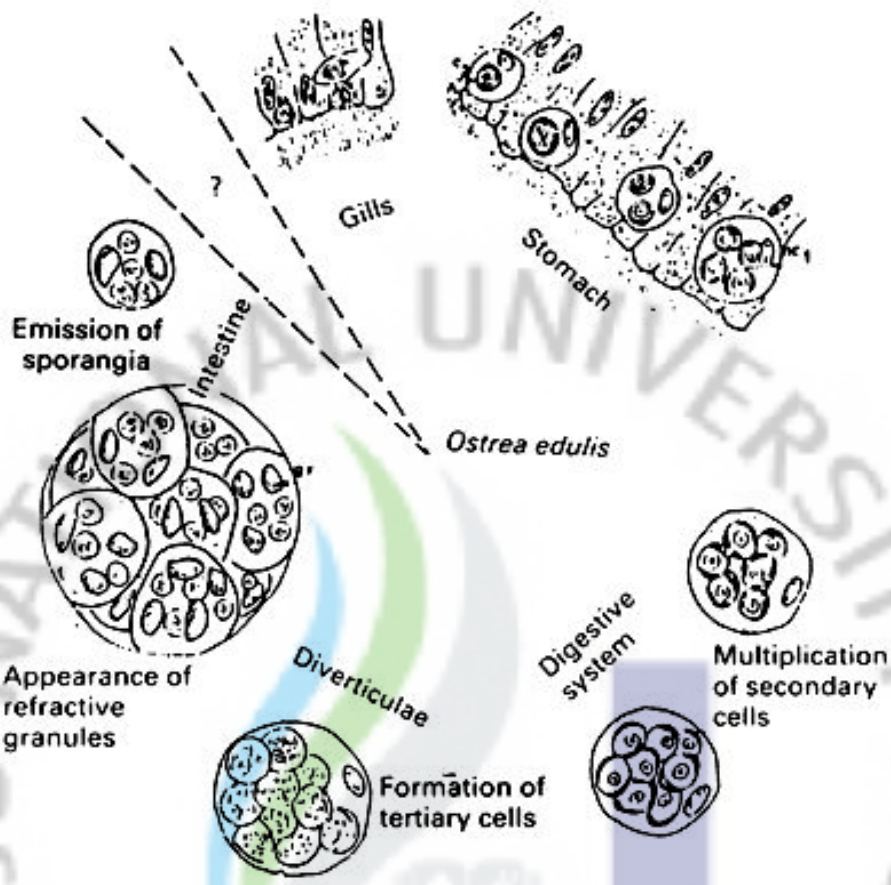

Figure 5-1 A tentative life cycle for *Marteilia refringens* in edible oyster (Grizel, 1974)

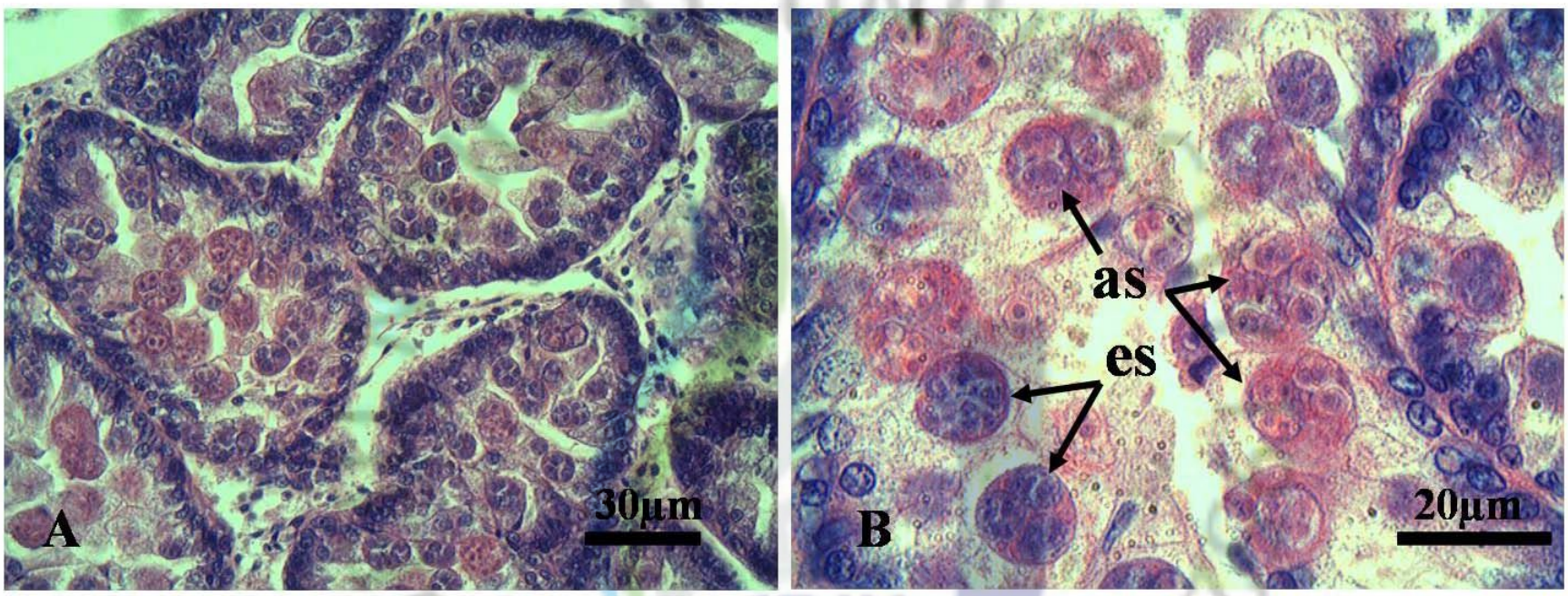

Figure 5-2 Histological section of Manila clam infected by *Marteilia* sp. in digestive gland (H&E). 5-2A: The round shape plasmodium were observed in digestive gland epithelium or in the lumen with the sloughing cells. 5-2B: The early stage (es) and advanced stage of *Marteilia* sp.

Reference

Abdallah, L.G.B., Menif, N.T.El. and Maamouri, F. 2009. The morphology and behavior of *Cercaria lata* Lespés, 1897 (Digenea, Faustulidae) from the Mediterranean clam *Tapes decussate* (L.). Journal of Helminthology 83, 69-76.

Andrews, J.D. 1996. History of *Perkinsus marinus*, a pathogen of oysters in Chesapeake Bay 1950-1984. Journal of Shellfish Research 15, 13-16.

Andrews, J.D. and Hewatt, W.G. 1957. Oyster mortality studies in Virginia II. The fungus disease caused by *Dermocystidium marinum* in oysters of Chesapeake Bay. Ecological Monographs 27, 1-25.

Audemard, C., Sajus, M., Barnaus, A., Sautour, B., Sauriau, P. and Berthe, F.J.C. 2004. Infection dynamics of *Marteilia refringens* in flat oyster Ostrea edulis and copepod *Paracartia grani* in a Claire pond of Marennes-Oléron Bay. Diseases of Aquatic Organism 61, 103-111.

Azevedo C., 1989, Fine structure of *Perkinsus atlanticus* n. sp. (Apicomplexa, Perkinsus) parasite of the clam *Ruditapes decussates* from Portugal. Journal of Parasitology 75, 627-635.

Azevedo, C. and Cachola, R. 1992. Fine structure of the Apicomplexa oocyst of *Nematopsis sp*. of two marine bivalve molluscs. Disease of Aquatic Organisms 14, 69-73.

Bataller, E. and Boghen, A.D. 2000. Elimination of the gill worm *Urastoma cyprinae* (Graff) from the eastern oyster *Crassostrea virginica* (Gmelin) using different salinity-temperature combinations. Aquaculture 182, 199-208.

Berthe, F.C.J., Le Roux, F., Adlard, R.D. and Figueras, A. 2004. Marteiliosis in molluscs: A review. Aquatic Living Resources 17, 433-448.

Berthelin, C., Kellner, K., Mathieu, M., 2000. Storage metabolism in the Pacific oyster (*Crassostrea gigas*) in relation to summer mortalities and reproductive cycle (West Coast of France). Comparative Biochemistry and Physiology 125B, 359-369

Bligh, E.G. and Dyer, W.J. 1959. A rapid method for total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37, 911-917.

Bondad-Reantaso, M.G., McGladdery, S.E., East, I. and Subasinghe, R.P. (eds.). 2001. Asia Diagnostic Guide to Aquatic Animal Diseases. FAO Fisheries Technical Paper No. 402, Supplements2. Rome, FAO. 240p.

Boucaud-Camou, È., Henry, M. 2003. The digestive system. In: An atlas of histology and cytology of marine bivalve mollusks. G. Henry. Ed. IFREMER. pp.65-71.

Bower, S.M., Blackbourn, J. and Meyer, G.R. 1992. Parasite and symbiont fauna of Japanese littlenecks, *Tapes philippinarum* (Adams and Reeve, 1850), in British Columbia. Journal of Shellfish Research 11, 13-19.

Brusca, R. C. and Brusca, G. J. 2003. Phylum Platyhelminthes. In: Invertebrates. 2nd ed. Sinauer Associates, Inc., Publishers. Massachusetts. pp285-317.

Buchanan, J.S. 1978. Cytological studies on a new species of rickettsia found in association with a phage in the digestive gland of the marine bivalve mollusk, *Tellina tenuis* (da Costa). Journal of Shellfish diseases 1, 27-43.

Burreson E.M., Alvarez R.S., Martínez V.V., Macedo L.A., 1994, *Perkinsus marinus* (Apicomplexa) as a potential source of oyster *Crassostrea virginica* mortality in coastal lagoons of Tabasco, Mexico. Disease of Aquaic Organism 20, 77-82.

Cáceres-Martínez, J., Vásquez-Yeomans, R. and Sluys, R. 1998. The Turbellarian *Urastoma cyprinae* from edible mussels *Mytilus galloprovincialis* and *Mytilus californianus* in Baja California, NW México. Journal of Invertebrate Pathology 72, 214-219.

Carballal, M.J., Díaz, S. and Villalba, A. 2005. *Urosporidium* sp. hyperparasite of the turbellarian *Paravortex cardii* in the cockle *Cerastoderma edule*. Journal of Invertebrate Pathology 90, 104-107.

Carballal, M.J., Iglesias, D., Santamarina, J., Ferro-Soto, B. and Villalba, A. 2001. Parasites and pathologic conditions of the Cockle *Cerastoderma edule* populations of the coast of Galicia (NW Spain). Journal of Invertebrate Pathology 78, 87-97.

Carrasco, N., López-Flores, I., Alcaraz, M., Furones, M.D., Berthe, F.C.J. and Arzul, I. 2007. First record of a Marteilia parasite (Paramyxea) in zooplankton populations from a natural estuarine environment. Aquaculture 269, 63-70.

Cartier, S., Pellerin, J., Fournier, M., Tamigneaux, E., Girault, L. and Lemaire, N. 2004. Use of an index based on the blue mussel (*Mytilus edulis* and *Mytilus trosssulus*) digestive gland weight to assess the nutritional quality of mussel farm sites. Aquaculture 241, 633-654.

Cavalier-Smith, T. and Chao, E. E. Y. 2003a. Phylogeny of Choanozoa, Apusozoa, and other Protozoa and early eukaryote megaevolution. Journal of Molecular Evolution 56, 540-563

Cavalier-Smith, T. and Chao, E. E. Y. 2003b. Phylogeny and Classification of Phylum Cercozoa (Protozoa). Protist 154, 341-358.

Cunningham, C.O. 2002. Molecular diagnosis of fish and shellfish diseases: Present status and potential use in disease control. Aquaculture 206, 19-55.

Cheng, T.C. 1988. Strategies employed by parasites of marine bivalves to effect successful establishment in hosts. American Fisheries Special Publication 18, 112-128.

Choi, K.S. 2008. Oyster capture-based aquaculture in the Republic of Korea. In: Capture-based aquaculture. Globle overview. A. Lovatelli and P.F. Holthus (eds.). FAO Fisheries Technical Paper. No. 508. Rome, FAO. Pp. 271-286.

Choi, K.S., Lewis, D.H., Powell, E.N., Ray, S.M., 1993. Quantitative measurement of reproductive output in the American oyster, *Crassostrea virginica* (Gmelin), using an Enzyme-Linked Immunosorbent Assay (ELISA). Aquaculture and Fisheries Management 24, 375-398.

Choi K.S., Park K.I., 1997, Report on the occurrence of *Perkinsus* spp. in the Manila clams, *Ruditapes philippinarum*, in Korean Journal of Aquaculture 10, 227-237.

Choi, K.S. and Park, K.I. 2001. Histopathology and spatial distribution of the protozoan parasite, *Perkinsus* sp. found in the Manila clam *Ruditapes philippinarum* distributed in Cheju, Korea. Korean Journal of Environmental Biology 19, 79-86 (in Korean with English abstract)

Choi, K,S., Park, K.I., Cho, M.J. and Soudant, P. 2005. Diagnosis, Pathology, and Taxonomy of Perkinsus sp. Isolated from the Manila clam *Ruditapes philippinarum* in Korean Journal of Aquaculture 18, 207-214.

Choi, K.S., Wilson, E.A., Lewis, D.H., Powell, E.N. and Ray, S.M. 1989. The energetic cost of *Perkinsus marinus* parasitism in oysters, quantification of the thioglycollate method. Journal of Shellfish Research 8, 125-131.

Chu, F.E. 1996. Laboratory investigations of susceptibility, infectivity, and transmission of *Perkinsus marinus* in oysters. Journal of Shellfish Research 15, 57-66.

Chu, F.E. and Greene, K.H. 1989. Effect of temperature and salinity on in vitro culture of the oyster pathogen, *Perkinsus marinus* (Apicomplexa: Perkinsea). Journal of Invertebrate Pathology 53, 260-268.

Couch, J.A. 1985. Prospective study of infectious and noninfectious diseases in oysters and fishes in three Gulf of Mexico estuaries. Disease of Aquatic Organisms 1, 59-82.

Coss C.A., Robledo J.A.F., Ruiz G.M., Vasta G.R., 2001, Description of *Perkinsus andrewsi* n. sp. isolated from the Baltic clam (*Macoma balthica*) by characterization of the ribosomal RNA locus, and development of a species specific PCR-based diagnostic assay. Journal of Eukaryote Microbiology 48, 52-61.

Cremonte, F., Balseiro, P. and Figueras, A. 2005. Occurrence of *Perkinsus olseni* (Protozoa: Apicomplexa) and other parasites in the venerid commercial clam *Pitar rostrata* from Uruguay, southwestern Atlantic coast. Disease of Aquatic Organisms 64, 85-90.

Cremonte, F., Kroeck, M.A. and Martorelli, S.R. 2001. A new monorchiid cercaria (Digenea) parasitizing the purple clam *Amiantis purpurata* (Bivalvia: Veneridae) in the southwest Atlantic ocean, with notes on its gonadal effect. Folia Parasitologica 48, 217-223.

Cremonte, F., Vázquez, N. and Ituarte, C. 2008. The development of *Gymnophallus australis* Szidat, 1962 (Digenea: Gymnophallidae) from the Patagonian coast (Argentina) from metacercaria to adult, with an amended diagnosis of Gymnophallus Odhner, 1905. Systemic Parasitology 69, 23-31.

Cunningham, C.O. 2002. Molecular diagnosis of fish and shellfish diseases: present status and potential use in disease control. Aquaculture 206, 19-55.

da Silva, P.M., Cremonte, F., Sabry, R.C., Rosa, R.D., Cantelli, L. and Barracco, M.A. 2009. Presence and histopathological effects of the *Parvatrema* sp. (Digenea, Gymnophallidae) in the stout razor clam *Tagelus plebeius* (Bivalvia, Psammobiidae). Journal of Invertebrate Pathology 102, 14-20.

Davey, J.T. and Gee, J.M. 1988. *Mytilicola intestinalis*, a copepod parasite of blue mussel. American Fisheries Society Special Publication 18, 64-73.

de Montaudouin, X., Kisielewski, I., Bachelet, G. and Desclaux, C. 2000. A census of macroparasites in an intertidal bivalve community, Arcachon Bay, France. Oceanologica Acta 23, 453-468.

Dridi, S., Romdhane, M.S., Elcafsi, M., 2007. Seasonal variation in weight and biochemical composition of the Pacific oyster, *Crassostrea gigas* in relation to the gametogenic cycle and environmental conditions of the Bizert lagoon, Tunisia. Aquaculture 263, 238-248.

Drummond, L., Mulcahy, M. and Culloty, S., The reproductive biology of the Manila clam, *Ruditapes philippinarum*, from the North-West of Ireland. Aquaculture. 254 (2006) 326-340.

Dubois, M., Gilles, K.A., Hamilton, J.K., Robers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28, 350-356.

Dungun, C.F. and Reece, K.S. 2006. In vitro propagation of two *Perkinsus* spp. Parasites from Japanese manila clams *Venerupis philippinarum* and description of *Perkinsus honshuensis* n. sp. The Journal of Eukaryotic Microbiology 53, 316-326.

Eble, A.F., Scro, R., 1996. General anatomy. In: V.S. Kennedy, R.I.E. Newell and A.F. Eble, Editors, The Eastern Oyster *Crassostrea virginica*, A Maryland Sea Grant Book, Maryland, USA (1996), pp. 19-73.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39, 783-791.

Figueras, A.J. and Montes, J. 1988. Aber disease of edible oysters caused by *Marteilia refringens*. American Fisheries Society Special Publication 18, 38-46.

Fisher, W.S. and Oliver, L.M. 1996. A whole-oyster procedure for diagnosis of *Perkinsus marinus* disease using Ray's fluid thioglycollate culture medium. Journal of Shellfish Research 15, 109-117.

Food and Agriculture Organization of the United Nations. 2009. Cultured aquatic species information programme *Ruditapes philippinarum* [www document]. URL http://fao.org/fishery/culturedspecies/Ruditapes_philippinarum/en

Ford S.E., 1996, Range extension by the oyster parasite *Perkinsus marinus* into the North Eastern United States: response to climate change? Journal of Shellfish Reseach 15, 45-56.

Gold-Bouchot, G., Simá-Alvarez, R., Zapata-Pérez, O. and Güemez-Ricalde, J. 1995. Histopathological effects of petroleum hydrocarbons and heavy metals on the American oyster (*Crassostrea virginica*) from Tabasco, Mexico, Marine Pollution Bullentin 31, 439-445.

Grizel, H., Comps, M., Bonami, J.R., Cousserans, F., Duthoit, J.L. and Le Pennec, M.A. 1974. Recherche sur l'agent de la maladie de la glande digestive de Ostrea edulis Linné. Bulletin de l'Institut des Pêches Maritimes du Maroc 240: 7-30.

Hamaguchi M., Suzuki N., Usuki H., Ishioka H., 1998, *Perkinsus* protozoan infection in short-necked clam *Tapes* (=*Ruditapes*) *philippinarum* in Japan. Fish Pathology 33, 473-480.

Hewatt, W.G. Andrews, J.D. 1954. Oyster mortality studies in Virginia: I. Mortalities of oysters in Trays at Gloucester Point, York River. Texas Journal of Science 6, 121-133.

Hine, P.M. and Thorne, T. 2000. A Survey of some parasites and diseases of several species of bivalve mollusk in northern Western Australia. Disease of Aquatic Organisms 40, 67-78.

Itoh, N., Komiyama, H., Ueki, N., Ogawa, K., 2004a. Early developmental stages of a protozoan parasite, *Marteilioides chungmuensis* (Paramyxea), the causative agent of the ovary enlargement disease in the Pacific oyster, *Crassostrea gigas*. International Journal of Parasitology 34, 1129-1135.

Itho, N., Momoyama, K. and Ogawa, K. 2005. First report of three protozoan parasites (a haplosporidian, *Marteilia* sp. and *Marteilioides* sp.) from the Manila clam, *Venerupis* (=*Ruditapes*) *philippinarum* in Japan. Journal of Invertebrate Pathology 88: 201-206.

Itho, N., Oda, T., Ogawa, K., Wakabayashi, H., 2002. Identification and development of a Paramyxean ovarian parasite in the Pacific oyster *Crassostrea gigas*. Fish Pathology 37, 23-28.

Itho, N., Oda, T., Yoshinaga, T., Okawa, K., 2003a. Isolation and 18S ribosomal DNA gene sequences of *Marteilioides chungmuensis* (Paramyxea), an ovarian parasite of the Pacific oyster *Crassostrea gigas*. Disease of Aquatic Organism 54, 163-169.

Itoh, N. Oda, T., Yoshinaga, T. and Ogawa, K. 2003b. DNA probes for detection of *Marteilioides chungmuensis* from the ovary of Pacific oyster *Crassostrea gigas*. Fish Pathology 38, 163-169.

Itho, N., Tun, K.L., Komiyama, H., Ueki, N. and Ogawa, K. 2004b. An ovarian infection in the Iwagaki oyster, *Crassostrea nippona*, with the protozoan parasite *Marteilioides chungmuensis*. Journal of Fish Diseases 27, 311-314.

Itoh, N., Momoyama, K. and Ogawa, K. 2005. First report of three protozoan parasites (a haplosporidian, *Marteilia* sp. and *Marteilioides* sp.) from the Manila clam, *Venerupis* (=*Ruditapes*) *philippinarum* in Japan. Journal of Invertebrate Pathology 88, 201-206.

Joaquim, S., Matias, D., Lopes, B., Arnold, W.S. and Gaspar, M.B. 2008. The reproductive cycle of white clam *Spisula solida* (L.) (Mollusca: Bivalvia): implications for aquaculture and wild stock management. Aquaculture 281, 43-48.

Johnson, M.A., Paulet, Y.M., Donval, A. and Le Pennec, M. 1996. Histology, histochemistry and enzymebiochemistry in the digestive system of the endosymbiont-bearing bivalve Loripes lucinalis (Lamarck). Journal of Experimental Marine Biology and Ecology 197, 15-38.

Jeong, W.G., Seo, J.M., Cho, S.M. and Park, C.I. 2005. Influence of environmental factors on the prevalence of the ovarian parasite, *Marteilioides chungmuensis*, in *Crassostrea gigas*, cultured in Pukman Bay, Tongyeong. The Korean Journal of Malacology 21, 33-33.

Kang, C.K., Park, M.S., Lee, P.Y., Choi, W.J., Lee, W.C., 2000. Seasonal variations in condition, reproductive activity, and biochemical composition of the Pacific oyster, *Crassostrea gigas* (Thunberg), in suspended culture in two coastal bays of Korea. Journal of Shellfish Research 19, 771-778.

Kang, D.H., Hyun, C.Y., Limpanont, Y. and Choi, K.S., 2007. Annual gametogenesis of the Chinese Anapella Clam *Coecella chinensis* (Deshayes 1855) at an upper intertidal sandy beach on the east coast of Jeju, Korea. Journal of Shellfish Research 26, 433-441.

Kang, D.H., Ngo, T.T.T., Koh, K.J. and Choi, K.S. 2004. Seasonal changes in Gonadal development of Manila clam, *Ruditapes philippinarum* from Shi-Heung-Ri, Jeju, Korea. Journal of Aquaculture 17, 81-88.

Kang, S.G., Choi, K.S., Bulgakov, A.A., Kim, Y., Kim, S.Y., 2003. Enzyme-linked immunosorbent assay (ELISA) used in quantification of reproductive output in the Pacific oyster, *Crassostrea gigas*, in Korea. Journal of Experimental Maine Biology and Ecology 282, 1-21.

Kim, B.K. 2009. Development of an immunological probe to measure reproductive effort of the Suminoe oyster, *Crassostrea ariakensis*. Master thesis. Cheju National University. 46 pp.

Kim, Y., Ashton-Alcox, K.A. and Powell, E.N. 2006. Histological techniques for marine bivalve mollusks: update. Silver Spring, MD. NOAA Technical memorandum NOS NCCOS 27. 76 pp.

Kim, Y. and Powell, E.N. 2004. Surf clam histopathology survey along the Delmarva mortality line. Journal of Shellfish Research 23, 429-441.

Kim, Y. and Powell, E.N. 2006. Relationships among parasites and pathologies in sentinel bivalves: NOAA status and trends "Mussel Watch" program. Bulletin of Marine Science 79, 83-112.

Kim, Y. and Powell, E.N. 2007. Distribution of parasites and pathologies in sentinel bivalves: NOAA status and trends "Mussel Watch" program. Journal of Shellfish Research 4, 1115-1151.

Kleeman, S.N., Le Roux, F., Berthe, F. and Adlard, R.D. 2002. Specificity of PCR and *in situ* hybridization assays designed for detection of *Marteilia sydneyi* and *M. refringens*. Parasitology, 125, 131-141.

Koukouzika, N., Raftopoulou, E.K. and Dimitriadis, V.K. 2009. Seasonal differences of lysosomal, lipid and lipofuscin parameters in the digestive gland of the mussel *Mytilus galloprovincialis*. Journal of Molluscan Studies 75, 261-267.

Krantz, G.E. and Jordan, S.J. 1996. Management alternatives for protecting Crassostrea virginica fisheries in Perkinsus marinus enzootic and epizootic areas. Journal of Shellfish Research 15, 167-176.

Lauckner, G. 1983. Diseases of Mollusca: Bivalvia disease of marine animals. In: Kinne,O.D (Ed.), Diseases of marine animals Volume II Introduction Bivalvia to Scaphopoda. Biologische Anstalt Helgoland, Hamburg. pp. 477-977.

Li, Q., Liu, W., Shirasu, K., Chen, W., Jiang, S., 2006. Reproductive cycle and biochemical composition of the Zhe oyster *Crassostrea plicatula* Gmelin in an eastern coastal bay of China. Aquaculture 261, 752-759.

Leander, B.S. 2007. Marine gregarine: evolutionary prelude to the apicomplexan radiation? Trends in Parasitology 24, 60-67.

Lee, M.K., Cho, B.Y., Lee, S.J., Kang, J.Y., Jeong, H.D., Huh, S.H., Huh, M.D. 2001. Histopathological lesions of Manila clam, *Tapes philippinarum*, from Hadong and Namhae coastal areas of Korea. Aquaculture 201, 199-209.

Lee, R.F., Heffernan, P., 1991. Lipids and proteins in eggs of eastern oysters (*Crassostrea virginica* (Gmelin, 1791)) and northern quahogs (*Mercenaria Mercenaria* (Linnaeus, 1758)). J. Shellfish Res. 10, 203-206.

Lee, S.H. and Chai, J.Y. 2001. A review of *Gymnophalloides seoi* (Digenea: Gymnophallidae) and human infections in the Republic of Korea. The Korean Journal of Parasitology 39, 85-118.

Lee, S.H., Sohn, W.M., Hong, S.J., Huh, S., Seo, M., Choi, M.H. and Chai, J.Y. 1996. A nationwide survey of naturally produced oysters for infection with *Gymnophalloides seoi* metacercariae.

Leethochavalit, S., Chalermwat, K., Upatham, E.S., Choi, K.S., Sawangwong, P. and Kruatrachue, M. 2004. Occurrence of *Perkinsus* sp. in undulated surf clams *Paphia undulate* from the Gulf of Thailand. Disease of Aquatic Organism. 60: 165-171.

Lester R.J.G., Davis G.H.G., 1981, A new *Perkinsus* species (Apicomplexa, Perkinsea) from the abalone *Haliotis ruber*. Journal of Invertebrate Pathology 37, 181-187.

Long, W.C., Mromage, E., Seitz, R.D., Kaattari, S., 2008. Quantifying fecundity in Macoma balthica using an enzyme-linked immunosorbent assay (ELISA). Aquatic Biology 3, 187-193.

López, C. and Darriba, S. 2006. Presence of *Marteilia* sp. (Paramyxea) in the razor clam *Solen marginatus* (Pennántt, 1711) in Galicia (NW Spain). Journal of Invertebrate Pathology 92, 109-111.

López-Flores, I., Garrido-Ramos, M.A., de la Herran, R., Ruiz-Rejón, C., Ruiz-Rejón, M. and Navas, J.I. 2008. Identification of *Marteilia refringens* infecting the razor clam *Solen marginatus* by PCR and in situ hybridization. Molecular and Cellular Probes 22, 151-155.

Lowry, O.H., Rosebrough, N.J., Lewis Farr, A., Randall, R.J., 1951. Protein measurement with the Folin phenol; reagent. J. Biol. Chem. 193, 265-275.

Mackin, J.G. 1961. Oyster disease caused by *Dermocystidium marinum* and other microorganisms in Louisiana. Publications of the institute of Marine Science 7, 133-229.

Mackin, J.G. and Boswell, J.L. 1956. The life cycle and relationships of *Dermocystidium marinum*. Proceeding of the National Shellfisheries Association 46, 112-115.

Mackin, J.G., Owen, H.M. and Collier, A. 1950. Preliminary note in the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassotrea virginica* (Gmelin). Science 111, 328-329.

Maeno Y., Yoshinaga T., Nakajima K., 1999, Occurrence of *Perkinsus* species (Protozoa, Apicomplexa) from Manila clam *Tapes philippinarum* in Japan. Fish Pathology 34, 127-131.

Massapina, C., Joaquim, S., Matias, D., Devauchelle, N., 1999. Oocyte and embryo quality in *Crassostrea gigas* (Portuguese strain) during a spawning period in Algarve, South Portugal. Aquatic Living Resource 12, 327- 333.

McLaughlin, S.M. and Faisal, M. 1999. A comparison of diagnostic assays for detection of *Perkinsus* spp. in the softshell clam *Mya arenaria*. Aquaculture 172, 197-204.

MOMAF, 2001. Development of optimal technology for sustaining production in shellfish farm. Technical report of the Ministry of Marine Affiars and Fisheries, Korea, pp. 1-930.

Montes J.F., Durfort, M. and Garćia-Valero, J. 1995. Cellular defence mechanism of the clam *Tapes semidecussatus* against infection by the protozoan *Perkinsus* sp. Cell & Tissue Research 279, 529-538.

Moore, M.N. 1988. Cytochemical responses of the lysosomal system and NADPH-ferrihemoprotein reductase in molluscan digestive cells to environmental and experimental exposure to xenobiotics. Marine Ecology Progress Series 46, 81-89.

Ngo, T.T.T., Berthe, F.C.J., Choi, K.S., 2003. Prevalence and infection intensity of the ovarian parasite *Marteilioides chungmuensis* during an annual reproductive cycle of the oyster *Crassostrea gigas*. Disease of Aquatic Organism 56, 259-267.

Ngo, T.T.T. and Choi, K.S. 2004. Seasonal changes of Perkinsus and Cercaria infections in the Manila clam *Ruditapes philippinarum* from Jeju, Korea. Aquaculture 239, 57-68.

Ngo, T.T.T., Kang, S.G., Choi, K.S., 2002. Seasonal changes in reproductive condition of the Pacific oysters, *Crassostrea gigas* (Thunberg) from suspended culture in Gosung Bay, Korea. Korean Journal of Environmental Biololy 3, 268-275.

Ngo, T.T.T., Kang, S.G., Kang, D.H., Sorgeloos, P., Choi, K.S., 2006. Effect of culture depth on the proximate composition and reduction of the Pacific oyster, *Crassostrea gigas* from Gosung Bay, Korea. Aquaculture 253, 712-720.

Norton J.H., Shepherd M.A., Perkins F.O., Prior H.C., 1993, *Perkinsus*-like infection in farmed golden-lipped pearl oyster *Pinctada maxima* from the Torres Strait, Australia. Journal of Invertebrate Pathology 62, 105-106.

Olivas-Valdez, J.A. and Cáceres-Martínez, J. 2002. Infestation of the blue mussel *Mytilus galloprovincialis* by the copepod *Pseudomyicola spinosus* and its relation to size, density, and condition index of the host. Journal of Invertebrate Pathology 79, 65-71.

The Office International des Epizooties (OIE). 2009. Chapter 2.4.4 Infection with *Marteilia refringens*. In: Manual of diagnostic tests for aquatic animals [www document]. URL http://www.oie.int

Ordás M.C. and Figueras A., 1998. In vitro culture of *Perkinsus atlanticus*, a parasite of the carpet shell clam *Ruditapes decussates*. Diseases of Aquatic Organisms 33, 129-136.

Park, K.I., Choi, J.W., Choi, K.S., 2005. Development of a *Saxidomus purpuratus* (Mollusca: Bivalvia) egg-specific antibody for the quantification of eggs using an enzyme-linked immunosorbent assay. Journal of Shellfish Research. 24, 1079-1085.

Park, K.I. and Choi, K.S. 2001. Spatial distribution of the protozoan parasite *Perkinsus* sp. found in the Manila clams, *Ruditapes philippinarum*, in Korea. Aquaculture 203: 9-22.

Park, K.I. and Choi, K.S.2004. Application of enzyme-linked immunosorbent assay for studying of reproduction in Manila clam *Ruditapes philippinarum* (Mollusca: Bivalvia) I. Quantifying eggs. Aquaculture 241, 667-687.

Park, K.I., Choi, K.S. and Choi, J.W. 1999. Epizootiology of *Perkinsus* sp. found in the manila clam, *Ruditapes philippinarum* in Komsoe Bay. Journal of the Korean Fisheries Society 32, 303-303.

Park, K.I., Figueras, A. and Choi, K.S. 2006a. Application of enzyme-linked immunosorbent assay (ELISA) for the study of reproduction in the Manila clam *Ruditapes philippinarum* (Mollusca: Bivalvia): II. Impacts of *Perkinsus olseni* on clam reproduction. Aquaculture 251, 182– 191.

Park, K.I., Ngo, T.T.T., Choi, S.D., Cho, M. and Choi, K.S. 2006b. Occurrence of *Perkinsus olseni* in the Venus clam *Protothaca jedoensis* in Korean waters. Journal of Invertebrate Pathology 93, 81-87.

Park, K.I., Paillard, C. Le Chevalier, P. and Choi, K.S. 2006c. Report on the occurrence of brown ring disease (BRD) in Manila clam, *Ruditapes philippinarum*, on the west coast of Korea. Aquaculture 255, 610-613.

Park, K.I., Park, J.K., Lee, J.H. and Choi, K.S. 2005. Use of molecular markers for species identification of Korean *Perkinsus* sp. isolated from Manila clams *Ruditapes philippinarum*. Disease of Aquatic Organisms 66, 255-263.

Park, K.I., Tsutsumi, H., Hong, J.S. and Choi, K.S. 2008. Pathology survey of the short-neck clam Ruditapes philippinarum occurring on sandy tidal flats along the coast of Ariake Bay, Kyushu, Japan. Journal of Invertebrate Pathology 99, 212-219.

Park, M.S. 2005. Survey on the ovarian parasite, *Marteilioides chungmuensis* in the cultured Pacific oyster, *Crassostrea gigas* in Korea. In: Diseases in Asian Aquaculture V. P. Walker, R. Lester and M.G. Bondad-Reantaso (eds.) Fish Health Section, Asian Fisheries Society, Manila. pp. 311-320.

Park, M.S. and Chun, S.K. 1989. Study on *Marteilioides chungmuensis* Comps et al., 1986 parasite of the Pacific oyster, *Crassostrea gigas* Thunberg. Journal of Fish Pathology 2, 53-70.

Park, M.S., Kang, C.K., Choi, D.L., Jee, B.Y., 2003. Appearance and pathogenicity of ovarian parasite *Marteilioides chungmuensis* in the farmed Pacific oysters, *Crassostrea gigas*, in Korea. Journal of Shellfish Research 22, 475-479.

Park, M.S., Lyu, H.Y., Lee T.S., 1999. Investigation on the cause of bad natural seed collection of the Pacific oyster, *Crassostrea gigas*: Relationships between the conditions of mother shell and the viability of the released eggs and larvae based on the pathological and embryological survey. J. Korean Fisheries Soc. 32, 62-67 (in Korean)

Perkin, F.O. and Menzel, R.W. 1966. Morphological and cultural studies of a motile stage in the life cycle of *Dermocystidium marinum.* Proceeding of the National Shellfisheries Association 56, 23-30.

Perkin, F.O. and Menzel, R.W. 1967. Ultrastructure of sporulation in the oyster pathogen *Dermocystidium marinum*. Journal of invertebrate pathology 9, 205-229.

Ray, S.M. 1952. A culture technique for diagnosis of infection with *Dermocystidium marinum*. Mackin, Owen and Collier in oysters. Science 116, 360

Ray, S.M. 1954. Biological studies of *Dermocystidim marinum*, a fungus parasite of oysters, Rice Institute Pamphlet. Special Issue, November, 1954. 114 p.

Ray, S.M. 1966. A review of the culture method for detecting *Dermocystidium marinum*, with suggested modifications and precautions. Proceeding of National Shellfisheries Association 54, 55-69.

Ray, S.M. and Chandler, A.C. 1954. Parasitological review. *Dermocystidium marinum*, A parasite of oysters. Experimental Parasitology 4, 172-200.

Rebstock, G.A. and Kang, Y.S. 2003. A comparison of three marine ecosystems surrounding the Korean peninsula: Responses to climate change. Progress in Oceanography 59, 357-379.

Robledo, J.A.F., Cáceres-Martínez, J., Sluys, R. and Figueras, A. 1994. The parasitic turbellrian *Urastoma cyprinae* (Platyhelminthes: Urastomidae) from blue mussel *Mytilus galloprovincialis* in Spain: Occurrence and Pathology. Disease of Aquatic Organism 18, 203-210.

Royer, J., Seguineau, C., Park, K.I., Pouvreau, S., Choi, K.S. and Costil, K. 2008. Gametogenic cycle and reproductive effort assessed by two methods in 3 age classes of Pacific oysters, *Crassostrea gigas*, reared in Normandy. Aquaculture 277, 313-32.

Saitou, N. and Nei, M. 1987 The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406-425.

Shaw, B.L. and Battle, H.I. 1957. The gross and microscopic anatomy of the digestive tract of the oyster Crassostrea virginica (Gmelin). Canada Journal of Zoology 35, 325-340.

Sohn, W.M., Na, B.K., Nyang, Y.S., Ching, H.L. and Lee, S.H. 2007. *Parvatrema chaii* n. sp. (Digenea: Gymnophallidae) from mice experimentally infected with metacercariae collected from surf clam, *Mactra veneriformis*. Korean Journal of Parasitology 45, 115-120.

Smit, S., Widmann, J. and Knight, R. 2007. Evolutionary rates vary among rRNA structural elements. Nucleic Acid Research 35, 3339-3354.

Soniat T.M., 1996, Epizootiology of *Perkinsus marinus* disease of eastern oysters in the Gulf of Mexico. Journal of Shellfish Research 15, 35-43.

Sparks, A.K. and Morado, J.F. 1988. Inflammation and wound repair in bivalve mollusks. American Fisheries Society Special Publication 18:139-152.

Sures, B. 2004. Environmental parasitology: relevancy of parasites in monitoring environmental pollution. Trends in Parasitology 20, 170-177.

Syasina, I.G., Vaschenko, M.A. and Zhadan, P.M. 1997. Morphological alterations in the digestive diverticula of *Mizuhopecten yessoensis* (Bivalvia: Pectinidae) from polluted areas of Peter the Great Bay, Sea of Japan. Marine Environmental Research 44, 85-98.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596-1599.

Thieltges, D.W. and Reise, K. 2006. Metazoan parasites in intertidal cockles *Cerastoderma edule* from the northern Eadden Sea. Journal of Sea Research 56, 284-293.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research 22, 4673-4680.

Thompson, R.J., Newell, R.I.E., Kennedy, V.S. Mann, R., 1996. Reproductive process and early development In: V.S. Kennedy, R.I.E. Newell and A.F. Eble, Editors, The Eastern Oyster *Crassostrea virginica*, A Maryland Sea Grant Book, Maryland, USA (1996), pp. 335-370.

Tun, K.L., Itoh, N., Shimizu, Y., Yamanoi, H., Yoshinaga, T., Ogawa, K., 2008a. Pathogenicity of the protozoan parasite *Marteilioides chungmuensis* in the Pacific oyster *Crassostrea gigas*. International Journal of Parasitology 38, 211-217.

Tun, K.L., Itoh, N., Shimizu, Y., Yamanoi, H., Yoshinaga, T., Ogawa, K., 2008b. Seasonality in the infecton and invasion of *Marteilioides chungmuensis* in the Pacific oyster *Crassostrea gigas*. Disease of Aquatic Organism 80, 157-165.

Tun K.L., Itoh, N., Ueki, N., Yoshinaga, T., Ogawa, K., 2007. Relationship between *Marteilioides chungmuensis* infection and reproduction in the Pacific oyster, *Crassostrea gigas*. Journal of Invertebrate Pathology 96, 205-212.

Tuntiwaranurak, C. Chalermwat, K., Upatham, E.S., Kruatrachue, M. and Azevedo, C. 2004. Investigation of *Nematopsis* spp. oocysts in 7 species of bivalves from Chonburi Province, Gulf of Thailand.

Usheva, L.N., Frolova, L.T., 2006. Morphofuctional changes of the digestive gland in the bivalve mollusk *Crenomytilus grayanus* (Dunker, 1853) in normal conditions and after parasitic invasion by trematodes. Russian Journal of Marine Biology 32, 96-105.

Usheva, L.N., Vaschenko, M.A. and Durkina, V.B. 2006. Histopathology of the digestive gland of the bivalve mollusk *Crenomytilus grayananus* (Dunker, 1853) from southwestern Peter the Great Bay, Sea of Japan. Russian Journal of Marine Biology 32, 166-172.

Valderrama, K., Oliva, M., Campos, B. and Brown, D. 2004. Parasitic castration of *Eurhomalea lenticularis* (Bivalvia: Veneridae) by a digenetic trematode: quantitative histological analysis. Diseases of Aquatic Organisms 59, 151-158.

Villalba, A., Casas, S.M., López, C., and Carballal, M.J. 2005. Study of perkinsosis in the carpet shell clam *Tapes decussates* in Galicia (NW Spain). II. Temporal pattern of disease dynamics and association with clam mortality. Disease of Aquatic Organisms 65, 257-267.

Villalba, A., Mourelle, S.G., Carballal, M.J. and López, M.C. 1993. Effects of infection by the protistan parasite Marteilia refringens on the reproduction of cultured mussels *Mytilus galloprovincialis* in Galicia (NW Spain). Diseases of Aquatic Organisms 17, 205-213.

Villalba, A., Reece, K.S., Ordás M.C., Casas, S.M. and Figueras, A. 2004. Perkinsosis in mollusks: A review. Aquatic Living Resource 17, 411-432

Virvilis, C. and Angelidis, P. 2006. Presence of the parasite Marteilia sp. in the flat oyster (*Ostrea edulis* L) in Greece. Aquaculture 259, 1-5.

Weis, P., Weis, J.S., Couch, J., Daniels, C., Chen, T., 1995. Pathological and Genotoxicological Observations in oysters (*Crassostrea virginica*) living on chromated copper arsenate (CCA)-treated wood. Marine Environment Research 39, 275-278.

Winstead, J.T., 1995. Digestive tubule atrophy in Eastern oysters, *Crassostrea virginica* (Gmelin, 1791), exposed to salinity and starvation stress. Journal of Shellfish Research 14, 105-111.

Wu, X.Z. and Pan, J.P. 2000. An intracellular prokaryotic microorganism associated with lesions in the oyster, *Crassostrea ariakensis* Gould. Journal of Fish Diseases 23, 409-414.

Yanagida, T., Shirakashi, S., Iwaki, T., Ikushima, N. and Ogawa, K. 2009. Gymnophallid digenean *Parvetrema duboisi* uses Manila clam as the first and second intermediate host. Parasitology International 58, 308-310.

Yang, E.J., Choi, J.K. and Hyun, J.H. 2008. Seasonal variation in the community and size structure of nano- and microzooplankton in Gyeonggi Bay, Yellow Sea. Estuarine, Coastal and Shelf Science 77, 320-330.

Yoon, H.S., Jung, H.T. and Choi, S.D. 2008. Suminoe oyster (*Crassostrea ariakensis*) culture in Korea. Journal of Shellfish Research 27, 505-508.

Yu, J.R., Chai, J.Y. and Lee, S.H. 1993. *Parvatrema timondavidi* (Digenea: Gymnophallidae) transmitted by a clam, *Tapes philippinarum*, in Korea. The Korean Journal of Parasitology 31, 7-12.

Zarlenga, D.S. and Higgins, J. 2001. PCR as a diagnostic and quantitative technique in veterinary parasitology. Veterinary Parasitology 101, 215-230.

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