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MASTER OF SCIENCE

DNA SEQUENCE VARIATION AMONG *IN VITRO* CULTURED *PERKINSUS OLSENI* ISOLATES DERIVED FROM MANILA CLAM *RUDITAPES PHILIPPINARUM* IN KOREA: COMPARISON OF FOUR COMMONLY USED DNA MARKERS OF *PERKINSUS* SPP.

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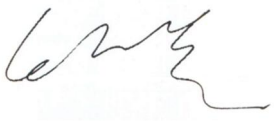

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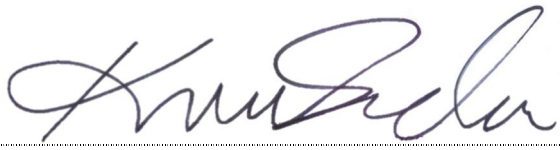

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ABSTRAT

Widely distributed in Korean waters, the OIE-listed protozoan parasite *Perkinsus olseni* has been associated with mass mortalities of Manila clam *Ruditapes philippinarum*. Intra-specific DNA sequence variation in *P. olseni* is believed to be linked to the phenotypic characteristics such as virulence, different levels of the infection intensities, and environmental tolerance. Previous study on spatial variation in *P. olseni* infection prevalence and intensity in clam populations in Korean waters suggested sequence variation in the different populations of *P. olseni.* Accordingly, we isolated *P. olseni* from different populations of Manila clam and cultured *in vitro* to examine genetic variabilities in the different isolates. To compare the DNA sequences, we analyzed *P. olseni* isolates using 4 types of DNA markers, including 18S ribosomal DNA (rDNA), actin gene DNA, internal transcribed spacer (ITS) and non-transcribed spacer (NTS). Sequence analysis of *in vitro* cultured *P. olseni* isolates revealed presence of a sequence polymorphism. NTS was most polymorphic, showing 14 polymorphic sites. On the other hand, 18S rDNA was least polymorphic, demonstrating only 3 polymorphic sites. Therefore, NTS is found to be the most appropriate among 4 DNA markers for identifying DNA sequence variation within the species. To examine intra-specific variation within the *P. olseni* isolates, pairwise distance of DNA sequences were calculated. As a result, NTS showed the highest variation (1.51%) and actin the lowest (0%) within the isolates in this study. Pairwise distance gap between *P. olseni* and *P. marinus* was most distant at NTS (24.9%), and least at 18S (0.8%). Phylogenetic analysis of 18S, ITS and NTS DNA showed two distinct clades while actin DNA showed monophyletic clade. In summary, 23 *in vitro* cultured *P. olseni* isolates from Korean waters differentiated into two strains, and this suggested possible phenotypic variation within the species.

국문요약

*Perkinsus olseni*는 해양 연체동물에 기생하는 원생동물 병원체로, 굴과 바지락 등을 포함한 상업적으로 중요한 이매패류의 대량폐사 원인생물로 알려져 있다. 우리나라 서해안 및 남해안에 서식하는 바지락에서도 높은 *Perkinsus* 감염율 및 감염도가 보고되었으며, 현재까지 국내에서는 *Perkinsus* 종의 구명, 분포, 지역별 감염율 및 감염도, *Perkinsus*가 바지락에 미치는 영향 등에 관한 연구가 진행되었다. *Perkinsus* 종 내에서 관찰되는 DNA 염기서열의 변이는 병독성 (virulence), 숙주의 면역작용 회피 반응 등과 같은 유전형질의 차이와 관련이 있는 것으로 추정된다. 우리나라 해역에서 관찰되는 지역별 바지락의 *Perkinsus* 감염율 및 감염도 차이는 우리나라에 분포하는 *P. olseni* 종 내에서의 DNA 염기서열 변이의 존재 가능성을 뒷받침 한다. 따라서, 이번 연구에서는 *P. olseni* 종 내에서의 DNA 염기서열 변이를 확인하고자 하였다. 또한, 이를 위하여 우리나라에서는 처음으로 *P. olseni*를 지역별 바지락으로부터 분리하여 *in vitro* culture 하였다. 18S ribosomal DNA (rDNA), actin gene DNA, internal transcribed spacer (ITS)와 non-transcribed spacer (NTS)의 네 가지 DNA marker를 분석에 사용하였다. 염기서열 분석결과, 모든 DNA marker에서 염기서열 다형성 (sequence polymorphism)이 관찰되었다. 이에 따라 18S rDNA, ITS, 그리고 NTS에서는 세가지 종류의 염기서열로, actin gene DNA에서는 두 가지 종류의 염기서열로 구분되었으며, NTS에서 가장 많은 염기서열 다형성이 확인되었다 (14개 염기서열 자리). 따라서, 종내 염기서열 변이 확인 등을 위한 분석에는 NTS가 가장 적절한 DNA marker인 것으로 판단된다. 염기서열간 pairwise distance를 계산한 결과, NTS에서 가장 큰 종 내 염기서열 변이를 보였으며 (1.51%), actin gene DNA에서는 변이가 없었다 (0%). 네 가지 DNA marker를 이용한 계통수 분석에서는 actin을 제외한 모든 marker에서, 이번 연구에서 분리한 23개의 지역별 *Perkinsus in vitro* culture 분리종이 지역별로 구분되지 않는 두 개의 clade로 구분되었다.

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1. Introduction

The genus *Perkinsus* includes protozoan parasite infecting commercially important marine molluscs including oysters, clams, abalones, scallops, cockles, and mussels (Villalba et al. 2004). Currently, seven species are considered valid in this genus, and *P. marinus* and *P. olseni* have been listed as a pathogen by World Organization for Animal Health (OIE) for being associated with mass mortalities of the hosts throughout the world. In Korea, *P. olseni* has been closely associated with mortalities of Manila clam, Ruditapes philippinarum, one of the most important shellfish resources. Choi and Park (1997) first reported *Perkinsus*-like organism in Manila clam, and it has been known to distribute widely along the west and south coasts of Korea with high infection prevalence and intensity (Park and Choi 2001). Molecular analysis using two DNA markers has revealed that *Perkinsus* sp. from west and south coasts of Korea was *P. olseni* (Park et al. 2005). Currently, Manila clam and Venus clam *Protothaca jedoensis* are only known host of *P. olseni* in Korea (Park and Choi 2001, Park et al. 2006b).

Perkinsus spp. have three main life stages, trophozoite, hypnospore and zoospore. The trophozoite is a non-motile spherical cell living in the host tissue ranging from 5 to 10㎛ (Choi and Park 2001), while size of the trophozoite cell varies among different species of *Perkinsus* (Villalba et al. 2004). The trophozoite multiplies in a vegetative form within the host tissues, and with the cell wall rupture, immature daughter cells are disseminated individually to become a mature trophozoite (Gogin and Lester 1995, Perkins 1996). In seawater or anaerobic condition, the trophozoite develops hypnospores, which is a resting spore characterized as increased cell size and thick cell wall. When hypnospores are located in aerobic condition with favorable temperature and salinity, hypnospore produce motile zoospores inside the spore and subsequently these zoospores are released to the environment

(Ray 1952, Casas and La Peyre 2013). The released zoospores are very small in size and equipped with two flagella used in swimming. It is believed that the motile zoospore infects the host organisms and develops into the trophozoite in the host tissue.

Infection with *Perkinsus* spp. has been diagnosed mostly using histology and Ray's fluid Thioglycollate medium (RFTM, Ray 1966). In RFTM, trophozoites of *Perkinsus* included in the host tissue form hypnospores, in which the anaerobic medium trigger the trophozoites to transform into the hypnospore. During incubation in RFTM, the hypnospores increase its size dramatically, from 5-10 μm to 50-100 μm. The cell wall of hypnospores can be stained well with th Lugol's iodine, which stains the hypnospores as dark brown or blue. Presence of hypnospores in the suspected host tissue can be easily detected under a low magnification microscope, after RFTM incubation and Lugol's staining. Density of the hypnospores in RFTM incubated suspected tissues can be determined by digesting the tissue in 2M NaOH, washed several times and counting the hypnospores cell number using an hemocyte cell counter (Choi et al. 1989). With application of molecular analysis of DNA markers using PCR and cloning, more species-specific and faster diagnostics have been developed to detect the *Perkinsus* infection. In addition, these marker DNAs have been widely used to understand phylogenetic affinities of *Perkinsus* species (Siddall et al. 1997, Noren et al. 1999, Kuvardina et al. 2002, Leander and Keeling 2004, Saldarriaga et al. 2003), or to understand genetic variation among or within *Perkinsus* species.

Commonly used DNA markers in *Perkinsus* diagnosis include ribosomal DNA (rDNA) and actin gene DNA. rDNA is a DNA sequence that encodes ribosomal RNA (rRNA). Several distinct rRNAs, together with proteins, form ribosomes on which the codons of mRNA are translated into amino acids in protein synthesis. The reason why rDNA is widely used as DNA markers is that different regions of rDNA repeat unit (Fig.1) evolve at very different rates (Hillis and Dixon 1991). For example, 18S rDNA has slow evolving rate, thus showing the less DNA sequence variation within the species. On the other hand, internal

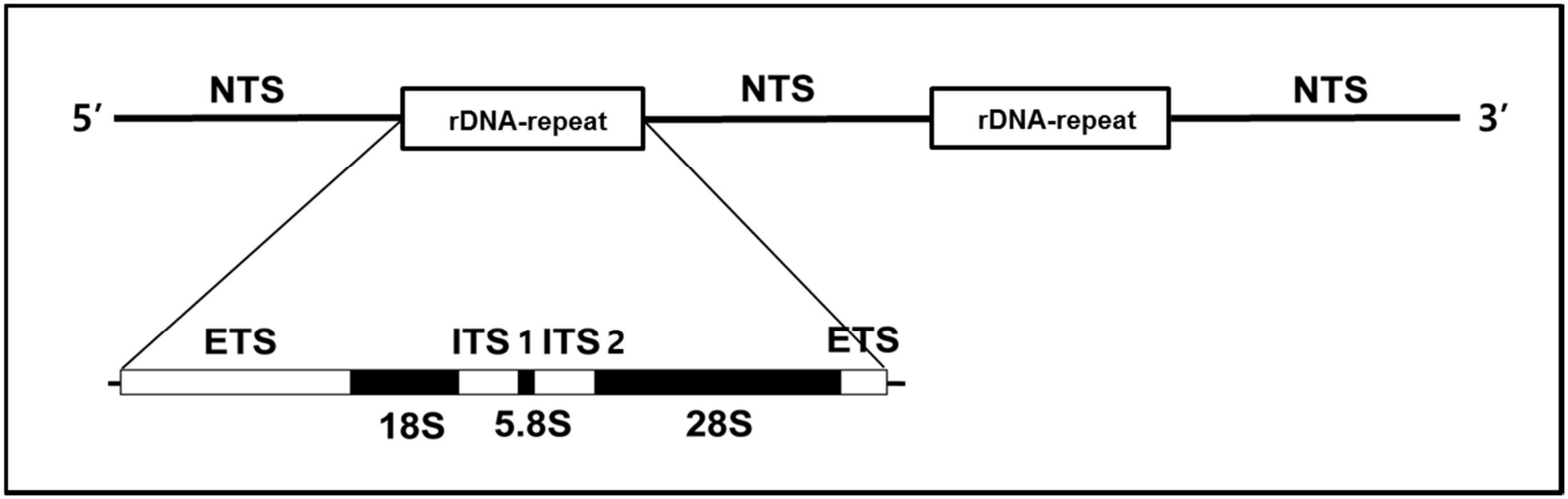

Fig. 1. Repeated unit and array of the rDNA of *Perkinsus* spp. NTS: non-transcribed spacer; ETS: external transcribed spacer; ITS: internal transcribed spacer.

transcribed spacer (ITS) region and non-transcribed spacer (NTS) region evolve the most rapidly among rDNAs. Therefore these spacer regions have been used to identify species or strains (Hillis and Dixon 1991).

As the component of microfilaments in the cytoskeleton, the actin protein is essential in eukaryotic cellular processes including cell motility (transport of macromolecules, phagocytosis, and chromosome movement), cell division, and cell growth. This functional efficiency in essential process does not allow actin proteins to evolve rapidly (Voigt, K. and Wöstemeyer 2000; Blanchoin et al. 2014). Therefore, the actin gene is considered to be highly conserved demonstrating the low level of DNA sequence variation within the species. This gene has been applied to examine phylogenetic position of *Perkinsus* (Reece et al. 1997), and species identification (Dungan et al. 2007; Casas et al. 2008; Moss et al. 2008; da Silva et al. 2014).

A sequence polymorphism is a variation in a DNA sequence that is presented at a particular nucleotide site, and occurs at a frequency of >1% in the population (Mir and Southern 2000). DNA sequence variation comprises substitution, insertion and deletion of nucleotide (Fig. 2). Substitution which is comprised of transition and transversion is defined as single or few base changes. Transition refers to the substitution of a purine for a pyrimidine, and transversion is the substitution of one purine nucleotide to another purine or pyrimidine to another pyrimidine (Fig. 3). Insertion and deletion of a single or few bases result in addition and missing of nucleotides, respectively. Contrary to a mutation of DNA sequence that directly associated with a change in phenotype, polymorphism in DNA sequence are not directly associated with phenotypic characteristics and do not code for proteins. However, polymorphism has been a convenient and useful genetic marker to examine phenotypic differences within a population.

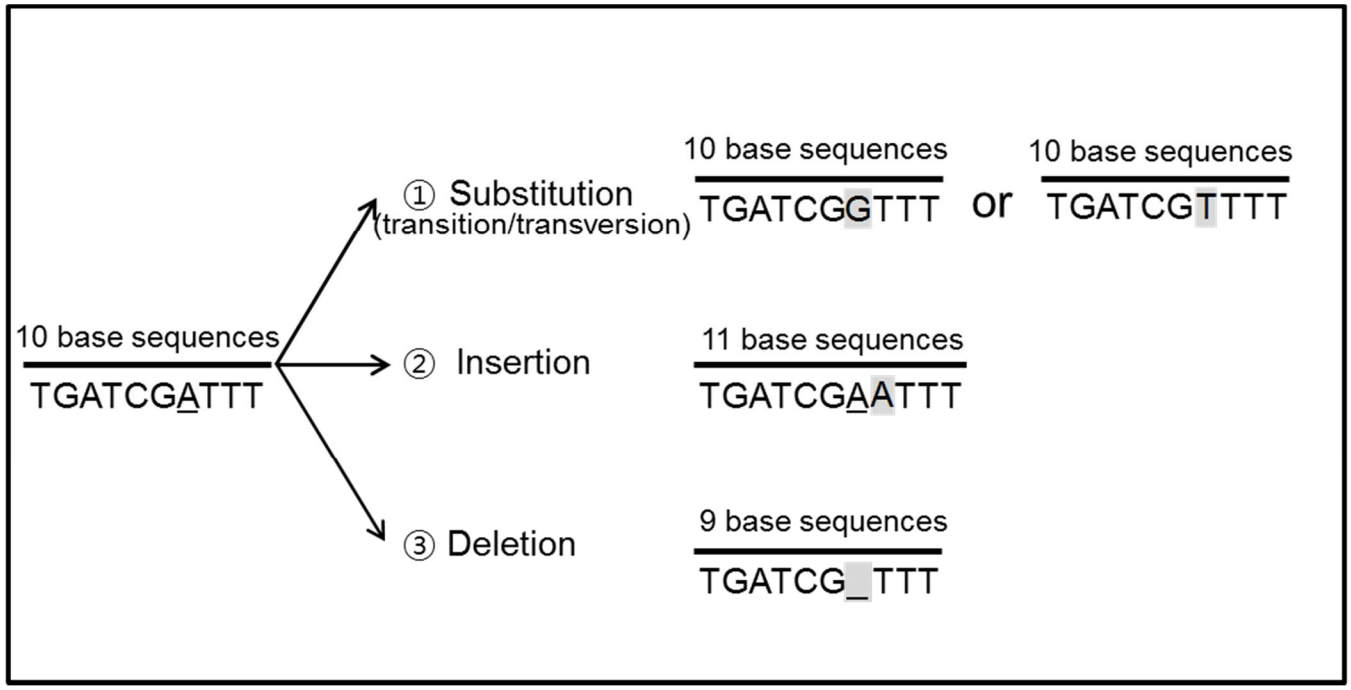

Fig. 2. Three types of DNA sequence variation. (1) Substitution. Underlined nucleotide 'A' from the original sequence is used as a base and changed nucleotides are presented in the grey box. Note the difference in the number of base sequence after being changed. Nucleotide 'A' is changed to 'G' (transition) or 'T' (transversion) with no changes in number of the sequences. See the difference between transition and transversion for Fig.3. (2) Insertion of the single nucleotide results in addition of one nucleotide. (3) Deletion. Underlined nucleotide 'A' from the original sequence is missing. Number of the base sequence is changed from 10 to 9.

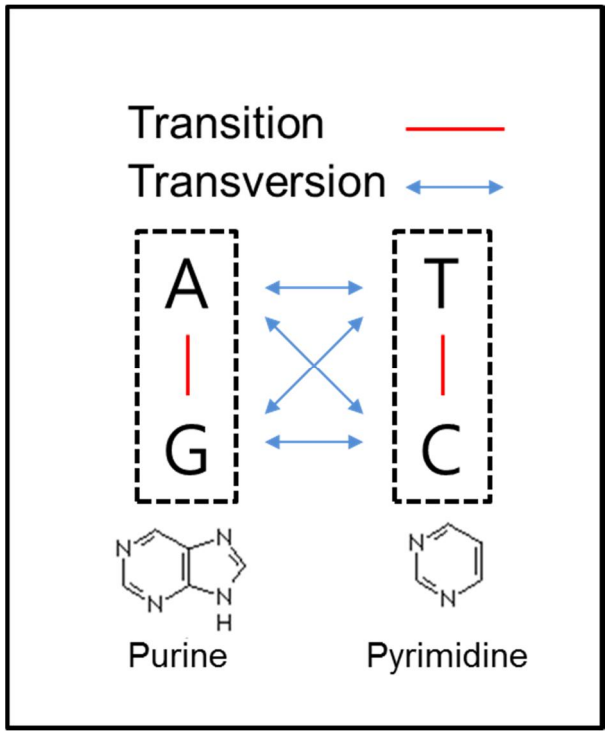

Fig. 3. Two types of DNA sequence substitution. Transitions are indicated in red (—) and transversions are in blue (↔). Transition refers to the substitution of a purine for a pyrimidine, and transition is the substitution of one purine nucleotide to another purine or one pyrimidine nucleotide to another pyrimidine.

Identifying DNA sequence variability of a parasitic pathogen is crucial in understanding different level of virulence or host-parasite interactions such as availability of evading the host's immune response (Schmid-Hempel 2009). So far, a few studies have demonstrated relationship between the DNA sequence variation and phenotypic characteristics in *Perkinsus*. Reece et al. (1997) analyzed four polymorphic loci of *in vitro* cultured *Perkinsus marinus* isolates from the eastern oyster, *Crassostrea virginica* collected from nine geographically distant locations. In their study, all loci examined were variable within the species, suggesting that the oysters are infected by multiple strains of *P. marinus*. Later, Reece et al. (2001) also reported the genetic variability among the geographic strains, using more than 150 cultures of *P. marinus* and eight polymorphic sites. Although other studies have revealed the occurrence of genetic variation within *Perkinsus* species (Robledo et al. 1999, Brown et al. 2004, Escobedo-Fregoso et al. 2012, Fernandez-Boo et al. 2015), no clear evidence of relationships between DNA sequence variability and phenotypic characteristics have been found.

One clue of possible genetic variation in *P. olseni* populations in Korean waters has been observed. According to a national survey of *Perkinsus* infection in Manila clam along the three coasts of Korean waters, spatial variation in *P. olseni* infection prevalence/intensity in clam populations has been reported (Yang 2011). Even an infection prevalence/intensity of different clam population from geographically distant, but same coast has shown a wide spatial variation (Yang 2011, Park et al. 2013). Although several factors such as clam size, density, water temperature and type of sediment have also been known to affect *Perkinsus* infection prevalence/intensity (Villalba et al. 2005, Park et al. 2006a, Yang et al. 2012), spatial variation can be one of the factors reflecting genetic differences as have been suggested by Reece et al. (2001).

Several marker DNAs have been widely used to solve phylogenetic affinities of *Perkinsus* species (Siddall et al. 1997, Noren et al. 1999, Kuvardina et al. 2002, Leander and

Keeling 2004, Saldarriaga et al. 2003), and to understand genetic variation among or within *Perkinsus* species. Since it is difficult to distinguish *Perkinsus* species by morphology (Goggin 1994) or geographical distribution, marker genes such as small sub-unit (SSU) rRNA and actin genes have been applied to resolve phylogenetic association of different *Perkinsus* species. However, these DNA markers were found to be insensitive to distinguish different species in the genus *Perkinsus* (Casas et al. 2004). In contrast, several studies have reported that ITS and NTS markers are sensitive and useful in molecular speciation among different species of *Perkinsus*, as well as in examining genetic variation within a population of *Perkinsus* species. Brown et al. (2004) identified multiple polymorphic sites in 12 genotypically and geographically diverse *P. marinus* isolates (Reece et al. 1997, 2001) based on sequencing of ITS. Similarly, Escobedo-Fregoso (2012) found polymorphism in ITS and NTS that can be used to discriminate genotype of *P. marinus* at population level.

Several studies have emphasized on usefulness of NTS sequences in identification of genetic variation in populations of *Perkinsus* species (Robledo et al. 1999, Escobedo-Fregoso et al. 2012). However, insufficient registered NTS sequences of genus *Perkinsus* in the GenBank hinder clearer identification of sequence variation within or among *Perkinsus* species. Additionally, although several markers have been applied, it is not practically explained to what extent these markers discriminate among *Perkinsus* species (Robledo et al. 1999).

Since the first establishment of in vitro culture of *Perkinsus* (La Peyre et al. 1993), the number of papers published on *Perkinsus* spp. increased dramatically (Fernandez-Robledo et al. 2014). This is in part because the ability to mass culture parasites *in vitro* enables essential biological, metabolic and morphological research that would otherwise be difficult or impossible to accomplish (La Peyre 1996). In order to obtain large quantities of the parasites for molecular analysis, continuous *in vitro* culture of *P. olseni* is essential. Although several *P. olseni* strains isolated from Manila clam have been cultured *in vitro* in

Europe and other countries, in vitro culture of P. olseni isolated from Manila clam in Korean waters has not been established yet.

A variety of culture media have been used to culture *Perkinsus* spp. *in vitro*. One of the *Perkinsus* in vitro culture media used in early phase was JL-ODRP-1 (La Peyre et al. 1993). The composition of the medium resembled to that of cell-free hemolymph (plasma) of oyster. JL-ODRP-1 was firstly used to propagate *P. marinus* and later for *P. chesapeaki* (McLaughlin and Faisal 1998). JL-ODRP-2 and JL-ODRP-2F which are modified version of JL-ODRP-1 have been used to *in vitro* culture *P. olseni* and *P. mediterraneus* (Casas et al. 2002, Casas et al. 2008, 2011). Currently, commercially available *in vitro* culture media for *Perkinsus* include Dulbecco modified Eagle's medium (DME), DME:Ham's nutrient mixture F-12 (DME:Ham's F-12), Leibovitz's L-15 medium, NTCT-135 and RPMI-1640, supplemented with 5-20% fetal bovine serum (FBS) or/and oyster plasma (La Peyre 1996). Dungan and Hamilton (1995) later evaluated efficiency of these commercial media, as well as Gauthier and Vasta (1995) also evaluated optimal concentration of FBS to be added in the medium. As a result, DME:Ham's F-12 (1:2) supplemented with 5% FBS was found to be the optimal combination.

The present study aimed to seek the genetic variation within *P. olseni* populations in Korean waters and to compare effectiveness of the four commonly used DNA markers in *Perkinsus* studies. For these purposes, 18S rDNA, actin, ITS and NTS DNA markers were applied to characterize genotypes of *in vitro* cultured populations of *P. olseni* isolated from clams from 6 different locations in Korean waters.

2. Materials and Methods

2.1. Sampling

Several studies carried out in Korea reported that the infection prevalence and intensity of P. olseni reach its annual peak in late summer or in early fall (Park and Choi 2001; Yang 2011). Accordingly, clams were collected in October 2013, to ascertain to obtain clams with the high prevalence and intensity during post-spawning season (Park et al. 2006a; Uddin et al. 2010). Initially, 40 clams were collected either from natural habitat or from commercial clam beds along the west, south, east coast and Jeju Island (Fig. 4). Among the 40 clams from each site, 30 clams were used for RFTM assay and 10 were used to isolate P. olseni for the *in vitro* culture.

2.2. RFTM assay

P. olseni infection prevalence and intensity level in clams was determined using RFTM assay (Ray, 1966) and Choi et al. (1989). The gill tissue was excised from body and placed in a tube with 5 ml of RFTM fortified with Penicillin-Streptomycin (200 mg/ml) and Nystatin (200 U/ml), as an antibiotic. After incubation for 7-10days in 25℃ at dark, the clam tissues containing induced *Perkinsus* hypnospore were digested with 2 M NaOH according to the method of Choi et al. (1989). The digested tissues were washed several times using phosphate buffered saline (PBS, pH 7.6) at 3,000 RPM. The number of hypnospores in the tube was then counted four times using hemocytometer, and calculated in the form of *Perkinsus* cells/g gill tissue weight.

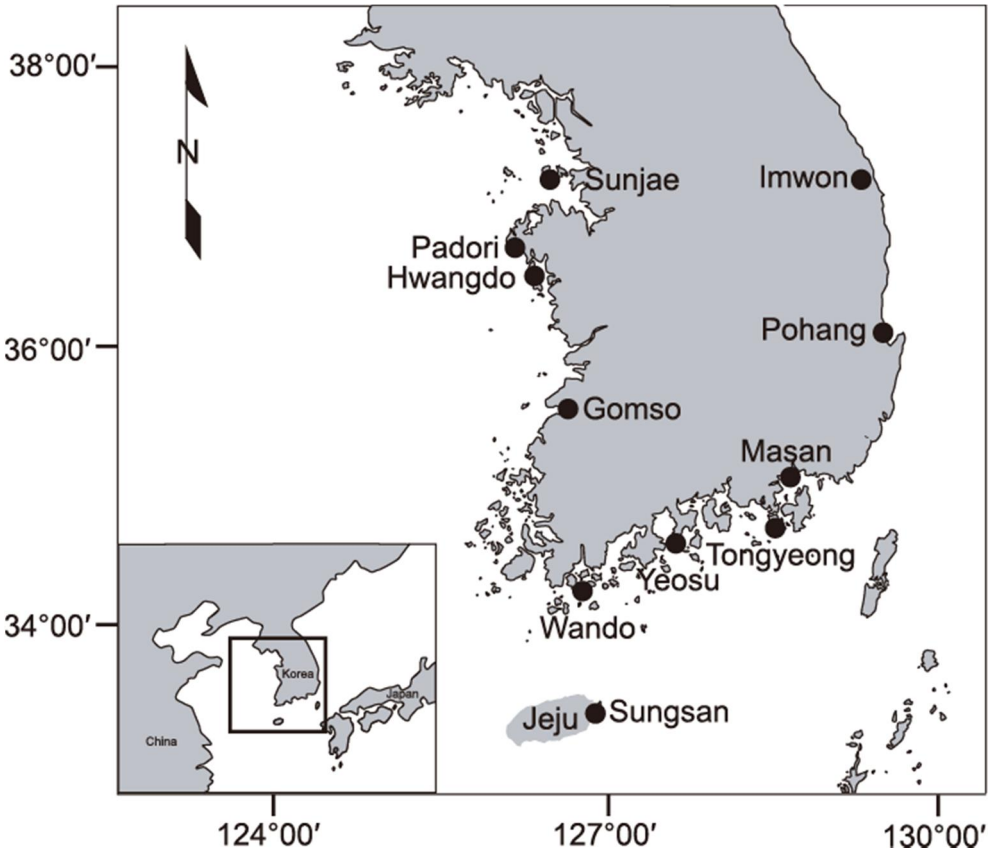

Fig.4. Map showing sampling sites of this study.

2.3. *In vitro* culture

2.3.1. Hypnospore cell induction

To establish and maintain *Perkinsus* cell culture *in vitro*, clam gill tissues was used as a source of the parasite. Each of the 10 gill tissue fragment was incubated in RFTM at 25 ℃ in the dark for two days, and the medium was fortified with antibiotics (Penicillin-Streptomycin 200 mg/ml, Nystatin 200 unit/ml) to prevent microbial contamination. To isolate the hypnospores from the gill tissues, tissues were cut in small pieces aseptically with a blade, and sieved through a sterile 100 μm nylon mesh.

2.3.2. Zoospore and trophozoite cell induction

The growth culture medium DMEM:Ham's F-12 (1:2) supplemented with 5% FBS, 1M HEPES, 1M $NaHCO_3$ and 200mM L-glutamine was used to establish and maintain *in vitro* culture of *P. olseni.* The hypnospores harvested from RFTM culture were carefully isolated from the tissue using aseptic forceps and inoculated in the growth medium fortified with antibiotics (Penicillin 500 units/ml, Streptomycin 500 μg/ml, Amphotericin B 1.25 μg/ml) at 25 ℃ in the dark to induce zoospores. After 7-10 days of incubation, the prezoosporangia were formed inside the hypnospores and the released zoospores were transferred into 2 ml of new same medium, and induced to develop trophozoites. From the primary cell culture composed of trophozoite cell population, 100 μl of cells were inoculated into 24-well plate containing the growth medium and serially diluted to obtain single or poly trophozoite cell (Fig. 5). Single or poly trophozoite cells were incubated until cell density reaches 10^{5-6} cells/ml in the new same medium. *In vitro* cultures initiated from the single cell, called single cloned isolate and those initiated from more than two cells were called poly cloned isolates.

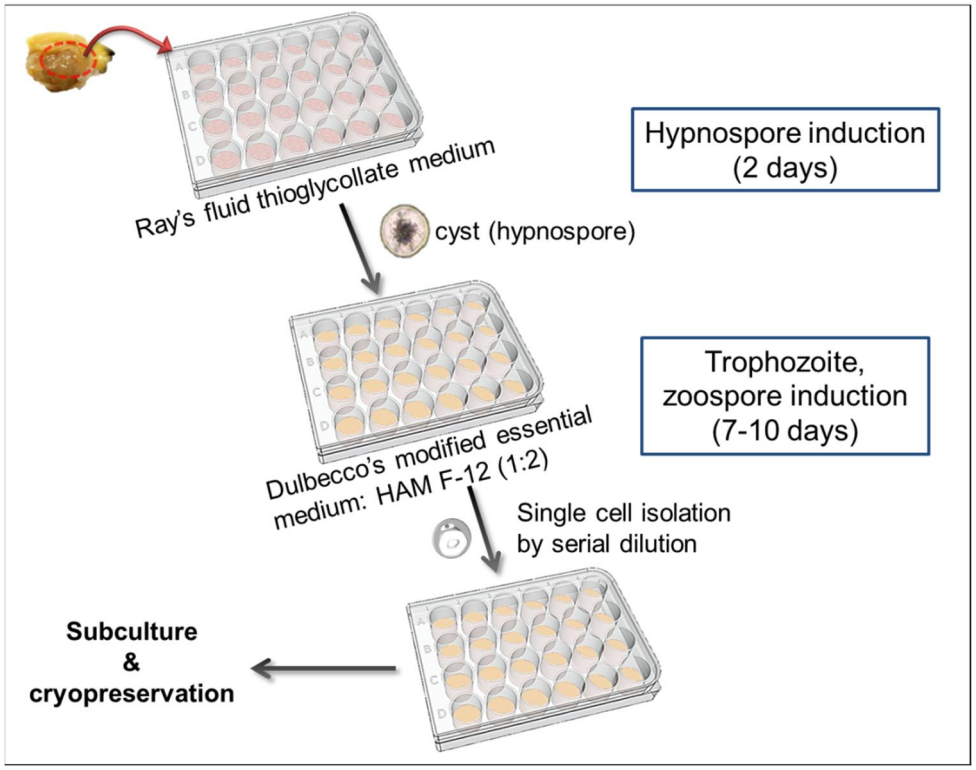

Fig. 5. A schematic process showing *in vitro* culture method of *P. olseni* in this study.

2.4. DNA extraction and sequencing

For DNA extraction, *in vitro* cultured *Perkinsus* cells were propagated in DMEM:F-12 (1:2) until the trophozoite reaches a density of 10^6 to 10^8 cells/ml. One ml of cell suspension was transferred to 1.5 ml microcentrifuge tube and centrifuged (2,000 RPM) to remove the medium. After washed with phosphate buffered saline (pH 7.2), the trophozoite cell pellet was re-suspended with tissue lysis buffer and proteinase K, and total DNA was extracted using DNeasy blood & Tissue Kit (Qiagen, Germany). A concentration of total DNA was measured using DS-11 Spectrophotometer (DeNovix, USA), and diluted at the concentration of 50 ng/μl.

Primer sets for an amplification of 18S ribosomal DNA, actin, ITS, NTS region used in this study are presented in Table 1. To minimize PCR amplification error and increase amplification efficiency, PCR was performed with Prime STAR HS polymerase (Takara) which possesses a 3'-5' exonuclease activity, resulting in a proofreading ability and a low error rate. A total of 50 μl reaction included 50 ng of template DNA, 5X PrimeSTAR buffer, 2.5 mM dNTPs, 25 pico-mole of primer set and 1.25 unit of DNA polymerase. PCR condition used in the analysis was as follows: initial denaturation at 98℃ for 3 min; 30 cycles of 98℃ for 10sec, 55℃ for 15sec and 72℃ for 1min (1min 50sec for 18S rDNA); final extension of 72℃ for 5min. The size of amplicons was confirmed with 1.2 % agarose gel electrophoresis and visualized by ethidium bromide under UV light.

The amplified reaction products were purified using AccuPrep PCR purification kit (Bioneer, Korea), and ligated into a cloning vector pUC118 (Takara, Japan). The vectors were transformed into *Escherichia coli* DH5–α, and plated onto LB agar plates containing 100μg/ml antibiotics. LB agar plates were incubated at 37℃ for 14 h and recombinant clones were identified by blue/white screening. 46 clones (2 clones each from 23 *Perkinsus* isolate) with inserts were purified using an Accuprep Plasmid Mini Extraction Kit (Bioneer, Korea) and sequenced (Macrogen Sequencing Service, Korea).

Table 1. DNA sequences of PCR primers used in amplifying 18S rDNA, actin gene, ITS and NTS region.

Gene	Sequences (5'-3')	Reference
ITS	Perkin ITSF: CCTAGAGGAAGGAGAAGTCGTAACA Perkin ITSR: GCTTANTTATATGCTAAATTCAGCG	Park et al., 2005
NTS	NTS F: AAGTCCTTAGGGTGCTGCTGGCTTCTT NTS R: TCACCTGGTTGATCCTGCCAGTAGT	Park et al., 2005
18S	Perkins-univ: CCTGGTTGATCCTGCCAGT Perkin SSU R: TGTTACGACTTCTCCTTCCTCTAAG	Itoh et al., 2013 Park et al., 2005
Actin	PerkinActin1 130F: ATGTATGTCCAGATYCAGGC PerkinActin1 439R: CTCGTACGTTTTCTCCTTCTC	Moss et al., 2008

2.5. Sequencing analysis and phylogenetic relationships

Sequences of *P. olseni* isolates obtained from *in vitro* culture were edited using DNAssist and analyzed for sequence polymorphism with DNaSP 5 software (Librado and Rozas 2009). For each DNA marker, every sequence was assigned a sequence type according to the polymorphism. The most common sequence was designated 'α', followed by 'β' and 'γ'. Sequences of α, β and γ obtained in this study and those from GenBank registered were aligned using the Clustal W (Thompson et al. 1994). To evaluate intra and inter-specific variation, pairwise distance was calculated using MEGA 6 (Tamura et al. 2013).

To understand phylogenetic relationship among the variants and other species of *Perkinsus*, 46 clones were listed by geographic origin and representative sequences were aligned with available *Perkinsus* spp. sequences. Phylogenetic distance was calculated using Tamura-Nei model for actin DNA, and Kimura-3-parameter for ITS, NTS and 18S DNA. The phylogenetic trees were computed with Neighbor Joining model using MEGA 6 software. Robustness of the trees was tested 2,000 times with bootstrap test.

3. Results

3.1. *In vitro* culture

Perkinsus infection status of at the 11 sampling sites and *in vitro* culture isolates obtained in this study are summarized in Table 2. Except for Padori, Imwon and Pohang where infection prevalence and intensity level of *Perkinsus* are known to be low (Yang 2011), remaining the 8 sites revealed high infection prevalence and intensity (>10^6 *Perkinsus* cells/g gill tissue weight). Twenty three isolates were obtained from 6 sampling locations where the level of *Perkinsus* infection prevalence and intensity were high. Up to 6 isolates were produced from each of 6 sites. Except for the 2 poly cloned isolates, all were single cloned isolates. From 5 sampling sites, isolation and *in vitro* culture of *P. olseni* were unsuccessful due to a contamination with fungal organisms during the incubation.

3.2. Sequence polymorphism in the 18S and actin

Polymorphic sites and obtained sequence types of 1773bp 18S rDNA sequences are summarized in Table 3. Among 46 sequenced DNA clones (i.e. 23 isolates x 2), 3 different sequence types were identified including 42 major type sequences (α type) and 4 variant type sequences (β and γ type). Transition or transversion which occurred 0.2 times per 100 base pairs were found at 3 different nucleotide sites.

The expected 330 bp amplicons of actin gene DNA were sequenced. Unlike the others, sequenced actin DNA clones consisted of two sequence types that were distinguished by transition at only one nucleotide position (0.3 times per 100 base pairs) (Table 4).

3.3. Intra and inter-specific variation in the 18S and actin

Although three types (α, β, γ) of sequences were identified by sequence polymorphism, 18S rDNA sequences obtained in this study were 99.9% identical to each

other. Sequence type of β and γ were 99.9% similar to that of GenBank deposited *P. olseni* (Table 5). Figure 6 shows 18S rDNA genetic distance (%) from α type in this study to *P. olseni* and *P. marinus*. 0.78% of pairwise distance gap was observed between *P. olseni* and *P. marinus*.

Two types of actin gene DNA sequences obtained in this study were 100% identical to each other when pairwise distance were calculated (Table 6). In other words, no intra specific sequence variation could be observed with this gene marker. However, pairwise distance gap between *P. olseni* and *P. marinus* were 11.8% which was second biggest among DNA markers used in this study (Fig. 6).

3.4. Phylogenetic analysis of the 18S and actin

Based on the neighbor joining analysis, the 18S rDNA sequences of *Perkinsus* sp. *in vitro* culture isolates from Korea were grouped into the *P. olseni* clade (Fig. 7). Within this, a clade consisted of α and β type sequence that we determined grouped together with *P. olseni* in *R. philippinarum* previously reported from Korea forming a sister clade with *P. mediterraneus* in *Ostrea edulis*. On the other hand, a clade consisted of β type sequence formed a monophyletic clade. The actin gene sequences obtained from *Perkinsus* sp *in vitro* culture isolates grouped into *P. olseni* clade which is divided into two sub-clades (Fig. 8).

Table 2. Infection status and *in vitro* cultured *Perkinsus olseni* isolates in this study. GTWT; gill tissue weight, SE; standard error.

Origin		N	Prevalence (%)	*Perkinsus* cells/g GTWT (Mean±SE)	Isolate	Clonality (# of isolates)
West coast	Sunjae	30	90.0	$1.00\times10^{6}\pm1.72\times10^{5}$	-	-
	Padori	30	13.3	$6.99\times10^{3}\pm4.22\times10^{3}$	-	-
	Hwangdo	30	100.0	$4.31\times10^{6}\pm4.73\times10^{5}$	HD11, HD12-a,b	single (3)
	Gomso	30	100.0	$1.62\times10^{6}\pm1.83\times10^{5}$	GS31-a,b, GS32, GS41-a,b,c	single (6)
South coast	Wando	30	100.0	$2.30\times10^{6}\pm3.07\times10^{5}$	WD01-a,b,c, WD05-a,b,c	single (6)
	Yeosu	30	86.7	$1.13\times10^{6}\pm2.28\times10^{5}$	YS41, YS51, YS51P*	single (2) or poly(1)
	Tongyeong	30	100.0	$3.21\times10^{6}\pm4.27\times10^{5}$	-	-
	Masan	30	100.0	$4.87\times10^{6}\pm8.43\times10^{5}$	-	single (3)
	Pohang	30	0.0	0.00	-	-
	Imwon	30	10.0	$1.74\times10^{3}\pm0.98\times10^{3}$	-	-
Jeju island	Sungsan	30	100.0	$2.10\times10^{6}\pm3.08\times10^{5}$	SS32, SS32P*	single (1) or poly(1)
				Total		23

Table 3. Polymorphic nucleotide sites of 18S rDNA (1173 bp) obtained from *in vitro* cultured *P. olseni* isolates in this study. Sequence of variant type 'α' was used as the base. Substitution are marked with asterisk; transition (*) and transversion (**)

Variant type	# DNA clones	Nucleotide sites			# Polymorphic sites/100bp
		763	1375	1716	
α	42	G	T	T	
β	3		C*	A**	0.2
γ	1	C**			

Table 4. Polymorphic nucleotide sites of actin gene DNA (330bp) observed in this study. Substitution are marked with asterisk; transition (*)

Variant type	# DNA clones	Nucleotide sites	# Polymorphic sites/100bp
		15	
α	40	T	0.3
β	6	C*	

Table 5. Mean pairwise genetic distances (percent sequence similarity) between 18S rDNA sequences obtained in this study and Genbank registered sequences of *Perkinsus* spp.

	α	β	γ	*P. olseni*	*P. mediterraneus*	*P. marinus*	*P. chesapeaki*	*P. qugwadi*
α	100	99.90	99.90	100	99.9-100	98.9-99.5	98.9-99.2	96.4
β		100	99.90	99.9	99.8-99.9	98.8-99.4	98.8-99.1	96.4
γ			100	99.9	99.8-99.9	98.8-99.4	98.8-99.1	96.4
P. olseni				100%	99.9-100	98.9-99.5	98.9-99.2	96.40
P. mediterraneus					>99.9	98.8-99.5	98.8-99.2	96.3-96.4
P. marinus						99.2-99.8	98.1-98.8	95.6-96.2
P. chesapeaki							99.6-99.9	95.9-96.2
P. qugwadi								100

Table 6. Mean pairwise genetic distances (percent sequence similarity) between actin gene DNA sequences obtained in this study and Genbank registered sequences of *Perkinsus* spp.

	α	β	*P. olseni*	*P. mediterraneus*	*P. marinus*	*P. honshuensis*	*P. chesapeaki*
α	100	100.00	>96.89	88.28-88.93	87.20-87.54	86.85	85.12-85.43
β		100	>96.89	88.28-88.93	87.20-87.54	86.85	85.12-85.43
P. olseni			>96.89	88.24-89.62	86.85-88.58	86.85-87.54	84.08-85.12
P. mediterraneus				>99.31	85.81-87.54	93.08-93.77	82.35-83.39
P. marinus					>97.92	84.43-85.81	83.39-84.43
P. honshuensis						100	81.66-82.35
P. chesapeaki							>98.62

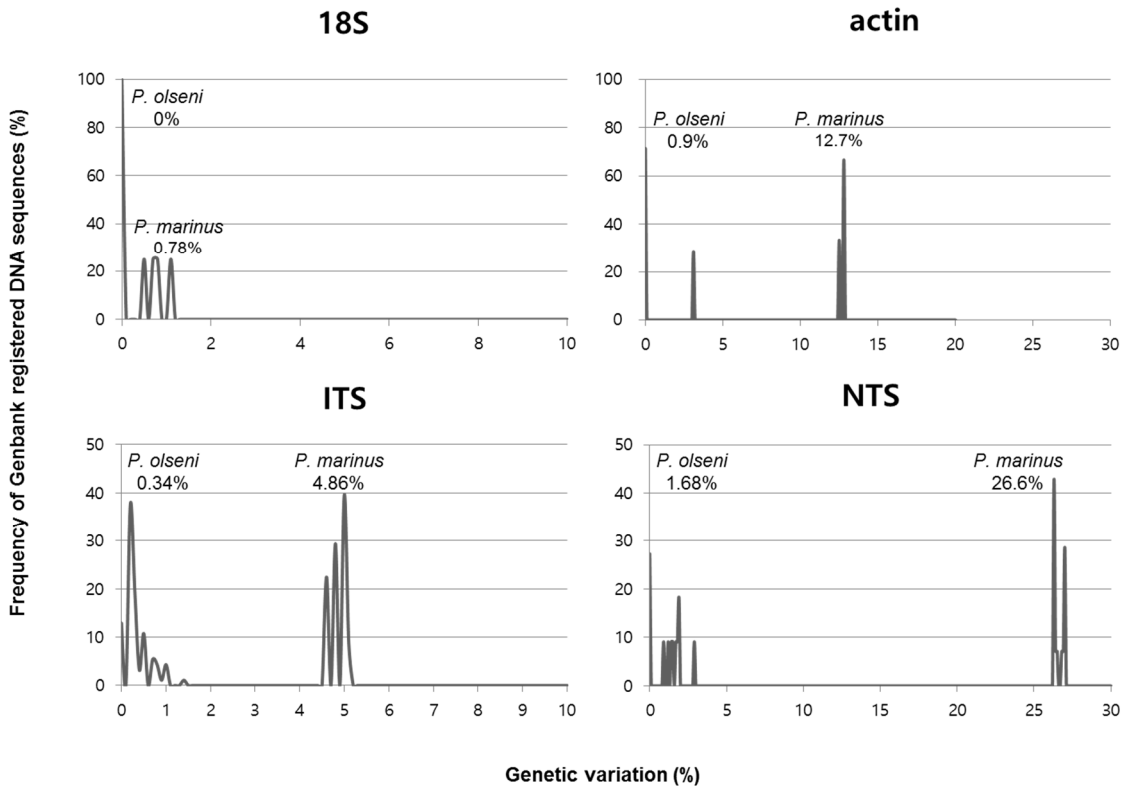

Fig. 6. Genetic variation between *P. olseni* and *P. marinus* inferred from pairwise distance gap. Numbers on each peak represent mean percentage gap between the α type DNA sequence obtained in this study and Genbank registered *P. olseni* or *P. marinus.*

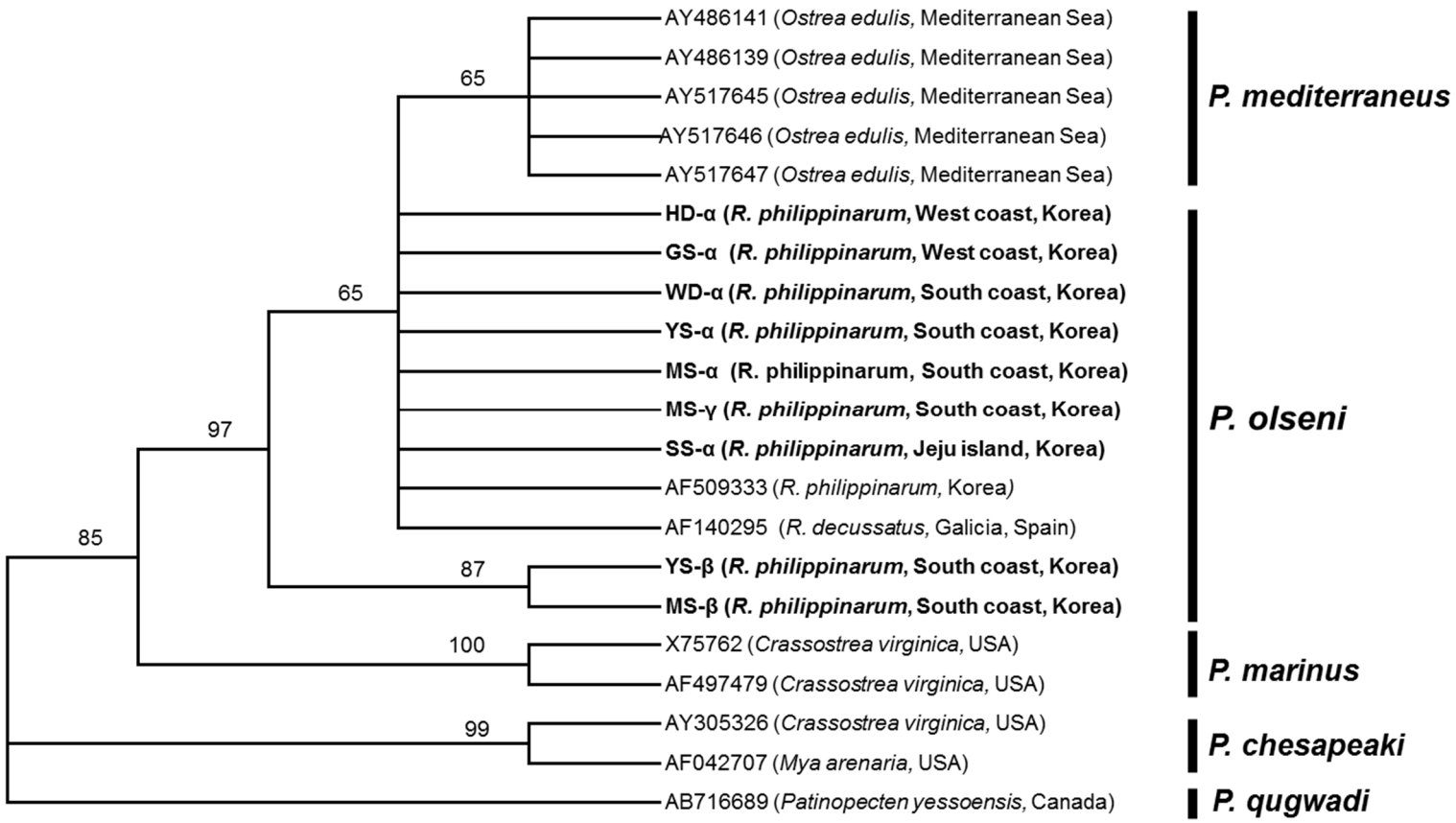

Fig. 7. Neighbor joining tree determined by analysis of the 18S rDNA sequences obtained in this study. Bootstrap support values are given above the branch respectively; only values >50% are shown. GenBank accession numbers are indicated in each branch followed by host of the parasite and geographic origin in parenthesis. The sequences obtained in the present study are indicated in bold, and the letter 'α', 'β', 'γ' refer to the variant type of each clonal isolates.

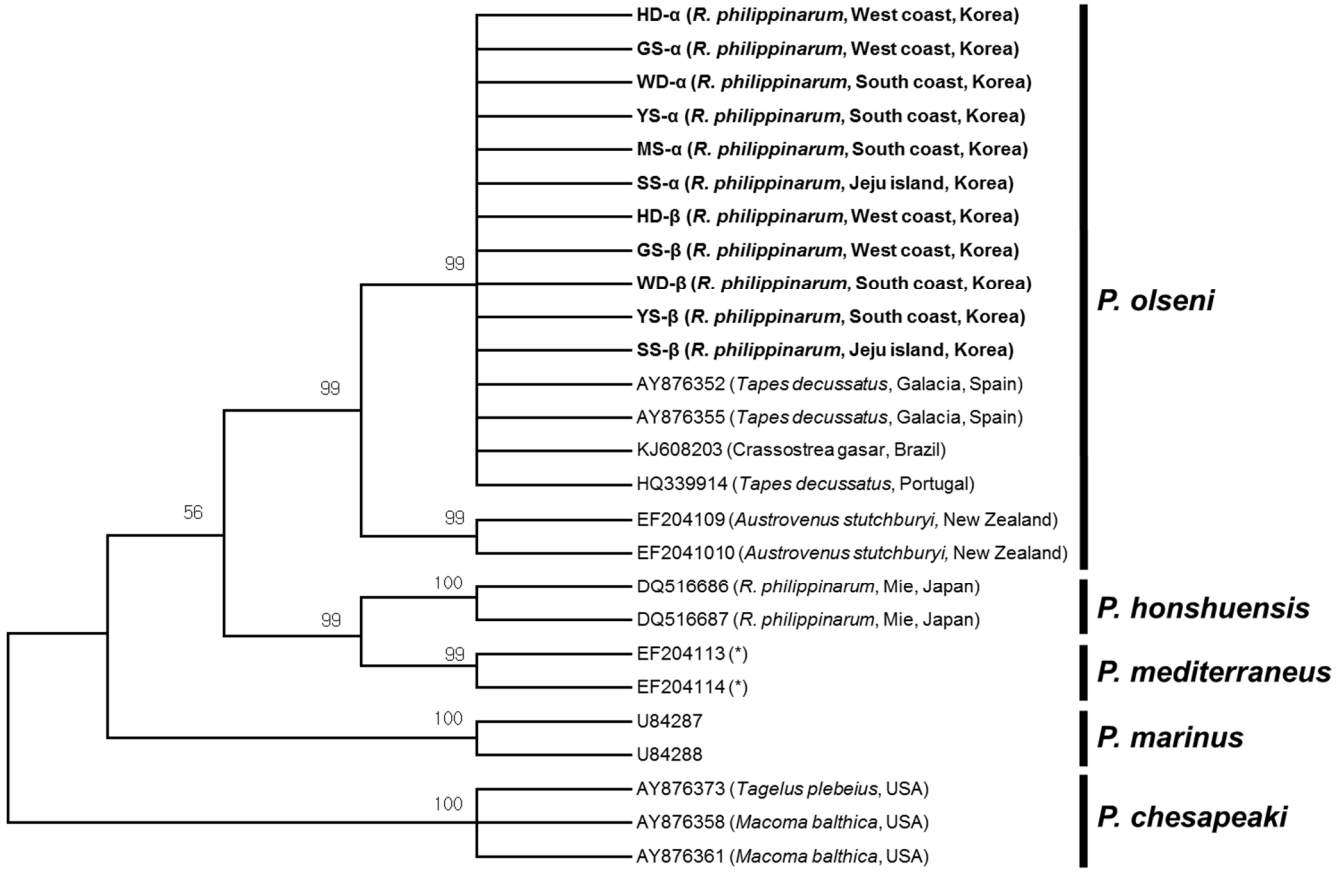

Fig. 8. Neighbor joining tree of the actin gene DNA resulting from the analysis with neighbor-joining method based on the Tamura-Nei model. Bootstrap values larger than 50% are shown above the branch. GenBank accession numbers are indicated in each branch followed by host of the parasite and geographic origin in parenthesis. The sequences obtained in the present study are indicated in bold, and the letter 'α', 'β', 'γ' refer to the variant type of each clonal isolates.

3.5. Sequence polymorphism in the ITS and NTS

Polymorphic sites and obtained sequence types of ITS are summarized in Table 7. Amplification of ITS region (ITS1-5.8S-ITS2) yielded 713-714 bp sequences. The ITS region included 183 or 184bp of ITS1, 159bp of 5.8S and 372bp of ITS2. Of the 46 ITS sequences obtained, 5.8S region sequences showed no variation at any position. On the other hand, ITS1 had insertion of a nucleotide at one position and ITS 2 showed transitions at three nucleotide sites. Total of four polymorphic sites which occurred 0.5 times per 100 base pairs were identified. These sequence mutation resulted in identifying three types of DNA sequences including 33 major type sequence (α type) and 13 variant type sequence (β and γ type).

Table 6 summarizes polymorphic sites and sequence type of NTS. Total of 46 sequences with 1170-1179bp were obtained. NTS was the most polymorphic of four DNAs applied in this study. 16 polymorphic nucleotide sites which occurred 1.3 times per 100bp revealed 3 types of NTS sequences including 41 major type sequence (α type) and 5 variant type sequence (β and γ type). The polymorphic sites included 11 transitions, 4 transversions and one insertion of nine nucleotides.

3.6. Intra and inter-specific variation in the ITS and NTS

ITS sequence variation of *P. olseni* isolates obtained in this study was 99.49-100% (Table 9). Sequences of α and β type were 100% identical to that of previously reported by Park et al. (2005). Genetic distance between *P. olseni* and *P. marinus* was 4.5% (Fig. 6).

Intra-specific variation of most variable NTS sequences obtained in this study ranged from 94.49% to 99.38% (Table 10). Compared to sequence of *P. olseni*, deposited at the GenBank, only type α sequence showed 100% similarity to the sequence of *P. olseni* previously reported from Korea (Park et al., 2005). The genetic distance between *P. olseni*

and *P. marinus* was the most wide among DNA markers used in this study showing 24.9% (Fig. 6).

3.7. Phylogenetic analysis of the ITS and NTS

Phylogenetic analysis of ITS region DNA demonstrated that Korean *in vitro* culture isolates were grouped into *P. olseni* clade according to Neighbor joining and Maximum likelihood methods (Fig. 9). Within this clade of clam host, the α and β type variants were grouped together and γ type sequence variant formed another host clade.

Similar results were observed in the phylogenetic analysis of NTS region DNA of Korean *in vitro* culture isolates (Fig. 10). *P. olseni* clade that consisted of clam host was clearly distinguished from that of other host. Within this clam host clade, Korean isolates were grouped into 2 clades

3.7. Locality of sequence type

Polymorphism types and their frequency of occurrence in the sequence of the rDNA from the 23 *in vitro* cultured *P. olseni* populations are listed in Table 11. From a single clonal culture, 2 different types of sequences were also identified. Interestingly, 15 isolate out of 23 isolates showed multiple clones in a single isolate. This phenomenon was most frequently occurred in ITS (8 isolates), while it was less frequent in NTS (i.e., occurred in 1 isolate).

Table 7. Polymorphic nucleotide sites of ITS (713-714bp) obtained in this study. Substitution are marked with asterisk; transition (*), and insertion with the grey column.

Variant type	# DNA clones	ITS1 (nucleotide sites)	ITS2 (nucleotide sites)			# Polymorphic sites/100 base pair
		76	382	396	618	
α	33	-	G	G	C	0.5
β	10	C				
γ	3	-	A*	A*	T*	

Table 8. Polymorphic nucleotide sites of NTS (1170-1179bp) observed in this study. Sequence of variant type 'α' was used as the base. Substitution are marked with asterisk; transition (*), transversion (**) and insertion with the grey column.

Variant type	# DNA clones	Nucleotide sites																# Polymorphic sites/100 base pair
		177	178	234	284	342	353	434	519	521	573	625	719	1036	1068	1108	1125	
α	41	C	T		T	T	G	T	A	A	C	C	T	C	C	A	A	
β	3	T*	C*	†Ins.	C*	C*	A*	C*	T**	T**	T*	T*	C*	T*	T*	T**	T**	1.3
γ	2	T*	C*	†Ins.	C*	C*	A*	C*	T**	T**	T*	T*						

†Ins. Insertion of nine nucleotides (5'-CCACTTGGC-3')

Table 9. Mean pairwise genetic distances (percent sequence similarity) between ITS sequences obtained in this study and Genbank registered sequences of *Perkinsus* spp.

	α	β	γ	*P. olseni*	*P. mediterraneus*	*P. marinus*	*P. honshuensis*	*P. beihaiensis*	*P. chesapeaki*
α	100	100	99.49	>98.65	95.07-96.58	94.94-95.45	93.26	89.38-94.44	87.35-88.03
β		100	99.49	>98.65	95.07-96.58	94.94-95.45	93.26	89.38-94.44	87.35-88.03
γ			100	98.48-99.49	94.81-96.33	94.77-95.28	92.99	89.21-94.27	87.18-87.86
P. olseni				>98.65	96.12-96.80	94.60-95.45	94.60-95.62	89.04-94.44	86.85-88.03
P. mediterraneus					>98.33	95.62-95.78	95.10-96.34	90.56-94.10	87.69-88.70
P. marinus						>99.33	94.60-95.11	89.21-92.92	86.85-87.69
P. honshuensis							100	90.05-93.09	87.18-88.20
P. beihaiensis								>94.60	84.82-87.86
P. chesapeaki									>98.15

Table 10. Mean pairwise genetic distances (percent sequence similarity) between NTS sequences obtained in this study and Genbank registered sequences of *Perkinsus* spp.

	α	β	γ	*P. olseni*	*P. marinus*
α	100	98.49	98.76	>97.07	73.05-73.67
β		100	99.38	97.52-99.02	72.96-73.58
γ			100	97.25-98.78	72.87-73.49
P. olseni				>97.07	72.52-74.18
P. marinus					>98.32

Table 11. Summary on polymorphism of rDNA of *P. olseni* populations analyzed in this study.

Site		Isolate	18S			Actin		ITS			NTS		
			α	β	γ	α	β	α	β	γ	α	β	γ
West coast	Hwangdo	HD11	2			1	1	1	1		2		
		HD12-a	2			2		1	1		2		
		HD12-b	2			2			2		2		
	Gomso	GS31-a	2			2		1	1		2		
		GS31-b	2			2		2			2		
		GS32	2			2			1	1	2		
		GS41-a	2			2			1	1	2		
		GS41-b	2			2		1		1	2		
		GS41-c	2			1	1	2					2
South coast	Wando	WD01-a	2			2		2			2		
		WD01-b	2			2		2			2		
		WD01-c	2			2		2			2		
		WD05-a	2			2		2			2		
		WD05-b	2			2		1	1		2		
		WD05-c	2			1	1		2		2		
	Yeosu	YS41	1	1		2		1		1	2		
		YS51	2			1	1	2			2		
		YS51P	2			2		2			2		
	Masan	MS02		2		2		2				2	
		MS03	1		1	2		2			2		
		MS04	2			2		2			1	1	
Jeju island	Sungsan	SS32	2			1	1	2			2		
		SS32P	2			1	1	2			2		
	Total		42	3	1	40	6	33	10	3	41	3	2

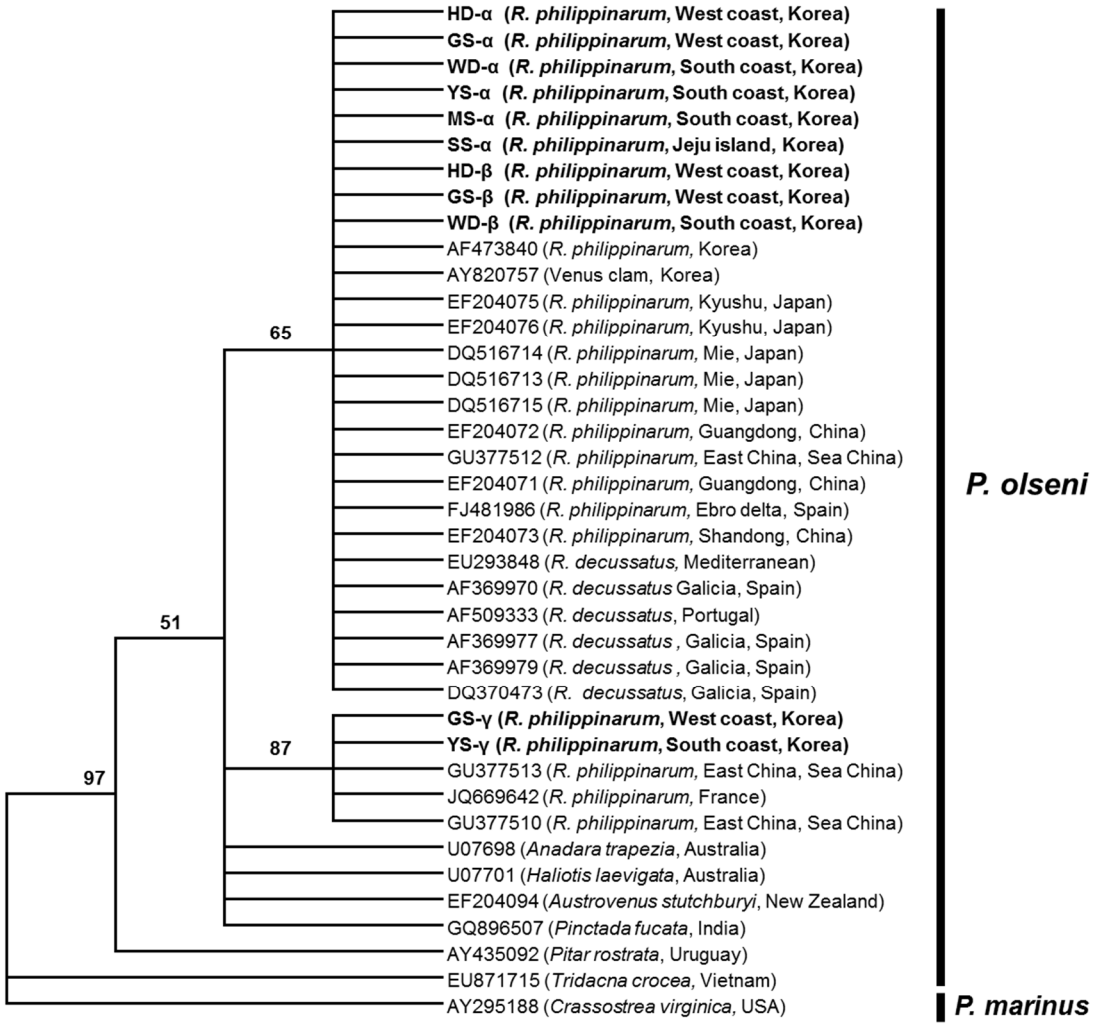

Fig. 9. Neighbor joining tree of the ITS region DNA resulting from the analysis with neighbor-joining method based on the Kimura-3-parameter model. Bootstrap values larger than 50% are shown above the branch. GenBank accession numbers are indicated in each branch followed by host of the parasite and geographic origin in parenthesis. The sequences obtained in the present study are indicated in bold, and the letter 'α', 'β', 'γ' refer to the variant type of each clonal isolates.

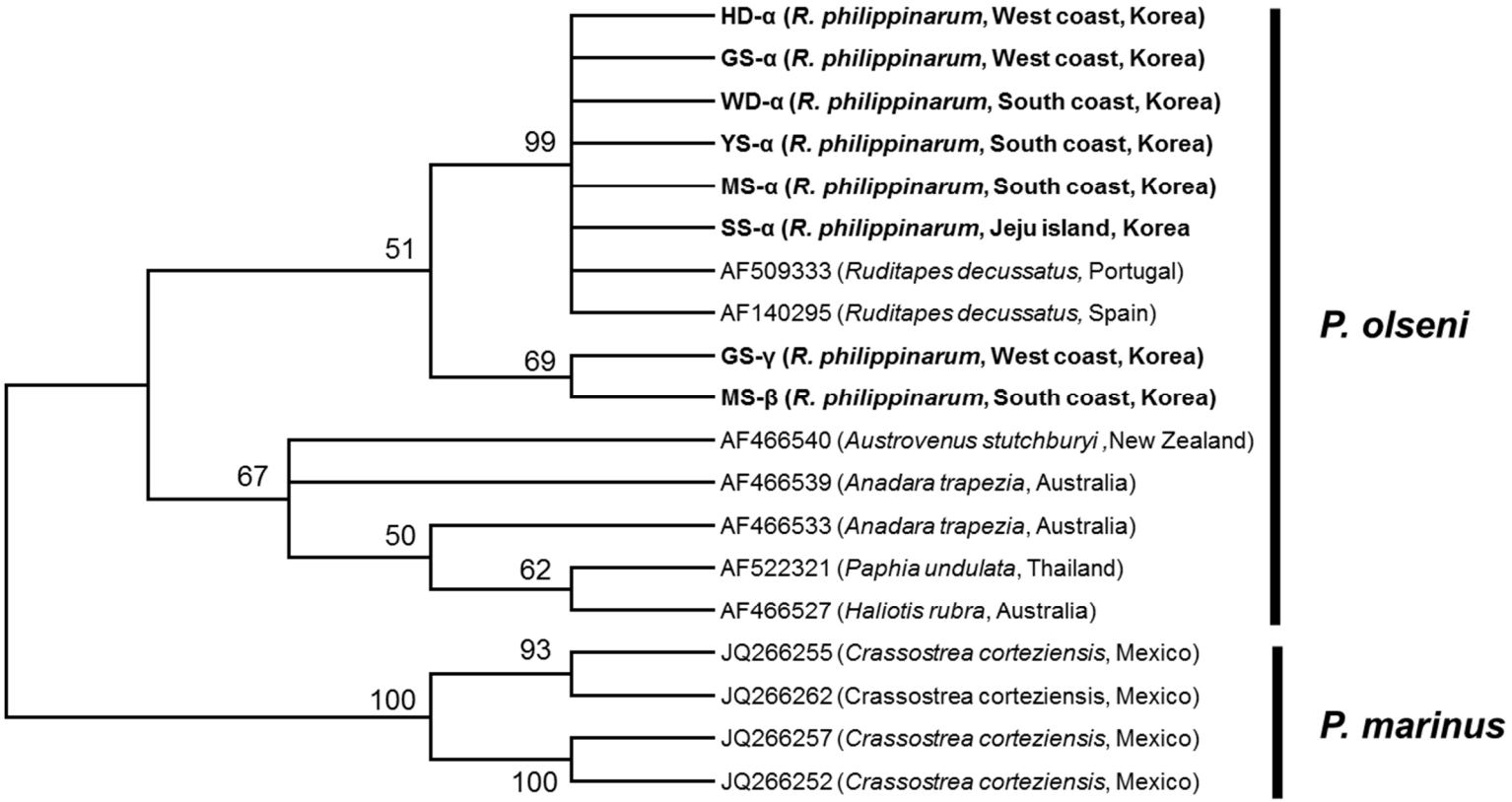

Fig. 10. Neighbor joining tree determined by analysis of the NTS region sequences obtained in this study. The sequences were analyzed with the Kimura-3-parameter model Bootstrap support values are given above the branch respectively; only values >50% are shown. GenBank accession numbers are indicated in each branch followed by host of the parasite and geographic origin in parenthesis. The sequences obtained in the present study are indicated in bold, and the letter 'α', 'β', 'γ' refer to the variant type of each clonal isolates.

4. Discussion

Successful *in vitro* proliferation of *P. olseni* enabled analysis on DNA sequence variation presented in Korean waters and inter-specific variation among genus *Perkinsus* due to the large quantities of the parasite obtained. The culture media DME:Ham's F-12 (1:2) has been widely used for *Perkinsus* spp. (Fernandez 2015) including *P.* olseni (Casas et al. 2002, Robledo et al. 2002, Dungan et al. 2007). In a previous study by Dungan et al. (2007), *P. olseni* has been readily propagated *in vitro* from New Zealand clam *Austrovenus stutchburyi* despite the low intensities of infections among the clams. This result is inconsistent with the present study. From the sampling sites showing low *Perkinsus* infection prevalence/intensity, *in vitro* propagation of the parasite was difficult due to the absence of zoospore and trophozoite formation from hypnospores.

18S rDNA and actin gene DNA are known to be highly conserved, whereas ITS and NTS region are variable due to rapid evolution (Hillis and Dixon 1991; Reece et al. 1997; Weider et al. 2005). Three polymorphic nucleotide sites were presented at 18S rDNA and two for actin. 90% of analyzed 18S rDNA clones, however, were sequence type α and 80% for actin suggesting the presence of major and minor type sequences. Although variant type α accounted for 72% in ITS showing more diversity than two conserved marker DNAs, relatively high ratio (81%) in NTS is an unexpected result. However, NTS was polymorphismic at 16 different sites indicating that this region is most variable among four markers used in this study.

According to phylogenetic affiliations of *P. olseni* isolates from *R. philippinarum* using 4 DNA markers , Korean *P. olseni* strains were classified into 2 clades except for actin

gene (Fig. 7, 9, & 10). In the 18S rDNA phylogenetic analysis, Korean *P. olseni* strains were separated into 2 clades (α, γ and β) based on the transversion (γ type) at nucleotide position of 763 and transition/transversion (β type) at nucleotide position of 1375 and 1716 (Fig. 7). The clade of α and γ type of *P. olseni* strains were genetically close to *P. mediterraneus* strains from *Ostrea edulis* with 65% bootstrap value, and these formed a sister clade with β type of *P. olseni* strains (65% bootstrap value) (Fig. 7). In addition, *P. olseni* strains were clearly distinguished from *P. marinus*, *P. chesapeaki* and *P. qugwadi*. This results are consistent with the previous study by Casas et al. (2004).

In contrast, the ITS and NTS sequences were able to resolve more detailed genotype variation of Korean *P. olseni* strains. As shown in Fig. 9, ITS tree of Korean *P. olseni* strains showed 2 clades (α,β and γ) and were grouped in a clade of *P. olseni* reported from Korea, Japan, China, Europe and Australia. Within the *P. olseni* clade, two main sub-clades could be distinguished based on the host animal, one of this sub-clade was *P. olseni* sequences from clam species (*R. philippinarum* and *R. decussatus*) and the other *P. olseni* sequences included different marine bivalves such as cockle, abalone, pearl oyster and venerid clams (Fig. 9). Similarly, NTS tree of Korean *P. olseni* also formed 2 clades (α and β,γ) with *P. olseni* reported from *R. philippinarum* and *R. decussatus* (Fig. 10). This *P. olseni* clade from clams formed a sister clade with other *P. olseni* reported from cockle, surf clam and abalone with 51% bootstrap value. These result suggest that *P. olseni* from Manila clams in Korean water has two genotypes based on the nucleotide polymorphism of 18S, ITS and NTS sequences, and differentiate these *P. olseni* strains from other host animals.

Several DNA markers such as 18S rDNA, actin, ITS and NTS have been commonly used to assess inter and intra-species variation (Casas et al. 2002; Dungan et al. 2002; Brown et al. 2004; Escobedo-Fregoso et al., 2012) and distinguish phylogeny of the protozoan parasites (Bachvaroff et al. 2001; Dungan and Reece 2006; Moss et al. 2008; Tang et al.

2012). In this study, we analyzed the genetic diversity of inter and intra-species in *P. olseni* and *P. marinus* using 4 rDNA markers. As a result, the genetic variation of conserved genes, 18S rDNA and actin between *P. olseni* and *P. marinus* was 0.8% and 11.8%, while it was 4.5% and 24.9% in the variable genes ITS and NTS (Fig. 6). Intra-species variations within *Perkinsus* spp. have been described in *P. marinus* from *Crassostrea virginica* and *C. corteziensis* (Brown et al., 2004, Escobedo-Fregoso et al., 2012). They demonstrated that nucleotide diversity between ITS and NTS was much higher in NTS, indicating that NTS regions is more useful for genotype identification and relatively ITS marker is a more effective in species-specific diagnosis. Therefore, among 4 DNA markers used in this study, NTS is most suitable for analyzing intra and inter specific sequence variation.

4. Conclusion

In this study, *in vitro* culture method of *P. olseni* has been successfully established which enabled DNA sequence variation analysis using four different DNA markers. Continuous and cryopreserved *P. olseni* isolates will be used not only to further understand relationship between DNA sequence variation and phenotypic characteristics of the parasite, but also to carry out research that would otherwise be difficult to achieve.

DNA sequence variation in *P. olseni* population isolated from Manila calm in Korea has been identified with three types of DNA sequence and two phylogenetically distinct clades. Geographic origin of each *in vitro* cultured isolates was not discriminated by the DNA sequence variability. These results suggest possible phenotypic variation of *P. olseni* within the same population, rather than variation which is discriminated by spatial distribution. Further studies are needed to understand relationship between genetic variation in DNA markers and phenotypic characteristics of *P. olseni*.

To compare the effectiveness of four commonly used rDNA markers of *Perkinsus* spp., 18S rDNA, actin gene DNA, ITS and NTS were applied in this study. Genetic variation between *P. olseni* and *P. marinus* was highest at NTS (24.9%) and least at 18S (0.8%). In other words, NTS DNA marker shows the highest chance of identifying sequence variation within the species. However, the number of NTS sequences registered in Genbank is low compared to ITS and 18S rDNA. Therefore we emphasize the usefulness of NTS when analyzing *Perkinsus* spp. using DNA markers.

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