수의학 박사 학위논문

Studies on the treatment, prevention and phylogenetic characterization of *Thelohanellus kitauei* and *Philasterides dicentrarchi*

장포자충(*Thelohanellus kitauei*)과 고병원성 스쿠치카증(*Philasterides dicentrarchi*)의 치료, 예방 및 계통분류학적 특성에 관한 연구

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By

Sang Phil Shin

August, 2013

Major in Veterinary Public Health

Department of Veterinary Medicine

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Abstract

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Dealing with animals in veterinary medicine, can be divided into companion animals and economic industry animals. This study covered with the parasitic diseases on companion and industirial animals and including method on the treatment, analysis of causative agent, epidemiological speculation and experiment for preventive medicine.

First, treatment of Koi carp infected *Thelohanellus kitauei* and its phylogenetic analysis. Koi carp *Cyprinus carpio haematopterus* fish was submitted to our laboratory for diagnostic examination and treatment. This fish was observed to have a cyst like tumor at the anus. Histopathological biopsy exhibited that the cyst contained a number of myxozoa. Based on the morphological features of its spores and the location of plasmodia, the pathogen in the cyst-like tumor was identified as *Thelohanellus kitauei*. The cyst was removed by surgical method. After surgery, low-concentration tricaine methanesulphonate (MS222) immersion and antibiotic

treatment were used. The surgical wound was completely healed and clinically normal 14 months post-surgery. The successful surgical removal of this type of the cyst suggests the possibility of treatment of *Thelohanellus kitauei* infection in Koi fish. Fresh mount and scanning electron microscopy (SEM) were used for morphological identification of the myxozoa present in the cyst. For the molecular identification and investigation of phylogenetic characteristics, the 18S rRNA gene of *T. kitauei* was amplified by optimized nested-PCR and sequenced. The sequence was compared with other 18S rRNA genes of *Thelohanellus* species and it was investigated the relationships between their host specificity and infection sites. Additionally, 18S rRNA of *T. kitauei* was compared with those of 67 *Myxobolus* spp., 11 *Henneguya* spp. and 6 *Thelohanellus* spp. Based on the 18S rRNA sequences, *T. kitauei* is most closely related to *T. hovorkai* (can infect intestine). Phylogenetic analysis revealed that *T. kitauei* was clustered with other *Thelohanellus* spp. infected Cyprininae. The result suggests that infection site and host specificity (subfamily level) are reflected in the genetic relationships among *Thelohanellus* species. Also phylogenetic analysis revealed that a Myxobolidae host switch event over fish family borders has occurred, after which they cospeciated with the host fish. Myxobolidae that infect Cyprinidae can be classified by two kinds of organ tropism, specific and non-specific infection site tropism (SIT and NSIT). Based on the phylogenetic tree, the infection site tropism and host specificity of *T. kitauei* originated from the ancient Myxobolidae, as exhibits NSIT and can infect Cyprinidae.

Second, identification of scuticociliates from seahorse and pathogenicity test. The Indo-Pacific seahorses, *Hippocampus kuda* which had been reared in one of

the private commercial aquaria for exhibition were sent to the laboratory for diagnosis. Feces and intestinal contents from 5 seahorses were examined microscopically and revealed numerous scuticociliates. The species of scuticociliate was identified by wet mount and SEM, PCR and sequencing. It was also investigated the possibility of cross infection through pathogenicity test. This study reports the first identification of scuticociliate *Philasterides dicentrarchi* from seahorse in Korea.

Third, preminary research for development of vaccine against *P. dicentrarchi* have been reported as a causative agent of scuticociliatosis in olive flounder (*Paralichthys olivaceus*) aquaculture of Korea. Although several attempts have been made to treat the disease with various chemotherapeutants, there are at present no available chemotherapeutants especially for systemic infections. Therefore, a vaccine against *P. dicentrarhi* infection would be highly desirable. To verify the possibility of vaccine candidates, a cDNA encoding cathepsin L-like cysteine protease (PdCtL) was identified from a cDNA library of *P. dicentrarchi* (synonym *Miamiensis avidus*) and 10 codons were redesigned to conform to the standard eukaryotic genetic code using PCR based site directed mutagenesis. The synthetic *P. dicentrarchi* procathepsin L (proPdCtL) was expressed at high levels in *E. coli* Rosetta (DE3) pLysS with pPET21a vector, and successfully refolded, purified and activated into a functional and enzymatically active form. The recombinant protease has optimal pH for protease activity and inhibited by cyteine protease inhibitors. These results suggest that the biochemical characteristics of the recombinant ciliate proPdCtL protein are similar to that of the cathepsin L-like cysteine protease, and that the PCR-based site-direct mutated ciliate gene was

successfully expressed in a biochemically active form.

This study approached the treatment and prevention of the diseases on Koi and olive flounder as companion and industrial animals in point of veterinary view, respectively and the causative agents was identified and analyzed through morphological and molecular methods.

Key words: *Thelohanellus kitauei,* surgery, phylogenetic characterization, *Philasterides dicentrarchi*, cross-infection, cathepsin L like cystein protease

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Abbreviations

HE	Hematoxylin and Eosin
MS222	Tricaine MethaneSulphonate
SEM	Scanning Electron Microscopy
NICEM	National Instrumentation Center for Environmental Management
FDA	Food and Drug Administration
PCR	Polymerase Chain Reaction
SOT	Specific Organ Tropism
NSOT	Non Specific Organ Tropism
ME	Minimum Evolution
CNI	Close Neighbor Interchange
MEGA	Molecular Evolutionary Genetics Analysis
BLAST	Basic Local Alignment Search Tool
IPTG	IsoPropyl β-D-1-ThioGalactopyranoside
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
DTT	Di ThioThreitol
EDTA	Ethylene Diamine Tetraacetic Acid
AMC	7-Amino-4-Methyl Coumarin
Z-Phe-Arg-AMC	carbobenZoxy-Phenylalanyl-Arginyl-AMC
PMSF	PhenylMethane Sulfonyl Fluoride
E64	L-trans-Epoxysuccinyl-Leu-amido-(4-guanidino) butane
Syn	Synonym
TBE	Tris-Borate-EDTA
PBS	Phosphate Buffered Saline
GSP	Gene Specific Primer
PdCtL	Philasterides dicentrarchi Cathepsin L
RACE-PCR	Rapid Amplification of CDNA Ends-PCR

General introduction

Carp are a large group of fish originally found in Central Europe and Asia. Various carp species were originally domesticated in East Asia. Natural color mutations of these carp would have occurred across all populations. Koi carp are ornamental varieties of domesticated common carp (*Cyprinus carpio*) that are kept for decorative purposes in outdoor or companion fish (4). *Thelohanellus* spp. are generally histozoic and highly host-specific fish parasites. A large number of host-specific *Thelohanellus* spp. that parasitize the Koi carp *Cyprinus carpio haematopterus* or common carp have been described (11). Fumagillin and vector control used to treat its infections. However, the effect of fumagillin is limited only to the early stages of infection (14). In addition, following rigorous testing required for U.S. Food and Drug Administration approval, it was deemed ineffective (3). Also vector control only use to decrease the prevalence in culturing system not individual fish (9). Thus, there is a need to the alternative treatment to overcome the disease.

In Korea farming of olive flounder, *Paralichthys olivaceus* is started in 1990s when wild population stocks were exhausted. Currently approximately 98 percent of the domestic need is produced in farms. The increase in production upsurged in 1998 and 2000, respectively (5) produced from about 300 commercial farms situated in Jeju Island, South Korea. However these farms experience increasingly high mass mortalities (7, 12). Scuticociliatosis has been recognized as an emerging problem that causes significant economic loss in aquaculture, through systemic infection (2, 7). *Philasterides dicentrarchi* causes fatal systemic scuticociliatosis in

cultured olive flounder (10). Several attempts have been made to treat scuticociliatosis disease with various chemotherapeutic agents (6, 13). These chemicals can eliminate the parasite; however it is not effective once the ciliates enter into the body (6). Also *P. dicentrarhi* showed intra-specific variances and surface antigen change (1, 8). It is considered as obstacles to development vaccine against *P. dicentrarchi*.

Now there are desire to find new target for the vaccine. Cysteine proteases of parasite is related with pathogenicity, immunoevasion and virulence (15) and was considered the anti-parasitic vaccine candidates.

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Literature Review

A. *Thelohanellus kitauei*

A.1. Taxonomy of Myxozoa

About 2184 species in 62 genera belong to the Myxozoa, an obligate parasitic group forming a separate phylum of multicellular metazoan parasites mainly of teleosts (57). Invertebrates like oligochaetes, bryozoans and polychaetes serve as secondary hosts. Despite being well-known as fish parasites, Myxozoa was also discovered in trematodes (73, 86), reptiles (55) and amphibians (60). Developmental stages were found in waterfowl (8), in nervous systems of mammalians (31) and myxospores were even detected in human feces (68). The members of the most abundant genus, *Myxobolus* (Myxobolidae), have recently been reviewed (24).

Previous study (84) suggested that the first myxozoans were coelozoic, inhabiting the gall bladder and later the urinary bladder of marine teleost fishes in the late Cretaceous period. Myxozoans later evolved to infect other tissues, with some forms becoming histozoic and also suggested that ancestral myxozoans were bipolarids (e.g. *Myxidium*, *Sphaeromyxa*), and that in fresh water they gave rise to the platysporinids (*Henneguyu*, *Myxobolus*). The class Myxosporea, until recently, contained two orders; the Bivalvulida (spores with 2 valves and generally 2 polar capsules) and the Multivalvulida (with spores containing greater than 2 valves and usually more than 2 polar capsules) (**Table I**). The order Multivalvulida (all marine histozoic forms) was derived from ancestors similar to Ceratomyxa (a coelozoic,

marine genus) (84). The class Malacosporea and the order Malacovalvulida for *Tetracapsula* spp. (family Saccosporidae), myxozoans that infect bryozoans (12). Some special features of these unusual myxozoans include a sac-like proliferative body and valve cells that do not cover the apex of the spore where the polar filament exits.

Table I. Abbreviated classification of the class Myxosporea (56)

Phylum Myxozoa		
	Class Myxosporea	
		Order Bivalvulida
		Suborder Variisporina
		Includes *Ceratomyxa, Chloromyxum, Hoferellus, Myxidium, Myxobilutus, Ortholinen, Parvicupsula, Polysporoplasma, Sinuolinea, Sphuerospora, Zschokkellu*
		Suborder Platysporina
		Includes *Myxobolus, Henneguya, Thelohunellus*
		Suborder Sphaeromyxina
		Includes *Sphaeromyxa*
		Order Multivalvulida
		Includes *Hexacupsula, Kudoa, Trilospora, Unicapsula*
	Class Malacosporea	
		Order Malacovalvulida
		Includes *Tetracapsula* (with 4 polar capsules)

A.2. Phylogenetic characterization of myxozoans

Myxozoans were thought to be protists for more than one hundred years until the 1990s due to the simplicity of their microscopic spores. Phylogenetic analysis of the first myxozoan 18S rRNA (91) then confirmed the marginalized suppositions

that Myxozoa are multicellular organisms (97, 106) and placed Myxozoans within the Metazoa. Phylogenetic analysis revealed the separation of freshwater and marine myxosporeans into two major branches (43, 44). Inner topologies within the marine and freshwater branches suggest that myxosporeans cluster in accordance with various characteristics. Previoous study analysed seven *Myxobolus* species grouped by spore morphology (81). In contrast, analysis of 10 different *Myxobolus* species revealed relationships according to the site of infection (4). Similarly, Eszterbauer (28) described two gill-infecting groups within *Myxobolus* spp. Other research group reported a relationship based on the site of infection (34). In their study, five urinary bladder infecting species assigned to different genera clustered together. Whipps et al. (107) found the site of infection to be an important criterion in relationships of some *Kudoa* species.

A.3. Life cycle of myxozoans

About 25 whole myxozoan life cycles have been elucidated. (43). With few exceptions, they include two alternating hosts, an aquatic invertebrate (oligochaetes or bryozoans) and a vertebrate host, mainly teleost fish (108). Waterborne actinospores (**Figure I**) released from oligochaetes infect the fish via gills or skin. Amoeboid sporoplasms containing the infective secondary cells leave the actinospore valve construct and actively penetrate the host integument. In the respective target tissue, they develop from trophozoits into sporogenic plasmodia. Coelozoic or histozoic parasitism occurs mostly extracellular, while some species penetrate host cells for multiplication. Some myxosporeans form extrasporogonic stages and multiply several times by internal cleavage. Most species tend to infect

specific kinds of organs, tissues or cell types (28, 66). During growth, somatic and generative nuclei are found. At the end of the intrapiscine development, myxospores composed of at least six cells are formed shedding at least two rigid (Myxosporea) or soft (Malacosporea) protective shell valves. They are formed in pansporoblasts inside plasmodia or develop in coelozoical or intercellular in pseudoplasmodia.

Although the existing knowledge on transmission is limited, mxospores are thought to be released from the fish hosts by death of the host, but there is an indication that release may also occur from live fish (72). They infect the intestine of invertebrate hosts by ingestion. Vegetative and sporogonic stages of actinosporeans are located intercellularly in the intestinal epithelium or in the coelom. Actinospores released from the oligochaete host inflate to develop their final habitus and initiate a new development in fish (**Figure I**). The release of myxo- and actinospores is often seasonal; therefore the whole life cycle may last for 1-2 years although the development takes only about 2 months in each host (25, 26, 27, 65).

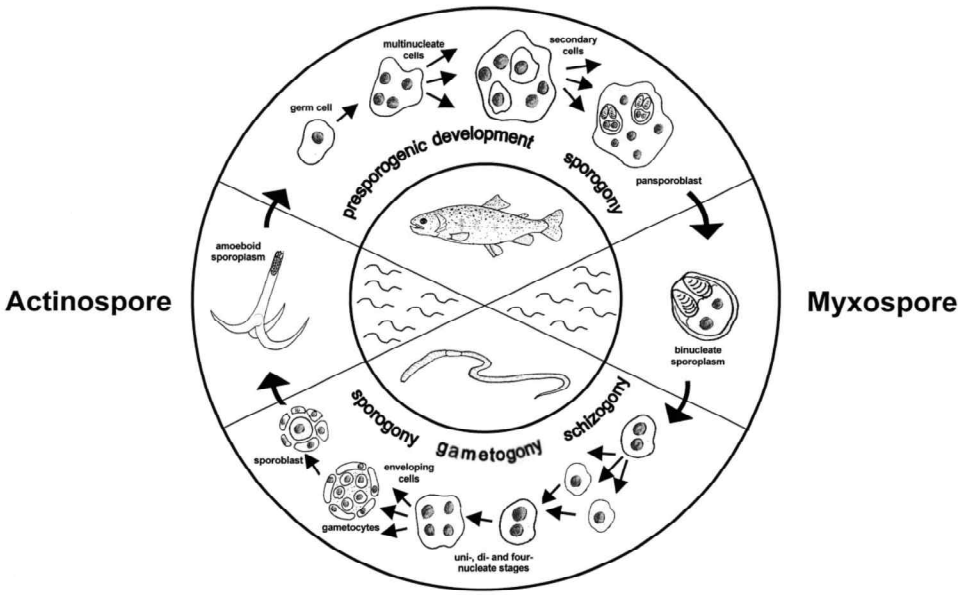

Figure I. The life cycle and development of myxzoans (43).

A.4. *Thelohanellus* spp.

The genus *Thelohanellu*s encompasses 75 described species (57) and includes those myxozoan parasites having a single polar capsule. Previous study included these myxozoans under the genus Myxobolus which possess tailless spores containing one iodinophilous vacuole and one or two polar capsules (100). In the year 1933, Kudo proposed to separate the unicapsulated species from the bicapsulated forms and established the new genus *Thelohanellus*, to which all unicapsulated *Myxobolus* species were transferred (48). General identifying characters of the genus *Thelohanellus* have been described like that a single pyriform, tear-shaped or subspherical polar capsule shifted toward longitudinal axis of spore, binucleate crescentic sporoplasm, mostly with spherical polysaccharide

inclusion; trophozoites large, polysporous, with pansporoblast formation; and strictly histozoic in fresh water fishes (56, 58, 85).

T. pyriformis was described from blood vessels of gills, spleen and kidney of *Tinca tinca* (100); also occurs in *Rutilus rutilus*, *Abramis brama* and some other cyprinids (22, 92). *T. jiroveci* from the gills of *Labeo bata* in India were reported macro- and microspores in the same plasmodium, a unique feature in myxosporea (49). *T. hovorkai*, common in *Cyprinus carpio* forming plasmodia in connective tissue of various organs (intestine, kidney, swimbladder, subcutis and brain); agent of haemorrhagic thelohanellosis (54, 67, 112). *T. kitauei* produces giant plasmodia in the intestine of common carp in Korea and Japan (23, 78, 79). *T. nikolskii* also common in carp (*Cyprinus carpio*), cyst-like plasmodia appear as bunch-of-grape-like structures on the fins, developing in close associations with cartilaginous fin rays (70). *T. wuhanensis* infecting skin and fin of fry of Allogynogenetic crucian carp (*Carassius auratus gibelio*) was isolated (109). *T. zahrahae* and *T. sienesis* were founded in gills of Java barb *Barbonymus gonionotus* and gold fish *Carassius auratus auratus* respectively (14, 99).

A.5. Occurrence of *T. kitauei*

T. kitauei was first reported from common carp *Cyprinus carpio* in Japan (47) and was subsequently identified as a new species due to its specific balloon like spore sack and intestinal parasitic habitat by Egusa and Nakajima (23). They also described morphological characteristics of *T. kitauei* spores and salient pathological changes in common carps (23). Previous studies have reported the similar parasites in china (30, 52, 110, 115). In the Korea, *T. kitauei* infection was first reported in

the farmed Israel carp (*Cyprinus carpio nudus*), and the infection *of T. kitauei* has been recognized as one of the most serious diseases occurring in inland farmed fish since 1988 (17). Infected fish evidence profound intestinal swelling and become emaciated due to the blockage of the intestinal tract by giant cysts, and the fish often ultimately die from the resultant enteritis (17, 23, 83). Moreover, it could cause mass mortality and severely damage the aquaculture industry (17, 23, 78, 79).

No detailed information is currently available regarding transmission mechanisms and the life cycle of *T. kitauei*, although the life cycle of *T. kitauei* may be similar to that of other myxosporean parasites, including an actinosporean stage in an alternate oligochaete host. Among *Thelohanellus* spp., the life cycles of *T. hovorkai* and *T. nikolskii*, which consist of myxosporean and actinosporean stages, have been confirmed at the molecular level, and the transmission and production of actinospores (aurantiactinomyxon) using the alternate oligochaete host have been experimentally demonstrated (3, 53, 98, 111). The high prevalence of actinosporean infections in oligochaete hosts such as *Branchiura sowerbyi* has been suggested to be one of the etiological factors responsible for disease outbreaks (112).

A.6. Control of *T. kitauei*

Although *T. kitauei* infection have occurred continuously and periodically since the first report of the disease in 1988, no detailed systematic strategies for its control have yet been developed (83). The antibiotic fumagillin has been described as an efficacious chemotherapeutant against myxozoan diseases, including haemorrhagic thelohanellosis and *T. kitauei* infection (77, 113). However, to date,

fumagillin treatment has not been practiced in aquaculture as there are unresolved problems with toxic side-effects to host fish, and the withdrawal time of the drug (54). Rhe et al (77) pointed out that fumagillin should be administered before the disease develops, as the efficacy of the drug is limited only to the early stages of infection. In addition, following rigorous testing required for U.S. Food and Drug Administration approval, it was deemed ineffective (32).

B. Scuticociliatosis

B.1. Taxonomy

The term scuticociliatosis covers diseases affecting a number of fishes, crustaceans, and molluscs species that caused by histophagous ciliates of the order Scuticociliatida which constitutes an abundant group inhabit eutrophic coastal and saprophytic maricultural waters. These ciliates are characterized by their high potential for systemic invasion destroying tissues that lead to high mortality in the host population (45) infecting a wide range of fishes. Previous study proposed a new order Scuticociliatida under class Ciliophora including the suborders Pleuronematina, Pseudocohnilembina, and Philasterina (89). Further revisions elevated scuticociliates to the level of subclass Scuticociliatia and the suborder Philasterina was up-ranked to an order as Philasterida (59). Since the criteria used hitherto to classify the families, genera, and species of scuticociliates have been variable, the taxonomy of these organisms is currently rather confusing (20). Since the taxonomy of scuticociliates is complicated, in several cases the involved parasite could not be identified upto species level (21, 105, 114).

B.2. Infection of scuticociliates

Scuticociliate species of the genera *Uronema*, *Miamiensis*, and *Philasterides*, previously regarded as environmental scavengers have become major opportunistic pathogens affecting marine fishes (36, 71, 96). Among these *Philasterides dicentrarchi*, is a serious problem, causing mass mortalities in cultivated marine fishes, such as olive flounder, turbot, and sea bass (2, 20, 21, 36, 39, 46, 114).

Other important pathogenic scuticociliate species of this order include *Uronema marinum*, *U. nigricans*, *Anophryoides haemophila* and *Mesanophrys* spp. (13, 15, 39, 69). Recently, three scuticociliate species *Pseudocohnilembus persalinus*, *U. marinum*, and *P. dicentrarchi* have been shown to be the major scuticociliates infecting farmed olive flounder in Korea (46); Other species like *U. nigricans* affect southern bluefin tuna (39, 71), and turbot (96); *Tetrahymena corlissi* infects guppy (37) and *Mesanophrys* spp infects sea horse (101). Infections of fish with *Uronema* sp. have been mainly reported in aquarium species. Infections of *U. nigricans* on fish in closed larvae-rearing systems and aquaria resulted in severe tissue damage and subsequent mortalities (9). Previous study on the scuticociliate infection in cultured flounder farms in Jeju Island of Korea have shown that the scuticociliates have a wide host range (40). In olive flounder scuticociliatosis first reported in Korea during 1990 has soon become a challenging disease with no effective control (16).

Table II. Occurrence of scuticociliate species and the hosts reported (33)

Parasite	Host	Country
Anophryoides haemophila	NA	Maryland
A. haemophila	*Homarus americanus*	Canada
Brooklynella hostilis	Marine fishes	New York
Chlamydonella derouxi	*Orthotrochilia pilula*	China
Cryptocaryon irritans	*Chelon labrosus*	Israel
Cryptosporidium molnari	*Sparus aurata*	Spain
Dysteria pusilla	*Haliotis discus*	China
Hyalophysa chattoni	NA	North Carolina
Ichthyophthirius multifiliis	Teleosts	UK
I. multifiliis	*Oncorhynchus mykiss*	Denmark
Mesanophrys chesapeakensis	*Callinectes sapidus*	Maryland
Philasterides dicentrarchi	*Psetta maxima, Scophthalmus maximu, Dicentrarchus labrax*	Spain
P. dicentrarchi	*Paralichthys olivaceous, S. maximus*	Switzerland
P. dicentrarchi	*Dicentrarchus labrax*	France
P. dicentrarchi, Miamiensis avi	*P. olivaceous*	Korea
Scuticicoliates[a]	*S. maximus, P. olivaceous*	Japan
Tetrahymena corlissi	*P. taeniolatus, P. reticulata*	
Scuticiciliate[a]	*S. maximus*	Portugal
Scuticociliates[a], *Mesanophrys*	*Nephrops norvegicus*	Scotland
Tetrahymena pyriformis	NA	Hungary
Tetramicra brevifilum	*S. maximus*	Spain
Trichodina jadranica	*Anguilla anguilla*	Denmark
Uronema marinum	P. olivaceous	Korea
U. marinum	Atlantic and Pacific marine fishes	New York
U. nigricans	*Thunnus maccoyii*	Australia
Uronema sp., M. avidus,	*Hippocampus erectus*	NA
Uronema spp.	*S. maximus*	Norway
U. marjnum, Sillqo ciliate	NA	Australia

B.3. Clinical sign and pathological finding

Main clinical signs and general histopathology features of infected fishes have been noted in the different outbreaks of scuticociliatosis. The most complete available information deals with the *P. dicentrarchi* infection (36), antigenicity (35), and invasion route (75). Several epidemiological, clinical, and pathological descriptions of scuticociliatosis outbreaks have been published (20, 21, 46, 96). The ciliates are characterized by their high potential for systemic invasion destroying tissues that lead to high mortalities of the host (46). When the disease

manifests the initial clinical symptoms include loss of scales, hemorrhagic lesion, bleached spots on the skin, and dermal necrotic lesions followed by dermal necrotic lesions, coalesced to form brownish musky clinical manifestations (5). Heavily infected fish are anorexic, weak, listless and appear emaciated with a large head with respect to the body. For fish experimentally infected with *Miamiensis avidus* (syn. *P. dicebtrarchi*), showed severe abdominal distension, dark body colour, increased opercula movement and convulsion. The ascitic fluid was red and large numbers of ciliates were swimming in the fluid. The intestine wall had become thin and transparent, and reddish fluid filled the intestine, and the liver and brain were red. The skin ulcer lesions also were detected. Most of the infected fish exhibited severe haemorrhages and ulcers on the fin, skin muscle and jaw. Masses of ciliates were observed in the fins, skin, brain and gills (94).

B.4. Economic loss

Scuticociliatosis has been recognized as an emerging problem that causes significant economic loss in aquaculture, through systemic infection (20, 40). *P. dicentrarchi* causes fatal systemic scuticociliatosis in several cultured marine fish including sea bass (20), olive flounder (46), turbot (2, 21, 36), and gilthead bass (20). So far in NW-Spain (Galicia) only *P. dicentrarchi* has been reported to cause mass mortality in turbot farms (59), although some authors have reported disease outbreaks caused by species of *Philasterides* and *Miamiensis* in turbot farms in Spain, Portugal, and France (2, 21, 36, 74). Scuticociliatosis first reported in olive flounder farm in Korea during 1990 (16) subsequently became an emerging problem in flatfish farming (39, 46). Recently, mass mortality due to histophagous

scuticociliate, *P. dicentrarachi* in olive flounder, *Paralichthys olivaceus* occurred in aquaculture farms of Jeju Island, Korea (40). The average seasonal prevalence of scuticociliatosis with *P. dicentrarchi* infections have showed from May to July higher than other season. The prevalence of mixed infection compiled with *Vibrio* spp. infection was coupled higher than that of scuticociliatosis alone (41). *U. marinum* as a facultative scuticociliate has also become a causative pathogen inflicting servere infection in olive flounder farms of Korea (39). The increasing frequency and severity of scuticociliatosis in olive flounder turbot culture, indicate the need to develop efficacious prevention and control strategies (33, 87).

B.5. Control stratagies

Formalin is commonly used to treat ectoparasitic infections (29), particularly protozoa, since it has powerful microbicidal and pesticidal effects; it binds to protein or dissolves lipids in parasite. Also, both of niclosamide and oxyclozanide also showed the greatest activity against *P. dicentrarchi* at low concentration (74). However, these chemicals are carcinogenic or highly toxic to human, animals and fish (1, 6, 95). In addition, there are at present no available chemotherapeutants especially for systemic infections (50). Therefore, a vaccine against scuticociliatosis would be highly desirable.

Previous studies on scuticociliatosis have demonstrated that immunization of turbot or olive flounder with the ciliate lysate or formalin-fixed ciliates has the ability to confer a certain degree of protection against challenge with scuticociliates (35, 51, 87). Therefore, development of effective vaccine may be the best way to control scuticociliatosis. However, *P. dicentrarchi* showed intraspecific variaition

and serotypes (10, 94). Previous study pointed out that *P. dicentrarchi* agglutinated by olive flounder antiserum could escape from the agglutinated sheath by change of surface antigens (50).

C. Cystein protease

C.1. General description

Peptide hydrolases (proteases) catalyse the cleavage of amide linkages in macromolecular proteins and oligomeric peptides. Proteases have been identified in biological systems from viruses to vertebrates. As genome sequencing projects are completed it has become clear that proteases comprise approximately 2% of all expressed genes with little variance between organisms (80). It is estimated that without proteases as biological catalysts it would take hundreds of years to hydrolyse a peptide bond; in comparison a protease can degrade as many as one million peptide bonds per second. Proteases range from monomers of 10 kDa to multimeric complexes of several hundred kDa. Catalysis can be initiated either within a polypeptide chain (endoprotease activity) or from amino or carboxyl ends (exopeptidase activity). Proteases have been divided into groups on the basis of the catalytic mechanism used during the hydrolytic process. The main catalytic types are serine, threonine, aspartate, metallo and cysteine proteases, other 'undefined' or cryptic proteases may also exist (80). Previous studies described a number of general parasite proteinases (61, 62, 63, 64, 102).

It is also now established that many parasite proteases are promising chemotherapeutic or vaccine targets (62, 63, 82). Interest in cysteine proteases as targets derives from the recognition that they are critical to the life cycle or pathogenicity of many parasites. This functional diversity is in turn derived from their unique nucleophilicity, adaptability to different substrates, and stability in different biological environments. Parasite derived cysteine proteases play key

roles in immunoevasion, enzyme activation, virulence, tissue and cellular invasion as well as excystment, hatching and moulting (80).

C.2. Classification

Cysteine proteases of parasitic organisms are divided into two main groups referred to as clans, CA and CD (7, 76). In 1879 the first cysteine protease was purified and characterised from Carica papaya, the papaya fruit, and was thus named papain. Papain was also the first cysteine protease structure to be solved. Since its discovery numerous proteases that have sequences in common with papain have been loosely called 'papain-like'. Papain-like, or Clan CA proteases, are further divided into families. Important parasite proteases are located to family C1 (cathepsin B and cathepsin L-like) and family C2 (calpain-like). Other clans and relevant families of pathogenic organisms include clan CB and CC (viral proteases) and clan CD (family C13; legumain-like) (**Figure II**) (80).

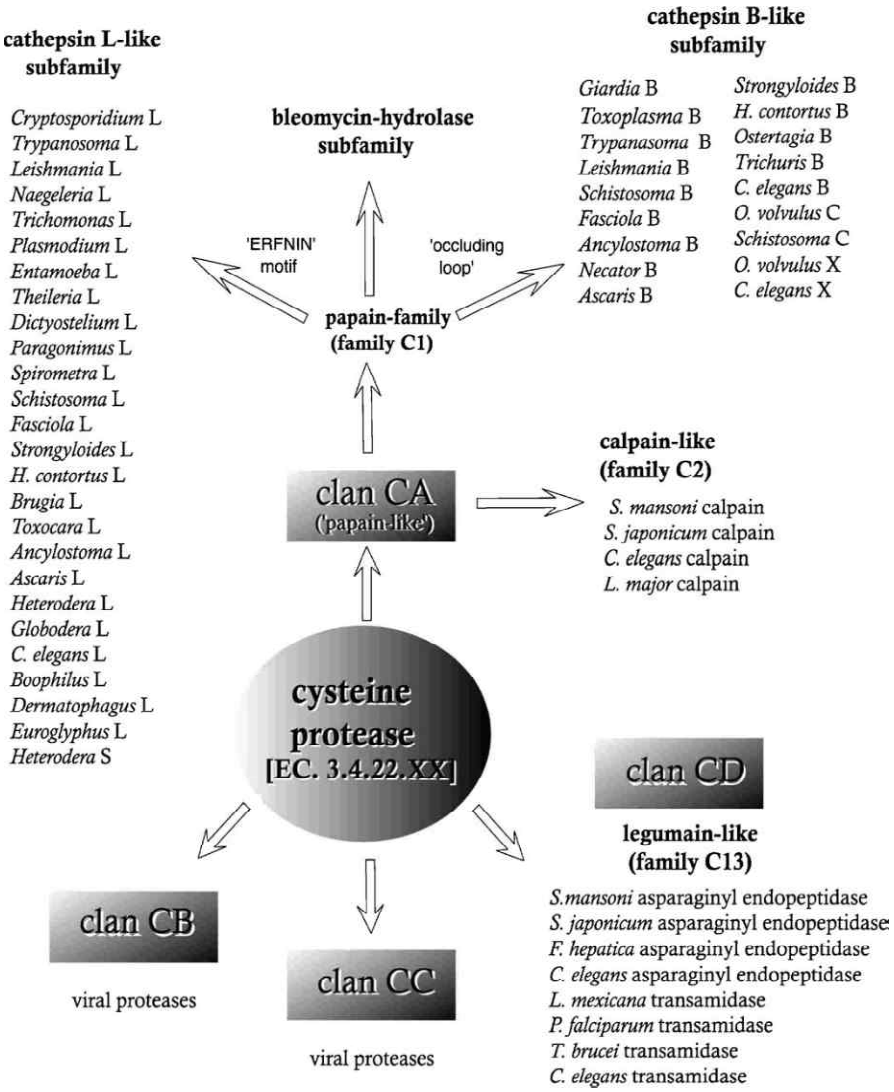

Figure II. Schematic diagram of the parasite cysteine protease (80)

C.3. Papain-like proteases

The majority of parasite cysteine proteases belong to the family C1 within clan CA (7). As genome sequencing projects are completed, a clearer picture is developing of molecular evolution of this protease family. Members of the CA clan are either targeted to intracellular vesicle compartments or are secreted, and thus

possess a leader peptide (104). Clan CA proteases are also characterised by their sensitivity to the general cysteine protease inhibitor, E64 (L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane). In addition, there are structural and sequence variances between cathepsin B and cathepsin L. Cathepsin Ls (including the closely related cathepsin S and cathepsin K) have a conserved inter-spaced motif in the proregion, Glu, X3, Arg, X2, (Ile/Val), Phe, X2, Asn, X3, Ile, X3, Asn ('ERFNIN'; named after the single letter code for amino acids; X is any amino acid) (42). Cathepsin Bs lack the ERFNIN motif but do have an inserted peptide loop in the catalytic domain, referred to as the 'occluding loop'. The cathepsin B and L subfamily can be further delineated by the length and sequence similarity within respective pro-regions as well as the number and order of cysteine residues involved in disulfide bond formation (80).

C.4. Possibility of vaccine

It has long been known that many cysteine proteases are unusually immunogenic. Antibodies directed against cysteine proteases can have an inhibitory affect on their proteolytic activity as seen with the anti-*Fasciola hepatica* cathepsin L antibodies (90), anti-*Dictyocaulus viviparus* cysteine protease and the anti-*Dermatophagoides pteronyssinus* Der p1 antibodies (11). Immunological protection using cysteine proteases has also been observed against *Plasmodium falciparum* SERA/SERP antigens in aotus monkeys and mice, the *Leishmania major* cysteine protease in mice, *Entamoeba histolytica* cysteine protease antigens *in vitro* and in animal models and *Schistosoma mansoni* anti-9B antibodies. An antibody mediated protection was reported against *Trypanosoma cruzi* and *L.*

mexicana. The *S. mansoni* calpain is implicated as a target of protective immunity and has been used as a vaccine in murine models; later studies identified the minimum protective oligopeptide epitope sequence of calpain as EWKGAWCDGS (38). The most encouraging work to verify the application of an anti-cysteine protease vaccine against parasitic organisms is that with *F. hepatica* and *Haemonchus contortus* (19, 88). Similar strategies may be effective against many parasitic organisms, particularly blood feeding parasites such as gastrointestinal nematodes, other hepatic fluke and the larvae and adult stages of arthropods such as ticks, mosquitoes and mites.

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Chapter I

Identification and phylogenetic characterization of *Thelohanellus kitauei* and its surgical treatment

Abstract

Koi *Cyprinus carpio haematopterus* fish was submitted to our laboratory for diagnostic examination. This fish was observed to have a cyst like tumor at the anus and it was removed by surgical treatment. For morphological identification, the cyst was examined by light (fresh mount), scanning electron microscopy (SEM) and histopathologic examination. Based on the morphological examination, we have identified the pathogen in the cyst-like tumor as *Thelohanellus kitauei* and the 18S rRNA gene of *T. kitauei* was amplified by optimized nested-PCR. The PCR product was sequenced and compared with other 18S rRNA genes of *Thelohanellus* species to investigate the relationships between their host specificities and infection sites. Based on the 18S rRNA sequences, *T. kitauei* is most closely related to *T. hovorkai* (can infect intestine). Phylogenetic analysis revealed that *T. kitauei* was clustered with other *Thelohanellus* spp. infected Cyprininae. Additionally, 18S rRNA of *T. kitauei* was compared with those of 67 *Myxobolus* spp., 11 *Henneguya* spp. and 6 *Thelohanellus* spp. Present study suggests that infection site and host specificity (subfamily level) are reflected in the genetic relationships among *Thelohanellus* species. Phylogenetic analysis of revealed that a Myxobolidae host switch event over fish family borders has occurred, after which they cospeciated with the host fish. Myxobolidae that infect Cyprinidae can be classified by two

kinds of organ tropism, specific and non-specific infection site tropism (SIT and NSIT). Based on the phylogenetic tree, the infection site tropism and host specificity of *T. kitauei* originated from the ancient Myxobolidae, as exhibits NSIT and can infect Cyprinidae. Lastly, the successful surgical removal of this type of the cyst suggests the possibility of treatment of *T. kitauei* infection in Koi.

Keywords: *Thelohanellus kitauei*, host specificity, specific infection site tropism, non-specific infection site tropism, surgical treatment

1.1. Introduction

The genus *Thelohanellus* Kudo 1933, encompasses 75 described species (21) some causing severe economic losses (11, 17, 24, 31, 32, 39). Most *Thelohanellus* species have been described based on spore morphology, however, it is clear that other features such as host and organ specificity are also useful for accurate identification in the absence of DNA sequence data (25) and correlate better with 18S ribosomal RNA (18S rRNA) compared to spore morphology (16). As molecular methods have become increasingly important in parasitological studies, the taxonomic classification of myxosporeans has been expanded via phylogenetic analyses (19). Presently, the application of morlecular analyses, particularly utilization of 18S rRNA gene sequences in the case of myxozoans, is the most sensitive approach for definitive species identification (15). For purposes of molecular identification, the partial sequences of the 18S ribosomal RNA genes of six *Thelohanellus* species (*T. kitauei*-China, *T. kitauei*-Korea, *T. hovorkai*-Hungary, *T. nikolskii*-Hungary, *T. nikolskii*-China, *T. sinensis*-China, *T. wuhanensis*-China, *T. wuhanensis*-China2, *T. wuhanensis*-China3 and *T. zahrahae*-Malaysia) have been deposited in GenBank under the accession numbers HQ115585, HM624024, DQ231155, DQ231156, GU165832, DQ452013, HQ613410, AY165181, JQ690370 and EU643622.

Surgery has been carried out in many fish species for a variety of purposes. Abdominal sarcoma and ovarian tumors in Koi were removed by surgical methods (18, 30). A seminoma was removed from a black sea bass by surgical methods (40). Pneumocystoplasty was used for overinflation of the swim bladder in a gold fish (3). Surgical excision of mycotic granulomas was performed in cuttlefish (13). In

tilapia, telemetry transmitters were implanted by surgical procedures (38).

In present study, a cyst was removed through surgical method and the causative agent was identified as *T. kitauei* through morphological examinations. The 18S rRNA gene of *T. kitauei* was sequenced and compared with the 18S rRNA genes of other *Thelohanellus* spp. and investigated the genetic homology and phylogenetic characteristics. Additionally, the 18S rRNA of *T. kitauei* was compared with the 67 *Myxobolus* spp., 11 *Heneguya* spp. and 6 *Thelohanellus* spp. to investigate the relationships among its host specificity, infection site tropism and geographical region.

1.2. Materials and methods

1.2.1. Fish

In April 2008, a Koi fish (*Cyprinus carpio haematopterus*) (length = 14.2 cm, body weight = 38.9 g) was submitted to our laboratory for diagnostic examination. This fish was observed to have a cyst-like tumor (diameter about 1 cm) at the anus **(Figure 1.1A)** and had begun to show lethargy, anorexia, depression and cystic obstruction at the anus.

1.2.2. Surgical treatment

Anesthesia was induced by immersing the fish in water containing 200 mg tricaine methanesulphonate (MS222, Woogenebng)/L for five minutes. Signs of surgical anesthesia were total loss of equilibrium, loss of reaction to pressure on the peduncle and mild movement of opercula. Anesthesia was maintained by dropwise administration of a solution of 100 mg/L MS222 onto the gill. The scales were

removed from the incision area with forceps. The skin was surgically prepared by gently swabbing with povidone iodine and was incised with a scalpel. A ventral midline abdominal incision was made, beginning immediately anterior to the anus and extending to 2 cm with scissors. The cyst was attached on the serosa of the intestine, like a polyp; it was dissected free from the intestine and the presence of other cysts was not found. The coelomic cavity was flushed with sterile saline. Muscle and skin were closed with 5-0 nylon together in a simple interrupted pattern. After surgery, bath application of oxytetracycline (50 mg/L) was carried out for 30 minutes to reduce the possibility of secondary infection. The fish was then immersed water in containing MS222 (20 mg/L) for 24 hours for sedation (34). Feed pellets were given to the fish after 3 days, and feces were checked on the following day to assess excretory function of the intestine. The skin and muscle sutures were removed 10 days after surgery

1.2.3. *Morphological identification of Thelohanellus kitauei*

Part of the removed cyst was wet-mounted and observed under the light microscope. For scanning electron microscopy (SEM), the specimen was homogenized and fixed in modified Karnovsky's fixative [2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2)], post-fixed in 1% osmium tetroxide in 0.05M sodium cacodylate buffer (pH 7.2) and dehydrated through an ethanol series (30%, 50%, 70%, 80%, 90%, 100%, 100%, 100% ethanol; 10 minutes at each concentration). After treatment of spores with hexamethyldisilazane (15 min, twice), specimens were dried at room temperature overnight. Spores were examined using a JEOL-5410 LV scanning electron

microscope at the National Instrumentation Center for Environmental Management (NICEM) at Seoul National University. A small fragment of the cyst was removed, fixed in 10% neutral phosphate-buffered formalin, routinely processed and embedded in paraffin. 5 μm sections were cut and stained with hematoxylin and eosin (HE) for histopathologic examination.

1.2.4. *DNA extraction*

The extracted plasmodium was homogenized and suspended in 180 μl of ATL buffer (Qiagen), to which 20 μl of proteinase K was added. After vortexing, the sample was incubated 55°C for 24 hours. Spores were centrifuged at low speed (230 *g*) for 5 min, collected and washed three times in phosphate buffered saline (PBS). Purified spores were suspended in 180 μl ATL buffer and the spore walls were broken by sonication. DNA was extracted using the DNeasy® Tissue Kit (Qiagen) following the manufacturer's protocol. Extracted DNA was stored at -20°C until use in PCR and sequencing reactions.

1.2.5. *18S rRNA PCR and nested PCR*

DNA was first amplified with the 18e and 18g' universal eukaryotic primer pair (2, 14): 18e: 5'-CTG GTT GAT TCT GCC AGT-3', 18g': 5'-CGG TAC TAG CGA CGG GCG GTG TG-3'. Following initial amplification, nested PCR amplification was performed using the MX5 and MX3 primer pair specific for the family Myxobolidae (2): MX5: 5'-CTG CGG ACG GCT CAG TAA ATC AGT-3', MX3: 5'-CCA GGA CAT CTT AGG GCA TCA CAG A-3'. 18S rRNA PCR was performed in 20 μl reaction mixtures containing DNA template (2 μl), 10 pmol

each primer and *i*-StarMaster mix (2.5 U *i-StarTaq* ^{TM} DNA Polymerase, 250 μM dNTP, 10 mM Tris-HCl, 2 mM Mg^{2+} solution, stabilizer and tracking dye) (Intronbio. Korea). Nested PCR was performed as described above except using 2 μl PCR product and specific primer. The amplification was carried out using a previously reported program (10). PCR and nested PCR products were analyzed using 2.0% agarose gel electrophoresis in 1% Tris-borate-EDTA (TBE) buffer. Gels were stained with ethidium bromide (0.5 μg/ml), visualized and photographed under UV illumination.

1.2.6. *Sequencing and analysis of sequence data*

The amplified PCR and nested PCR products of *T. kitauei* isolated from koi were sequenced using the BigDyeTM terminator cycle sequencing kit (Macrogen Genomic Division, Korea). Electrophoresis of sequencing reactions was performed using the Automatic Sequencer 3730x1 DNA analyzer (Applied Biosystems, USA). The obtained 18S rRNA gene sequence of *T. kitauei* was compared with similar sequences of described myxozoans using a BLAST (megablast) search for highly similar sequences in the NCBI nonredundant nucleotide (nr/nt) database. Sequence identities to the 18S rRNA sequence of *T. kitauei* were determined for the other *Thelohanellus* spp. (*T. kitauei*-China HQ115585, *T. kitauei*-Korea HM624024, *T. hovorkai*-Hungary DQ231155, *T. nikolskii*-Hungary DQ231156, *T. nikolskii*-China GU165832, *T. sinensis*-China DQ452013, *T. wuhanensis*-China HQ613410, *T. wuhanensis*-China2 AY165181, *T. wuhanensis*-China3 JQ690370 and *T. zahrahae*-Malaysia EU643622). These sequences were aligned with ClustalW and analyzed with the MEGA4 (Molecular Evolutionary Genetic analysis, 4.0) software program.

The sequences were downloaded and aligned using the ClustalW application of MEGA4 (37) and cropped to yield sequences of equal length (1580 bp, including gaps). These sequences were used to construct a phylogram.

1.2.7. Phylogenetic analysis

Preliminary analysis included 241 of the similar sequences identified by the BLAST search. From this initial data set, the number of sequences was reduced to 102, which included the *Myxobolus* spp., *Henneguya* spp. and *Thelohanellus* spp., as well as sequences from representative members of well-defined groups of closely related myxobolids. *Tetracapsuloides bryosalmonae* (U70623) served as an outgroup to root the phylogenetic analysis.

The sequences were downloaded and aligned using the ClustalW application of MEGA4 (37) and cropped to yield sequences of equal length (1620 bp, including gaps). These sequences were used to construct a phylogram. Phylogenetic analyses were inferred using the minimum evolution (ME) algorithm in MEGA 4.0. ME distance analysis was performed using a close-neighbor-interchange (CNI) search level 1, with evolutionary distanced determined by maximum composite likelihood. The initial tree for ME analysis was generated using a neighbor-joining algorithm with pairwise gap deletion. Clade support for the analysis was assessed by bootstrapping (1,000 replicates for ME). The nucleotide sequences used here for the phylogenetic tree are available in GenBank, and the corresponding countries, host species, infection sites and accession numbers are provided **(Table 1.1)**.

1.3. Results

1.3.1. Surgical removal of the cyst

The fish recovered from the anesthesia and surgical procedure without complications. However, immediately on recovery from anesthesia, the fish displayed an excited condition, with fast swimming and leaping out of the water. After treatment with a low concentration of MS222 (20 mg/L), the fish was sedated. Feed pellets were given to the fish 3 days following surgery, and the next day feces were present on the bottom of the aquarium. Ten days after surgery, the fish was anesthetized for suture removal. Much of the suture material was hanging loose from the skin, and it was removed. The incision had healed well; however, the posterior part of the skin had not completely healed **(Figure 1.1B)**. The fish made a complete recovery and was clinically normal 14 months after surgery **(Figure 1.1C)**.

1.3.2. Morphological identification

The spores of *T. kitauei* were pyriform in shape, with a sharply tapered anterior and a rounded posterior. The dimensions of the spores were 24-27 μm in length and 7-10 μm in width. The length, including the spore envelope, was 27-33 μm. A single polar capsule, pyriform in shape, converged slightly anteriorly and occupied about three-fifths of the space. The polar filaments were tightly coiled in 9-12 turns perpendicular to the axis of the capsule **(Figure 1.2A)**. In the scanning electron microscope, several wavy ridges were discernable on the posterior portion of the spore surface, and the single polar capsule with an almost smooth surface was also clearly observed. The shell valve of the spore was thick and smooth **(Figure 1.2B)**. Based on histopathologic result, the cyst was encapsulated and consisted of

multiple lobules separated by connective tissue **(Figure 1.3A)**. The lobules indicated plasmodia of *T. kitauei*. The tissue contained numerous pyriform spores. Two circular nuclei were present in the sporoplasm, the posterior part of which contained an iodinophilous vacuole **(Figure 1.3B)**. Occasionally, connective tissue was expanded by infiltration with numerous lymphocytes, plasma cells and macrophages and a few heterophils.

1.3.3. *Homology of Thelohanellus kitauei with other Thelohanellus sp.*

The universal 18e/18g' primers and the specific primer pair MX5/MX3 successfully amplified approximately 1900- and 1600-bp fragments of the 18S rRNA gene from *T. kitauei*, respectively. The edited alignment was 1561 bp in length; this sequence was deposited in GenBank under the accession number GQ396677. The sequence showed 99-100% nucleotide similarity with *T. kitauei*, 97% with *T. hovorkai*, 92-94% with *T. wuhanensis*, 89-90% with *T. nikolskii*, 90% with *T. sinensis* and 86% with *T. zahrahae* **(Table 1.2)**. Multiple alignment revealed three variable regions within the 18S rRNA locus among the *Thelohanellus* spp. The first variable region revealed multiple bases differing between *T. kitauei* and sequences of the other *Thelohanellus* spp. However, the second and third variable regions revealed that of the six species, *T. kitauei* and *T. hovorkai* are the most highly homologous **(Figure 1.4)**.

1.3.4. *Phylogenetic characterization about host specificity and infection site tropism*

Phylogenetic analyses among *Theohanellus* spp. were based on the edited

alignment, which was 1580 bp in length. This phylogenetic tree distinguished as host fish subfamily. *T. kitauei* was clustered with other two *Thelohanellus* spp. (*T. hovorkai* and *T. wuhanensis*) this clade was placed with the Cyprininae **(Figure 1.5)**. *T. zahrahae* showed long distance from the other *Thelohanellus* spp. and was located in Barbinae. Other phylogenetic analyses among Myxobolidae were based on the edited alignment, which was 1620 bp in length and contained 104 myxosporean taxa. This phylogenetic tree **(Figure 1.6)** distinguished two main groups as host fish family. While one Myxobolidae group infects Cyprinidae, the other group infects Salmonidae, Mugilidae and Ictaluridae. The Myxobolidae group that infects Cyprinidae can be classified according to two kinds of infection site tropism. The first group shows specific infection site tropism (SIT) and infects specific sites, such as gills or muscle. The second group shows non-specific infection sites tropism (NSIT), as it can infect gills, intestine, swim bladder and fins. *T. kitauei* was clustered with other three *Thelohanellus* spp. (*T. hovorkai*, *T. nikolskii*, *T. wuhanensis*), and this clade was placed with the NSIT Myxobolidae group. However, the genus *Thelohanellus* is polyphyleptic appearing on three branches and there are not any relationship between geographical region and phylogenetic cluster.

1.4. Discussion

After identifying a cyst in the fish, one of the goal in present study was to identify the causative organism. Differential diagnoses for this type of cyst include 2 *Thelohanellus* spp (*T. kitauei* and *T. hovorkai*). These *Thelohanellus* spp. need to be differentiated morphologically as they have both been reported (31) as intestinal

parasites in fish. Previous study (1) has noted the location-dependent variability of *Thelohanellus* spp. That is, *Thelohanellus spp.* has specific infection sites tropism, for example *T. kitauei* has an obligate gut tropism, and *T. hovorkai* exhibits connective tissue (including intestinal connective tissue) tropism, *T. wuhanensis* was isolated from skin (41), *T. nikolskii* was identified in the scale and fin rays (27), *T. sinensis* and *T. zahrahae* was isolated from the gill (5, 36). Therefore, in addition to differences in spore morphology, data on host and infection site specificity have become very useful tools species identification (1, 11, 26). Therefore, the diagnostic tests used to differentiate between the 2 *Thelohanellus* spp in the present report included light microscopy and scanning electron microscopy. With these tools, *T. kitauei* could be identified exactly and it was differentiated from *T. hovorkai* on the basis of published physical differences between these species (6, 42). For example *T. kitauei* are pyriform and characterized by a relatively small sporoplasm and a large polar capsule (pyriform), whereas *T. hovorkai* are ovoid and characterized by large sporoplasm and a small polar capsule (subspherical). Measurements of *T. kitauei* spores indicate that they are between 23 to 29 μm in length and 8 to 11 μm in width, whereas a single polar capsule is 14 to18 μm by 6 to 9 μm. However, *T. hovorkai* spores are reported to be 20.0 to 22.5 μm in length, 12.5 to 14.0 μm in width, ovoid, pointed cranially and enclosed by a loose membranous envelope. A single polar capsule, 10.5 to11.5 μm in diameter, is also present. Also, a *T. hovorkai* infection is characterized by extensive hemorrhage because dispersion of mature spores of *T. hovorkai* from the plasmodia [plasmodial stage be situated in the tissue and has vegetative nuclei (spores)] into the neighboring tissues causes the destruction of capillary networks (43). This results

in hemorrhage and edema in the skin lesion. However, koi did not show any sign of hemorrhage in the present report, further supporting the identification of the spore as *T. kitauei*. The fish in this report had an anal cyst, that are consistent with a previous report of *T. kitauei* (31).

In the koi in the present report, histopathological findings indicated plasmodia (vegetative form of the parasite) resulting in a host reaction, including inflammation, and a cyst consisting of numerous plasmodia, which was obstructing the anus. A previous report (31) comparing infections from *T. kitauei* and *T. hovorkai* found that *T. kitauei* to cause intestinal cysts which do not penetrate the serosa whereas infections from *T. hovorkai* have been shown to infiltrate the serosa (26, 31). Thus, on the basis of previous report (31), *T. kitauei* is known to obstruct the lumen of the intestine. In contrast, in the present report, *T. kitauei* could also infect serosa and cause obstruction of the anus.

Fumagillin is the only drug that has been found to be effective for the treatment of myxosporean or *Thelohanellus* spp infections (33). However, previous report explained that after extensive tests required by the Food and Drug Administration (FDA), this drug was deemed to be ineffective (12) and FDA approved this use for the prevention of *nosema* in honey bees. The efficacy of the drug is dependent on the stage of the infection. Fish with gross signs of the disease do not recover completely (33). Lesions size and number of spores are decreased but not removed. In the present study, the cyst caused by *T. kitauei* in a koi was removed by use of surgery. The operation was successful, and the fish recovered from the surgical procedure. The positive outcome of this surgery suggests the possibility of an alternative treatment for *T. kitauei* infection. *T. kitauei* was identified in the cyst

with morphological examination. The results of these analyses provided some basic information about the relationships between organ and tropism for this parasite.

As described above, spore morphology has been used as a basic feature to identify myxosporean species. Molnár (23) suggested that host and infection site specificity are important characters for specific assignment. Several studies have confirmed the importance of these features and found them to correlate with the results of molecular data based on the comparison of 18S rRNA sequences (16). For molecular identification, the 18S rRNA gene of *T. kitauei* was sequenced and it was compared with 18S rRNA genes of other *Thelohanellus* spp. In comparing with other isolates of *T. kitauei*, our sample revealed 100% similarity with HQ115585 while showed 3 different nucleotides with HM 624024. Three nucleotides differences among *T. kitauei* isolates was revealed at 374/523, 536/685 and 1174/1323 nt. Liu et al (20) explained it was caused by base calling error or intraspecific variance. The *T. kitauei* isolates showed similar pathogenicity, chinese isolate was found in intestinal wall and this isolate also showed giant cyst in intestine (20). In comparing with other *Thelohanellus* spp. *T. kitauei* was closest to *T. hovorkai.* Additionally, the multiple alignment revealed *T. kitauei* and *T. hovorkai* are the most highly homologous. *T. kitauei* has an obligate gut infection, and *T. hovorkai* exhibits a various organs including intestine in the common carp (11, 43). However, the other four parasites do not infect intestine. *T. wuhanensis* has been isolated from skin of the allogynogenetic silver crucian carp (*Carassius auratus gibelio*) (41). *T. nikolskii* has been identified in the scale and fin rays of the common carp (*Cyprinus carpio*) and european wild carp (*Cyprinus carpio carpio*) (22, 27). *T. sinensis* has been isolated from the gill of the goldfish (*Carassius*

auratus auratus) (5), and Székely et al. (36) found *T. zahrahae* in the gill of the Java barb (*Barbonymus gonionotus*). Based on phylogenetic tree result, *T. kitauei* was clustered with other *Thelohanellus* spp. infected Cyprininae while *T. zahrahae* revealed long distance from the other *Thelohanellus* spp. Java barb has included family Cyprinidae and subfamily Barbinae while the other fish have belonged to Cyprinidae and subfamily Cyprininae (35). These results suggest that infection site and host specificity (subfamily level) are reflected in the genetic relationships among *Thelohanellus* species. Previous studies mainly explained the relationship between host specificity and infection sites of *Myxobolus* spp. at species and tissue level (4, 9). However, the host specificity and infection site of *Thelohanellus* spp. was explained at subfamily and organ level in the present study.

To investigate in detail the relationship between infection site tropism and host specificity, *T. kitauei* was compared with 102 Myxobolidae (*Myxobolus* spp., *Henneguya* spp. and *Thelohanellus* spp.) considering the countries, host spp. (at the level of the family) and infection sites. It is revealed that the genetic separation based on host specificity (family level) is a more ancient evolutionary feature than infection site tropism. First, the phylogenetic tree was divided by the family of fish host, such as Salmonidae, Mugilidae, Ictaluridae or Cyprinidae and then the Myxobolidae group that infects Cyprinidae was divided by the two kinds of infection site tropism characteristics, such SIT and NSIT.

The NSIT Myxobolidae group was divided by the genus of parasites, such as *Henneguya*, *Thelohanellus* and *Myxobolus*, and then the last clade revealed that a various infection site, such as gills, intestine, skin, swim bladder and fins. *Thelohanellus* spp. (except *T. zahrahae*) have previously been clustered with the

NSIT Myxobolidae group, and as described above, *Thelohanellus* spp. infect a various organs of the Cyprininae host fish (5, 11, 27, 36, 41, 43). These previous reports correspond to our phylogenetic result. However, our phylogenetic data, as with previous study (20) clearly demonstrate the polyphyletic nature of *Thelohanellus*, although this is not a completely haphazard pattern because many *Thelohanellus* sp. cluster together. The species of the polyphyletic genus *Thelohanellus* probably arose from Myxobolidae ancestors that infect to Cyprinidae one times in myxosporean evolution. Based on this result, it is revealed that the organ tropism and host specificity of the *T. kitauei* was originated from the ancient NSIT Myxobolidae that infectious to Cyprinidae.

The SIT Myxobolidae group was classified by two specific infection site tropisms. One group infects the gills of the host fish, and the other group infects the muscle. As Molnár et al. (25) demonstrated, in the case of species infection of the skeletal muscle of cyprinids, molecular biological methods are essential validating the identity of the species. Eszterbauer (9) described the molecular evidence of tissue specificity among gill-infecting *Myxobolus* spp. Camus and Griffin (4) compared the 18S rRNA genes of *Myxobolus* spp., and the phylogenetic tree was generated by maximum parsimony analyses and minimum evolution analyses. Particularly, the phylogenetic tree generated by maximum parsimony was the most similar to the phylogenetic tree in present study. However while they did focus on the level of the species of host fish or simply tissue tropism, this study explained the relationships between organ specificity and the level of the family of host fish.

Host and parasite lineages evolved and adapted together over long periods. This process is known as coevolution or cospeciation (29). Basically, the close

relationships between hosts and their parasites resulted in the parasites being considered more or less host phenotypic characters, which should lead parasite phylogeny to mirror host phylogeny (Farenholz's rule) (8). Several different fish host and parasite associations have contributed to the study of coevolution, e.g., copepods and teleost fish (28), *Lamellodiscus* and Sparidae (7) and *Gyrodactylus* and six fish families (44). Based on phylogenetic results, it was suggested that after a host switch event over fish family borders (or contact with a new host), ancient Myxobolidae cospeciated with the host fish because host specificity can lead to cospeciation of host and parasite.

The phylogenetic tree revealed by this study demonstrated that infection site tropism and host specificity may play an important role in the genetic relationships among myxosporean spp. The phylogenetic relationships among the Myxobolidae (including *Thelohanellus* spp.) examined suggest that when the parasites contact new host fish, they adapt to be able to infect to new fish, and then the parasites cospeciate with the new host. The infection site tropism and host specificity of *T. kitauei* originated from an ancient NSIT ancient Myxobolidae that was able to infect Cyprinidae. This study will provide important information on the genetic relationships between infection site tropism and host specificity of myxosporean spp.

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Table 1.1. Sequence data of Myxobolidae used for phylogenetic comparison in this study

Species	Host species (scientific name)	Host family	Infection site	Country	Accession NO.
Thelohanellus wuhanensis-China3	Allogynogenetic gibel carp (*Carassius auratus gibelio*)	Cyprinidae	Skin	China	JQ690370
Thelohanellus wuhanensis-China	Crucian carp (*Carassius carassius*)	Cyprinidae	n.d.	China	HQ613410
Thelohanellus kitauei -China	Common carp (*Cyprinus carpio*)	Cyprinidae	Intestine	China	HQ115585
Thelohanellus kitauei -Korea	Israel carp (*Cyprinus carpio nudus*)	Cyprinidae	Intestine	Korea	HM624024
Thelohanellus wuhanensis-China2	Allogynogenetic gibel carp (*Carassius auratus gibelio*)	Cyprinidae	Gills	China	AY165181
Thelohanellus kitauei (present study)	Koi carp (*Cyprinus carpio haematopterus*)	Cyprinidae	Intestine	Korea	GQ396677
Thelohanellus nikolskii	Common carp (*Cyprinus carpio*)	Cyprinidae	Fins	China	GU165832
Thelohanellus zahrahae	Java barb (*Barbonymus gonionotus*)	Cyprinidae	Gills	Malaysia	EU643622
Thelohanellus sinensis	Gold fish *(Carassius auratus auratus)*	Cyprinidae	Gills	China	DQ452013
Thelohanellus nikolskii	Common carp (*Cyprinus carpio*)	Cyprinidae	Fins	Hungary	DQ231156
Thelohanellus hovorkai	Common carp (*Cyprinus carpio*)	Cyprinidae	Intestine	Hungary	DQ231155
Myxobolus toyamai	Common carp (*Cyprinus carpio*)	Cyprinidae	Gills	Japan	FJ710802
Myxobolus longisporus	Koi carp (*Cyprinus carpio haematopterus*)	Cyprinidae	Gills	China	AY364637
Myxobolus koi (USA)	Common carp (*Cyprinus carpio*)	Cyprinidae	Gills	USA	FJ841887
Myxobolus koi (Japan)	Common carp (*Cyprinus carpio*)	Cyprinidae	Gills	Japan	FJ710800
Myxobolus wulii	Gold fish (*Carassius auratus auratus)*	Cyprinidae	Gills	Japan	EF690300

(Continued)

Myxobolus ampullicapsulatus GF	Gold fish (*Carassius auratus auratus)*	Cyprinidae	Gills	China	DQ339482
Myxobolus honghuensis	Allogynogenetic gibel carp (*Carassius auratus gibelio*)	Cyprinidae	Gills (pharynx)	China	JF340216
Myxobolus ampullicapsulatus GC	Allogynogenetic gibel carp (*Carassius auratus gibelio*)	Cyprinidae	Gills (pharynx)	China	HM188545
Myxobolus pellicides	Common creek chub (*Semotilus atromaculatus*)	Cyprinidae	Gills	Canada	AF378339
Myxobolus pendula	Common creek chub (*Semotilus atromaculatus*)	Cyprinidae	Gills	Canada	AF378340
Myxobolus dykovae	Tinfoil barb (*Barbonymus schwanenfeldii*)	Cyprinidae	Gills	Malaysia	EU643627
Myxobolus bilobus	Golden shiner (*Notemigonus crysoleucas*)	Cyprinidae	Gills	Canada	DQ008579
Myxobolus sp. 2 GC-2011	White bream (*Blicca bjoerkna*)	Cyprinidae	Gills	Hungary	JF311898
Myxobolus intimus RO	Common roach (*Rutilus rutilus*)	Cyprinidae	Gills	Hungary	AY325285
Myxobolus intimus ASP	Asp (*Aspius aspius*)	Cyprinidae	Gills	Hungary	JF311899
Myxobolus dujardini	European chub (*Leuciscus cephalus*)	Cyprinidae	Gills	Hungary	DQ439804
Myxobolus hungaricus	Common bream (*Abramis brama*)	Cyprinidae	Gills	Hungary	AF448444
Myxobolus sp. 1 GC-2011	White bream (*Blicca bjoerkna*)	Cyprinidae	Gills	Hungary	JF311900
Myxobolus obesus	Common bleak (*Alburnus alburnus*)	Cyprinidae	Gills	Hungary	AY325286
Myxobolus sp. Hungary-EE-2003	Common bream (*Abramis brama*)	Cyprinidae	Gills	Hungary	AY325283
Myxobolus leptobarbi	Mad barb (*Leptobarbus hoevenii*)	Cyprinidae	Muscle	Malaysia	EU643623
Myxobolus stanlii	Largescale stoneroller (*Campostoma oligolepis*)	Cyprinidae	Muscle	USA	DQ779995
Myxobolus terengganuensis	Silver shark minnow (*Osteochilus hasselti*)	Cyprinidae	Muscle	Malaysia	EU643629
Myxobolus pseudodispar WB	White bream (*Blicca bjoerkna*)	Cyprinidae	Muscle	Hungary	AF380143

(Continued)

Myxobolus pseudodispar CB	Common bream (*Abramis brama*)	Cyprinidae	Muscle	Hungary	AF380144
Myxobolus pseudodispar RD	Common rudd (*Scardinius erythrophthalmus*)	Cyprinidae	Muscle	Hungary	AF380142
Myxobolus pseudodispar RO	Common roach (*Rutilus rutilus*)	Cyprinidae	Muscle	Hungary	AF380145
Myxobolus sp. CMW-2004	Northern pikeminnow (*Ptychocheilus oregonensis*)	Cyprinidae	Muscle	USA	AY591531
Myxobolus cyprini	Common carp (*Cyprinus carpio*)	Cyprinidae	Muscle	Hungary	AF380140
Myxobolus artus	Common carp (*Cyprinus carpio*)	Cyprinidae	Muscle	Japan	FJ710799
Myxobolus musculi (Portugal)	Iberian barbel (*Luciobarbus bocagei*)	Cyprinidae	Muscle	Portugal	JQ388891
Myxobolus musculi (Hungary)	Barbel (*Barbus barbus*)	Cyprinidae	Muscle	Hungary	AF380141
Myxobolus erythrophthalmi	Rudd (*Scardinius erythrophthalmus*)	Cyprinidae	Kidney	Hungary	EU567311
Myxobolus ellipsoides	European chub (*Leuciscus cephalus*)	Cyprinidae	Fins	Hungary	DQ439813
Myxobolus alburni	Common bleak (*Alburnus alburnus*)	Cyprinidae	Fins	Hungary	EU567313
Myxobolus leuciscini	European chub (*Leuciscus cephalus*)	Cyprinidae	Gills	Hungary	DQ439811
Myxobolus wootteni	Common roach (*Rutilus rutilus*)	Cyprinidae	Fins	Hungary	DQ231157
Myxobolus gayerae	European chub (*Leuciscus cephalus*)	Cyprinidae	Intestine	Hungary	DQ439809
Myxobolus cycloides	European chub (*Leuciscus cephalus*)	Cyprinidae	Swim bladder	Hungary	DQ439810
Myxobolus caudatus	Barbel (*Barbus barbus*)	Cyprinidae	Fins and scales	Hungary	JQ388889
Myxobolus impressus	Common bream (*Abramis brama*)	Cyprinidae	Gills	Hungary	AF507970
Myxobolus muellericus	European chub (*Leuciscus cephalus*)	Cyprinidae	Gills	Hungary	DQ439808
Myxobolus diversicapsularis	Common roach (*Rutilus rutilus*)	Cyprinidae	Gills	Hungary	GU968199

(Continued)

Myxobolus rotundus	Common bream (*Abramis brama*)	Cyprinidae	Gills	Hungary	EU710583
Myxobolus parviformis	Common bream (*Abramis brama*)	Cyprinidae	Gills	Germany	AY836151
Myxobolus basilamellaris	Common carp (*Cyprinus carpio*)	Cyprinidae	Gills	Hungary	AF507971
Myxobolus musseliusae	Common carp (*Cyprinus carpio*)	Cyprinidae	Gills	Japan	FJ710801
Myxobolus pavlovskii SC	Silver carp (*Hypophthalmichthys molitrix*)	Cyprinidae	Gills	Hungary	HM991164
Myxobolus pavlovskii BC	Bighead carp (*Aristichthys nobilis*)	Cyprinidae	Gills	Hungary	AF507973
Myxobolus cyprinicola	Common carp (*Cyprinus carpio*)	Cyprinidae	Intestine	Hungary	DQ439805
Myxobolus dispar	Common carp (*Cyprinus carpio*)	Cyprinidae	Gills	Hungary	AF507972
Myxobolus algonquinensis	Common shiner (*Notropis cornutus*)	Cyprinidae	Ovary	Canada	AF378335
Myxobolus margitae	Common bleak (*Alburnus alburnus*)	Cyprinidae	Gills	Hungary	EU598803
Myxobolus feisti	Common roach (*Rutilus rutilus*)	Cyprinidae	Gills	Hungary	JN252487
Myxobolus macrocapsularis	Common bream (*Abramis brama*)	Cyprinidae	Gills	Hungary	AF507969
Myxobolus muelleri	European chub (*Leuciscus cephalus*)	Cyprinidae	Gills	Hungary	DQ439806
Myxobolus bramae	Common bream (*Abramis brama*)	Cyprinidae	Gills	Hungary	AF507968
Myxobolus bliccae WB	White bream (*Blicca bjoerkna*)	Cyprinidae	Gills	Hungary	HM138770
Myxobolus bliccae VB	Vimba bream (*Vimba vimba*)	Cyprinidae	Gills	Hungary	HM138772
Henneguya cutanea	Common bream (*Abramis brama*)	Cyprinidae	Skin	Hungary	AY676460
Henneguya doneci GC	Allogynogenetic gibel carp (*Carassius auratus gibelio*)	Cyprinidae	Gills	China	HM146129
Henneguya doneci GF	Gold fish (*Carassius auratus auratus*)	Cyprinidae	Gills	China	EU344899

(Continued)

Henneguya salminicola	Sockeye salmon (*Oncorhynchus nerka*)	Salmonidae	Muscle	Canada	AF031411
Henneguya zschokkei	Mountain whitefish (*Prosopium williamsoni*)	Salmonidae	Muscle	Canada	AF378344
Henneguya nuesslini	Brook Trout (*Salvelinus fontinalis*)	Salmonidae	Skin	Germany	AY669810
Henneguya adiposa	Channel catfish (*Ictalurus punctatus*)	Ictaluridae	Fins	USA	EU492929
Henneguya ictaluri	Channel catfish (*Ictalurus punctatus*)	Ictaluridae	Gills	USA	AF195510
Henneguya exilis	Channel catfish (*Ictalurus punctatus*)	Ictaluridae	Gills	USA	AF021881
Henneguya gurlei	Brown bullhead (*Ameiurus nebulosus*)	Ictaluridae	Fins	USA	DQ673465
Henneguya sutherlandi	Channel catfish (*Ictalurus punctatus*)	Ictaluridae	Skin	USA	EF191200
Henneguya pellis	Blue catfish (*Ictalurus furcatus*)	Ictaluridae	Skin	USA	FJ468488
Myxobolus episquamalis (Korea)	Common mullet (*Mugil cephalus*)	Mugilidae	Skin (Scale)	Korea	JF810537
Myxobolus episquamalis (Tunisia)	Common mullet (*Mugil cephalus*)	Mugilidae	Skin (Scale)	Tunisia	AY129312
Myxobolus bizerti	Common mullet (*Mugil cephalus*)	Mugilidae	Gills	Tunisia	AY129318
Myxobolus spinacurvatura	Common mullet (*Mugil cephalus*)	Mugilidae	Mesenteric vessel	Tunisia	AF378341
Myxobolus ichkeulensis	Common mullet (*Mugil cephalus*)	Mugilidae	Gills	Tunisia	AY129315
Myxobolus supamattayai	Blue spot mullet (*Valamugil seheli*)	Mugilidae	Skin (Scale)	Thailand	HQ166720
Myxobolus muelleri	Thinlip mullet (*Liza ramada*)	Mugilidae	Mesenteric vessel	Tunisia	AY129314
Myxobolus exiguus	Thinlip mullet (*Liza ramada*)	Mugilidae	Intestine	Tunisia	AY129317
Myxobolus cerebralis	Rainbow trout (*Oncorhynchus mykiss*)	Salmonidae	Brain	USA	EF370481
Myxobolus insidiosus	Chinook salmon (*Oncorhynchus tshawytscha*)	Salmonidae	Muscle	USA	U96494

(Continued)

Myxobolus neurobius (Hungary)	Rainbow trout (*Oncorhynchus mykiss*)	Salmonidae	Nerve	Hungary	AF085180
Myxobolus arcticus (USA)	Sockeye salmon (*Oncorhynchus nerka*)	Salmonidae	Brain	USA	AF085176
Myxobolus insidiosus	Chinook salmon (*Oncorhynchus tshawytscha*)	Salmonidae	Muscle	USA	EU346374
Myxobolus squamalis	Chinook salmon (*Oncorhynchus tshawytscha*)	Salmonidae	Skin (Scale)	USA	U96495
Myxobolus fryeri	Coho salmon (*Oncorhynchus kisutch*)	Salmonidae	Nerve	USA	EU346370
Myxobolus sp. OMM1995	Masu salmon (*Oncorhynchus masou masou*)	Salmonidae	Nerve	Japan	AB469985
Myxobolus neurobius (Norway)	Brown trout (*Salmo trutta*)	Salmonidae	Nerve	Norway	AB469986
Myxobolus arcticus (Japan)	Masu salmon (*Oncorhynchus masou masou*)	Salmonidae	Brain	Japan	AB469990
Myxobolus arcticus (Canada)	Sockeye salmon (*Oncorhynchus nerka*)	Salmonidae	Brain	Canada	HQ113227
Myxobolus neurotropus	Rainbow trout (*Oncorhynchus mykiss*)	Salmonidae	Brain	USA	DQ846661
Myxobolus kisutchi	Chinook salmon (*Oncorhynchus tshawytscha*)	Salmonidae	Brain	USA	AB469989
Tetracapsuloides bryosalmonae	Rainbow trout (*Oncorhynchus mykiss*)	Salmonidae	Kidney	France	U70623

* Not determined.

Table 1.2. Identity of *Thelohanellus kitauei* 18S rRNA sequence to other *Thelohanellus* spp. 18S rRNA

Species	DNA sequence identity (%) to *T. kitauei* - Present study	GenBank NO.
T. kitauei-China	1301/1301 (100%)	HQ115585
T. kitauei-Korea	1552/1555 (99%)	HM624024
T. hovorkai-Hungary	1474/1516 (97%)	DQ231155
T. nikolskii-Hungary	1393/1559 (89%)	DQ231156
T. nikolskii-China	1418/1584 (90%)	GU165832
T. wuhanensis-China	1475/1562 (94%)	HQ613410
T. wuhanensis-China2	634/686 (92%)	AY165181
T. wuhanensis-China3	1472/1558 (94%)	JQ690370
T. sinensis-China	652/728 (90%)	DQ452013
T. zahrahae-Malaysia	1008/1174 (86%)	EU643622

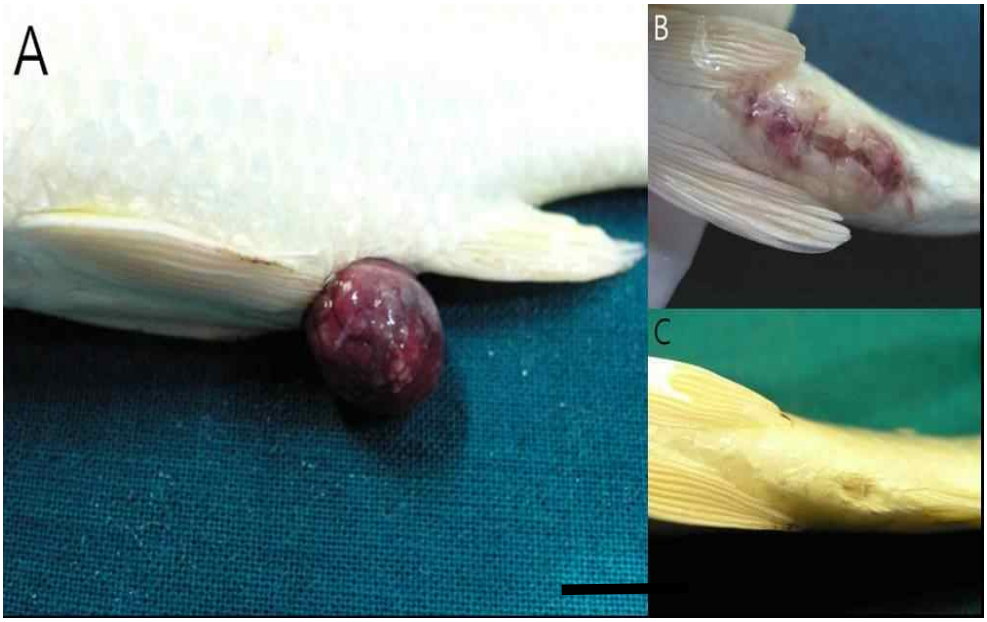


Figure 1.1. Surgical removal of anal cyst caused by *Thelohanellus kitauei* from koi *Cyprinus carpio koi* and incision wound healing. (A) Koi *Cyprinus carpio koi* fish was observed to have a cyst-like tumor at the anus. Bar = 1 cm. (B) After suture removal, the incision healed well; however, the posterior part of the skin had not completely healed. (C) The incision wound was a completely recovered 14 months after surgery.

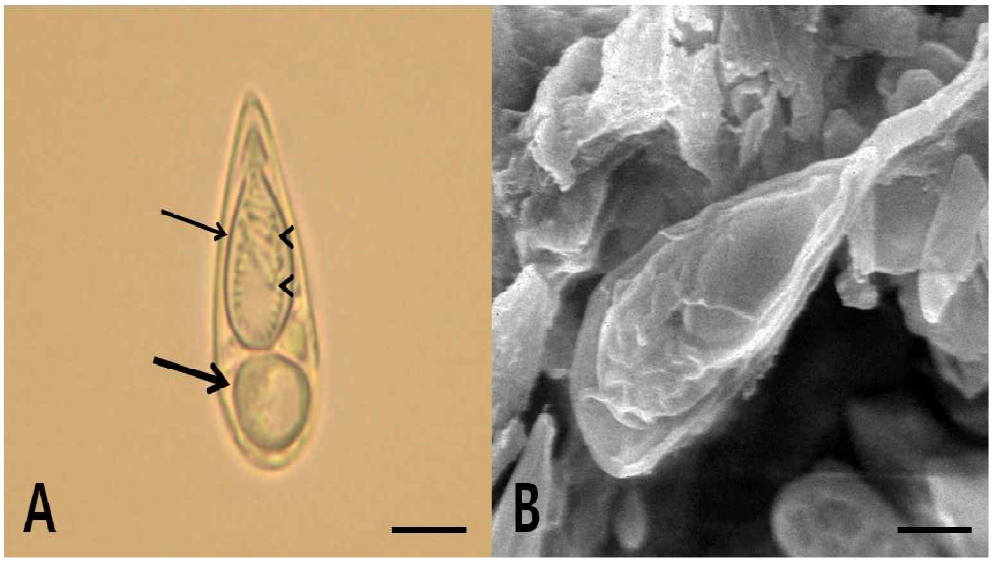


Figure 1.2. Portion of the anal cyst from the koi in Figure 1 after surgical excision. (A) Light micrograph of a wet-mount section of the excised cyst. Notice the Thelohanellus kitauei spore containing polar capsule (arrow) and sporoplasm (bold arrow). The polar filament was coiled in 12 turns perpendicular to the axis of the capsule (arrow heads). Bar = 10 μm. (B) Scanning electron micrograph of a section of the anal cyst. Notice the T kitauei spore exhibits a pyriform body shape with several wavy ridges discernable on the caudal portion. Bar = 5 μm.

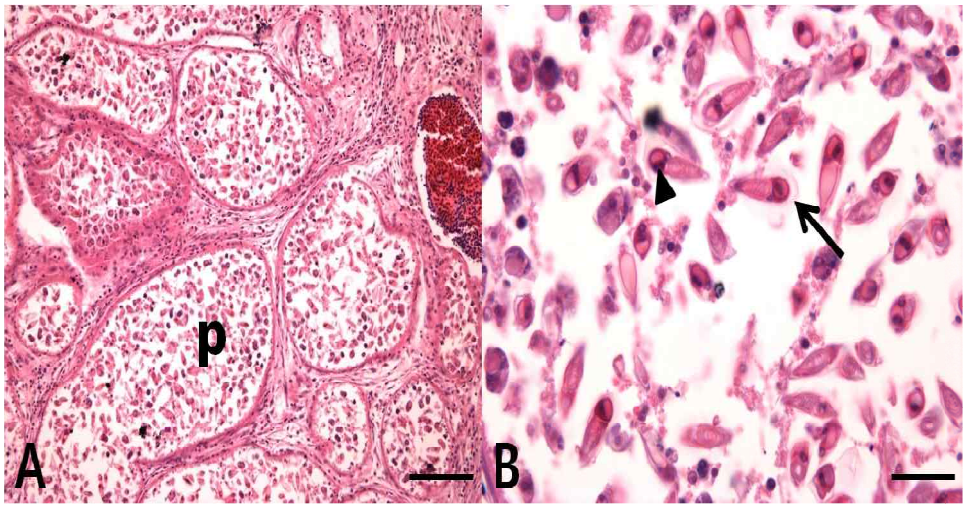


Figure 1.3. Light micrographs of a biopsy specimen obtained from a nodular anal mass in an 8-month-old koi (Cyprinus carpio) with lethargy, anorexia, signs of depression, and anal obstruction. (A) Plasmodia (p) surrounded by connective tissue. H&E stain; bar = 150 µm. (B) A plasmodium filled with many enveloped spores (arrow) with 2 circular nuclei present in the sporoplasm (arrowhead). H&E stain; bar = 30 µm.

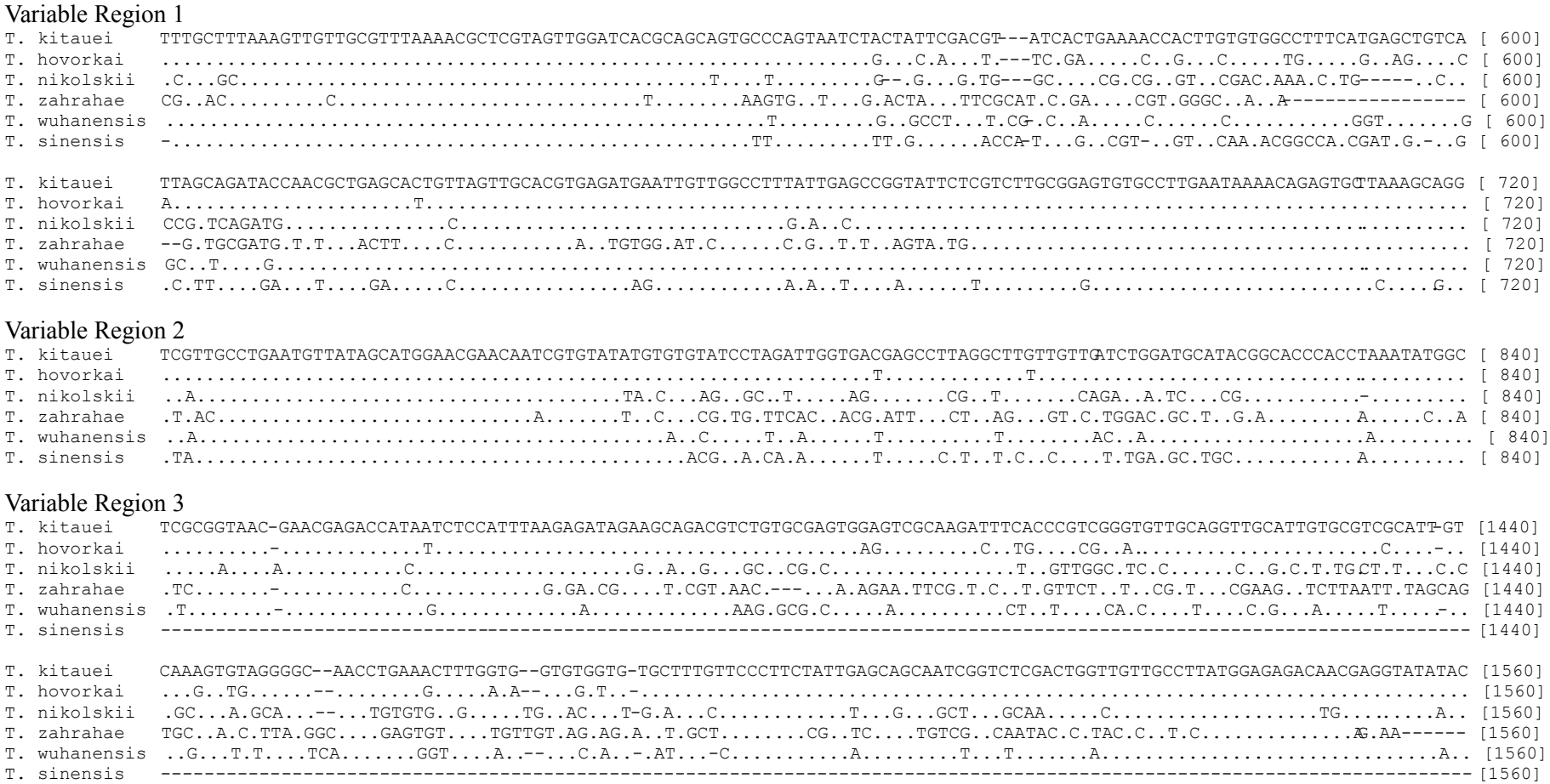


Figure 1.4. Alignment of 18S rRNA sequences emphasizing three variable regions. No data are available for T. sinensis for variable region 3. Period(.) designate conserved regions; dashes (-) designate gaps or missing data.

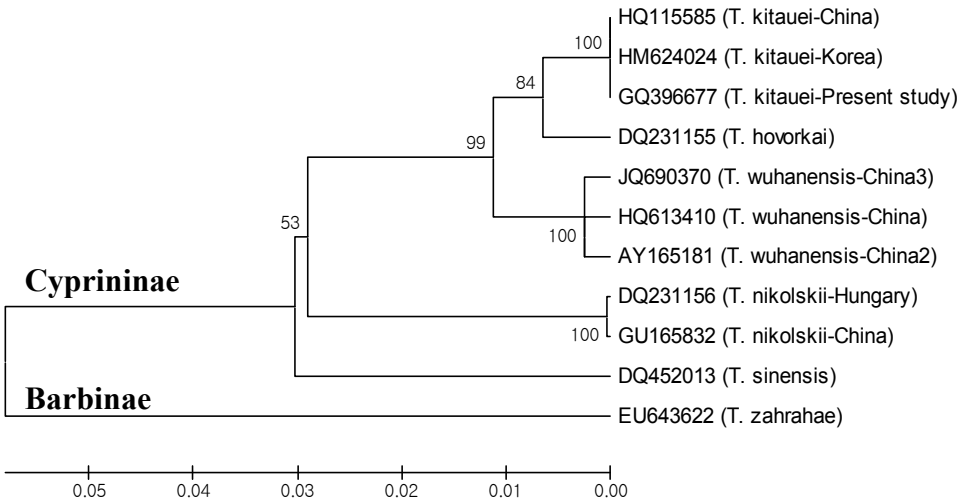
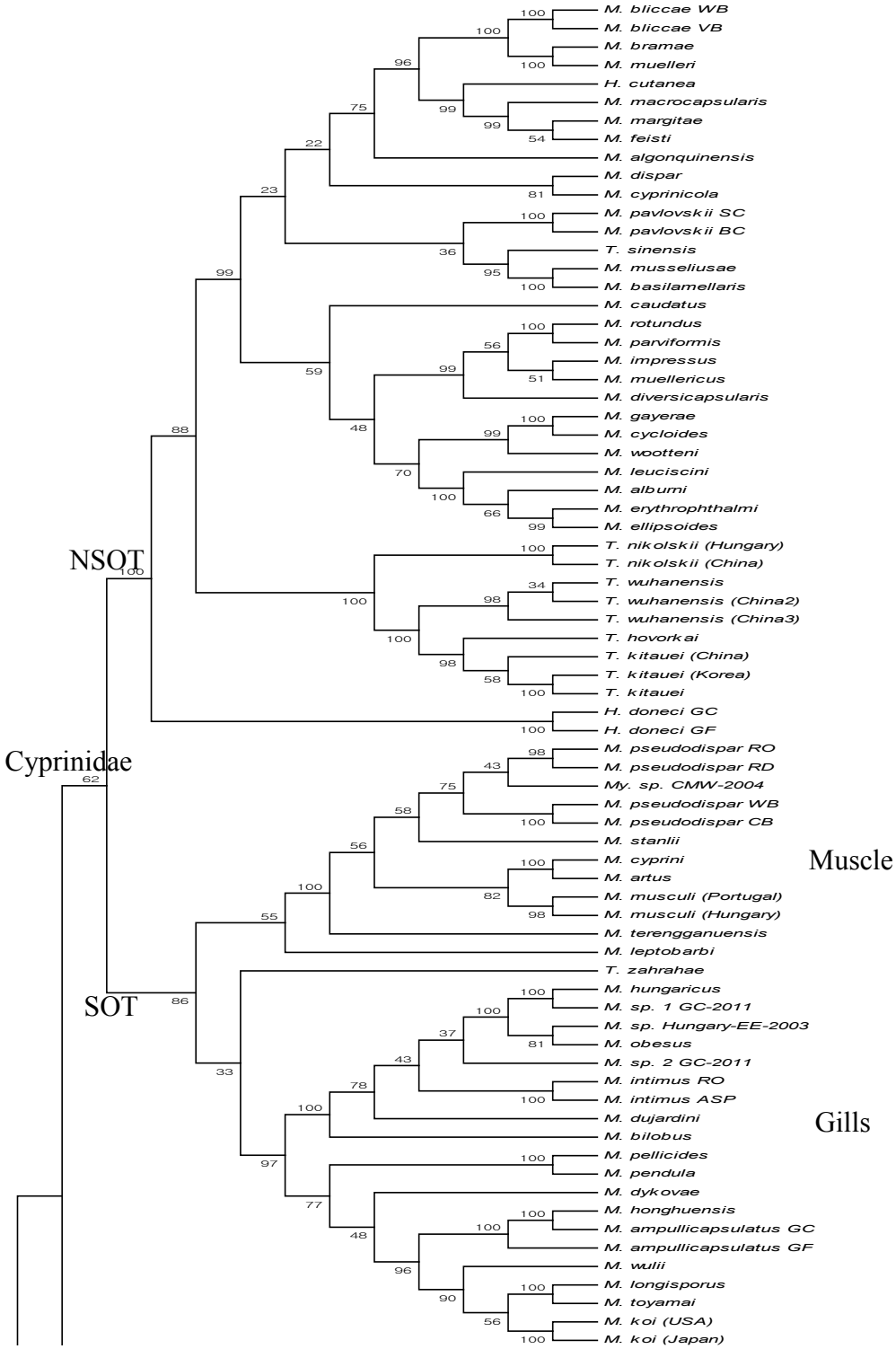


Figure 1.5. Phylogenetic tree generated by minimum evolution analyses of the 18S rRNA sequences of *Thelohanellus* spp. Numbers at nodes indicate bootstrap confidence values (1,000 replicates). Units of evolutionary distance are in the number of base substitutions per site.


M. bliccae WB
M. bliccae VB
M. bramae
M. muelleri
H. cutanea
M. macrocapsularis
M. margitae
M. feisti
M. algonquinensis
M. dispar
M. cyprinicola
M. pavlovskii SC
M. pavlovskii BC
T. sinensis
M. musseliusae
M. basilamellaris
M. caudatus
M. rotundus
M. parviformis
M. impressus
M. muellericus
M. diversicapsularis
M. gayerae
M. cycloides
M. wootteni
M. leuciscini
M. alburni
M. erythrophthalmi
M. ellipsoides
T. nikolskii (Hungary)
T. nikolskii (China)
T. wuhanensis
T. wuhanensis (China2)
T. wuhanensis (China3)
T. hovorkai
T. kitauei (China)
T. kitauei (Korea)
T. kitauei
H. doneci GC
H. doneci GF
M. pseudodispar RO
M. pseudodispar RD
My. sp. CMW-2004
M. pseudodispar WB
M. pseudodispar CB
M. stanlii
M. cyprini
M. artus
M. musculi (Portugal)
M. musculi (Hungary)
M. terengganuensis
M. leptobarbi
T. zahrahae
M. hungaricus
M. sp. 1 GC-2011
M. sp. Hungary-EE-2003
M. obesus
M. sp. 2 GC-2011
M. intimus RO
M. intimus ASP
M. dujardini
M. bilobus
M. pellicides
M. pendula
M. dykovae
M. honghuensis
M. ampullicapsulatus GC
M. ampullicapsulatus GF
M. wulii
M. longisporus
M. toyamai
M. koi (USA)
M. koi (Japan)
NSOT
Cyprinidae
SOT
Muscle
Gills

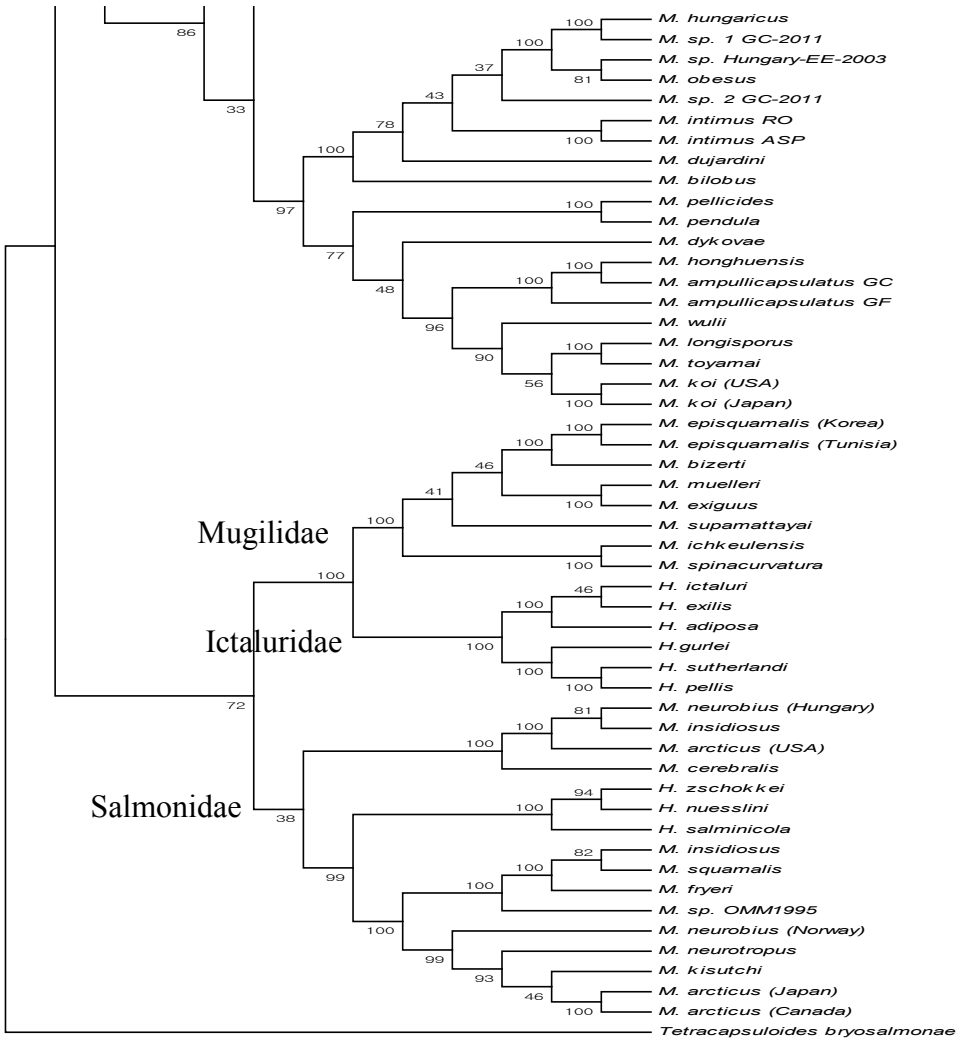


Figure 1.6. Phylogenetic tree generated by minimum evolution analyses of the 18S rRNA sequences of myxosporeans, rooted at *Tetracapsuloides bryosalmonae*. Numbers at nodes indicate bootstrap confidence values (1,000 replicates). Units of evolutionary distance are in the number of base substitutions per site. (*H. Heneguya; M. Myxobolus; T. Thelohanellus*).

Chapter II

Identification of scuticociliate *Philasterides dicentrarchi* from Indo-Pacific seahorses *Hippocampus kuda*

Abstract

The Indo-Pacific seahorses, *Hippocampus kuda* which had been reared in one of the private commercial aquaria for exhibition were sent to the laboratory for diagnosis. Feces and intestinal contents from 5 seahorse were examined microscopically and revealed numerous scuticociliates. We identified the species of scuticociliate by light (wet mount) and scanning electron microscopy (SEM), PCR and sequencing. This paper reports the first identification of scuticociliate *Philasterides dicentrarchi* from seahorse in Korea.

Key words: Indo-Pacific seahorse, *Philasterides dicentrarchi*, scanning electron microscopy, sequencing, pathogenicity test

2.1. Introduction

Scuticociliatosis is a disease caused by histophagus ciliates or free-living scuticociliates, facultative parasites of the order Scuticociliatida belonging to the genera *Uronema*, *Miamiensis*, *Tetrahymena*, and *Philasterides* (2, 3, 5, 20). These opportunistic histophagus parasites are characterized by their high potential for systemically invading the host and occasionally pathogenic causing high mortalities (10, 13). Mortalities occurred in cultured marine fish (3, 4, 6, 16) like olive flounder *Paralichthys olivaceus* (7, 10, 11, 12) and in seahorse *Hippocampus erectus* (20). Scuticociliates primarily infect the gills, skin, and sometimes systemically affect the internal organs and brain of fish resulting to high mortality. It is also associated with various pathological changes, including bleeding cutaneous ulcers, dystrophic and necrotic effects in muscles, hypochromic anemia and encephalitis associated with softening or liquefaction of brain tissues (6).

Scuticociliatosis has been recognized as an emerging problem that causes significant economic loss in aquaculture, through systemic infection (8) The causative agents of scuticociliatosis in Korea have been isolated and identified as *Uronema marinum* (7), *Pseudocohnilembus persalinus* (11), *Philasterides dicentrarchi* (10). *P. dicentrachi* and *Miamiensis avidus* have caused mass mortality in turbot farms in Spain, Portugal, and France (1, 4, 6, 18).

Seahorses are especially popular in some Asian countries due to strong demand from the Chinese traditional medicine and the souvenir trade on a global scale (15, 22). Recently, the Indo-Pacific seahorse *Hippocampus kuda* (Family Syngnathidae) that is widely distributed throughout the tropical Indo-Pacific Region (14) become popular ornamental fish species and artificially cultured in Jeju Island, South Korea.

The present study deals with the numerous scuticociliates obtained from the intestinal contents and feces of a moribund 5 seahorses sample based on light (fresh mounts) and scanning electron microscopy (SEM), PCR, sequencing. The results revealed that the ciliates identified was *P. dicentrarchi*. In addition, based on the experimental infection to olive flounder (*Paralichthy olivaceus*), the possibility of cross infection was sugested between seahorse and olive flounder.

2.2. Materials and methods

2.2.1. Samples collection

In April 2008, 12 pieces of seahorse, *Hippocampus kuda* (average total length = 13.9 cm, average body weight = 10.9 g) were bought from a commercial hatchery in Jeju Island, South Korea for public exhibition in one of the private commercial aquaria in Seoul. After one month, some of the seahorses with no visible external lesion started to show lethargy, anorexia and died one by one with an interval of four to five days. In May 2008, 5 moribund seahorses were submitted to our laboratory for diagnostic examination. Theses seahorses showed sloughing of epidermis, depigmentation of skin and abdominal distension. In order to rule out disease causing factors of seahorses, fungal and parasitic infection was checked using wet mount of the skin-scratch, fecal, and internal contents. Moreover viral diseases, such as betanodavirus, birnavirus, iridovirus, and flounder-herpesvirus which caused mass mortality in cultured marine fish in Korea, were also tested by using PCR assay. For bacterial isolation, the bacterial strain was investigated via culturing and using VITEK II system (BioMerieux, France). Fungal, bacterial and viral diseases were all negative in tested methods and water parameters, such as

dissolved oxygen, temperature, pH and ammonia showed normal range value.

2.2.2. *Light (wet mount) and scanning electron microscopy (SEM) examinations*

Feces and intestinal contents were wet mounted and observed under a microscope. For SEM, these specimens were fixed in modified Karnovsky's fixative, post fixed in 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2), dehydrated through an ethanol serial solutions of 30%, 50%, 70%, 80%, 90%, 100%, 100%, 100% for 10 minutes per each concentration. After treatment of ciliates with hexamethyldisilazane (15 min, two times), specimen drying was performed at room temperature overnight. Ciliates were examined using a JEOL-5410 LV scanning electron microscope (NICEM, Korea).

2.2.3. *Molecular analysis of parasites*

Feces and intestinal contents total DNA was extracted with DNeasy® Tissue Kit (QIAGEN, USA) following the manufacturer's protocol. Extracted DNA was stored at -20℃ until use for PCR and sequencing. Total 18S rRNA PCR and nested PCR were modified as previously described (10, 11). Universal 18S rRNA primer for scuticociliates, *U. marinum,* and *P. dicentrarchi* specific primers set were used for detection of seahorses pathogen. 18S rRNA PCR was performed in 20 μl reaction mixtures containing DNA template (2 μl), a 10 pmol concentration each primer (Bioneer, Korea) and *i*-StarMaster mix PCR Kit (2.5 U *i-StarTaq* ™ DNA Polymerase, 250 μM dNTP, 10 mM Tris-HCl, 2 mM Mg^{2+}solution, stabilizer and tracking dye) (Intronbio, Korea). Nested PCR was performed as described above except using 2 μl PCR product and specific primer. Primers used in the

present study were summarized in **Table 2.1**. The amplification was carried out in a T-personal 48 Biometra thermocycler (Biometra, Germany) with the following parameters: an initial denaturation step of 95°C, 2 min; 35 serial cycles of a denaturation step of 95°C, 30 s, annealing at 50°C, 30 s extension at 72°C, 2 min; and final extension step of 72°C, 10min. PCR and nested PCR products were analyzed by 2.0% agarose gel electrophoresis in 1% Tris-borate-EDTA (TBE) buffer. Gels were stained with ethidium bromide (0.5 μg/ml), visualized and photographed under UV illumination. For definitive identification, amplified PCR product of scuticociliate isolated from seahorses was sequenced using BigDyeTM terminator cycle sequencing kit (Macrogen Genomic Division, Korea). Electrophoresis of sequencing reactions was completed using Automatic Sequencer 3730x1 DNA analyzer (Applied Biosystems, USA). 18S rRNA gene sequence of isolate (SNUSS001) was compared and realigned using the multiple alignment algorithms in the MegAlign package Windows Version 3.12e (DNASTAR, USA) along with the three known scuticociliate species in Korea to give a phylogenetic tree. The Genebank accession numbers of the known scuticociliates sequence used in this study were *P. dicentrarchi* (AY642280), *P. persalinus* (AY835669), *U. marinum* (AY551905).

2.2.4. *Pathogenicity test*

To test pahtogenicity of the ciliates, 3 groups (A, B and C) of 15 olive flounder (*Paralichthy olivaceus*) fish weighing 1 to 3 g were used and the fish of group A and B were exposed to ciliates at a concentration of 1.27×10^4 and 8.94×10^3/ml using immersion method. The fish of control (group C) were exposed to 1 ml PBS

containing no ciliates. These fish were fed commercial dry pellets. Dead and moribund fish were removed from the tank and clinical signs and mortality for the duration of the experiment were recorded.

2.3. Results

2.3.1. Morphological examination

The ciliate in wet mount **(Figure 2.1A)** and by SEM **(Figure 2.1B)** examinations showed ovoid, elongated and egg shape. While the anterior part was tapered, the posterior part was rounded with a contractile vacuole and a long caudal cilium. The ciliate body was covered with numerous cilia. Posterior part was occupied with many food vacuoles.

2.3.2. Molecular examination

Using universal primer, PCR results revealed between 1500 and 2072 bp and nested PCR product (326 bp) results matched the size of the 18S rRNA gene segment of *P. dicentrarchi*. Using *U. marinum* primer, result of the band showed to be negative **(Figure 2.2)**. The result of the PCR product (1666 bp) sequenced showed 99% nucleotide similarity with *P. dicentrarchi*, 93% with *P. persalinus*, and 91% with *U. marinum*. Phylogenetic tree analysis was constructed based on 18S rRNA gene and revealed that this strain is *P. dicentrarchi* **(Figure 2.3)**. This sequence (1666 bp) was deposited in GenBank under the accession number GU572375.

2.3.3. Pathogenicity test

The cumulative mortalities of fish infected with *P. dicentrarchi* are shown in Fig. 4. The fish of group A and B have started to die after 3 days post immersion, and mortality reached 100% by 14 days. While the cumulative mortality for both groups were 100%, only one fish died in the control group for the duration of the experiment **(Figure 2.4)**. Most of died fish exhibited depigmentations, haemorrhages and ulcers on the skin and fins. *P. dicentrarchi* was re-isolated from died fish.

2.4. Discussion

In the morphological examinations, the present results shared similar characteristics with the scuticociliates identified from weedy sea dragon *Phyllopteryx taeniolatus* (21) and olive flounder (19). Based on these descriptions, the ciliates were suggested as of the genus *Philasterides*. Further confirmation using nucleotide sequencing showed to be closely related to *P. dicentrarchi* and genetically distinguishable from the other two types of scuticociliates used in this study. In other study, the *P. dicentrarchi* partial nucleotide sequence also showed high identity (99%) with the sequence of *M. avidus* (syn. *P. dicentrarchi*) that was isolated from farmed turbot (17) and *M. avidus* is a senior synonym of *P. dicentrarchi* (9).

In the present study, seahorses comes from the aquaculture system in Jeju Island, Korea, where scuticociliates infection has already been a problem in many olive flounder cultured system in Jeju Island (10). Some of these seahorse culture systems were located near the olive flounder aquaculture systems where outbreak of *P. dicentrarchi* species has already occurred. Based on our experimental

infection result, *P. dicentrarchi* showed high pathogenicity and it is corresponding with previous report which showed higher pathogenicity than other scuticociliates isolated from this fish in Korea (19). The species of *P. dicentrarchi* is an opportunistic histophagus or facultative parasitic ciliate that is also the notorious culprit of high or mass mortality in other cultured marine fish including turbot *Psetta maxima* (6) and seabass (3). In addition, *P. dicentrarchi* species can be infected via water to fish (19). Cross-infection between cultured olive flounder and seahorse might be possible in this present study due to the pathogenicity and similarity of species isolated and identified.

This pathogen was identified as *P. dicentrarchi* in the intestines and feces of the seahorses that caused scuticociliatosis through the basis of morphological and molecular examination, therefore, it was concluded that this pathogen causes the mortality of seahorses that was used for public exhibition in the aquarium.

2.5. References

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Table 2.1. Oligonucleotide primers used for PCR and nested PCR in this study

Primer	Nucleotide sequence (5'-3')	Target region	Pathogens
Uni-F	AACCTGGTTGATCCTGCCAG	1757bp, 1760bp	*U. marinum* (1757bp)
Uni-R	TGATCCATCTGCAGGTTCAC		*P. dicentrarchi* (1760bp)
Um-F	CTTCTGTACAGTCTCATTTC	722bp	*U. marinum*
Um-R	AACGCCAATTAAAGATCAAC		
Pd-F	CGGACCGGCTTATAAAACTGG	326bp	*P. dicentrarchi*
Pd-R	GTAACGCCAATTGTGCATCAAC		

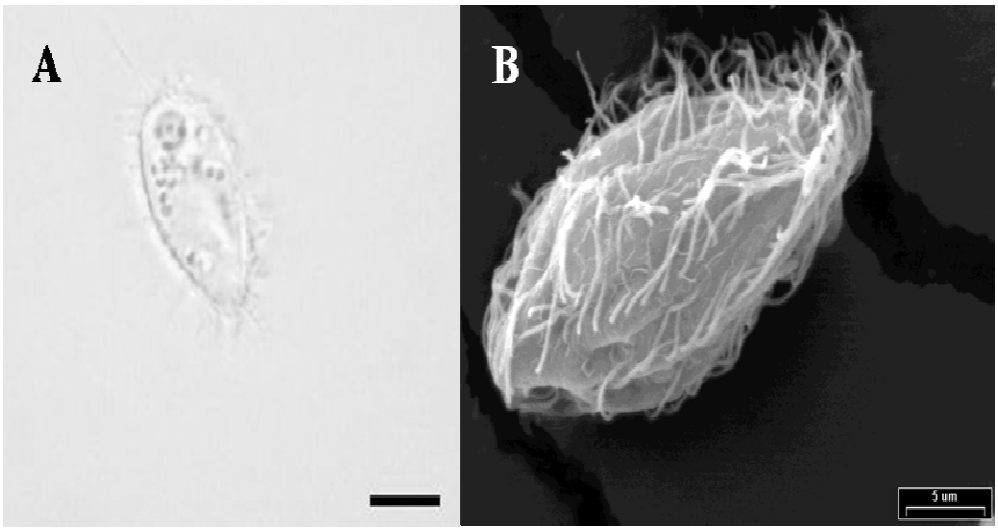

Figure 2.1. (A) Light microscopy (wet mount) of the scuticociliate. Ciliate containing numerous food vacuoles. A contractile vacuole and a long caudal cilium observed at posterior part (1000x). (B) Scanning electron micrograph. Ciliate had an ovoid body shape and covered with cilia over the body. Scale bar = 5 μm.

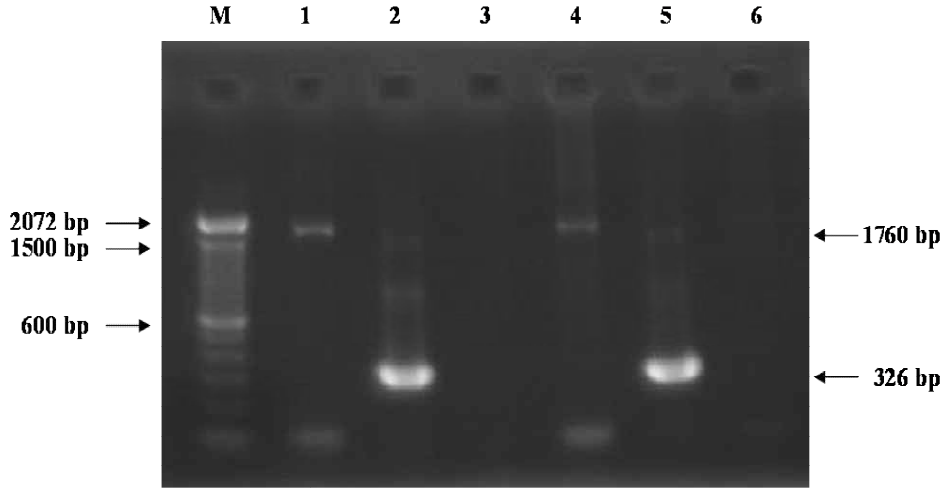

Figure 2.2. Amplification products obtained using the 18S rRNA PCR and nested PCR assays for detection of scuticociliates species in seahorses feces (Lane 1-3) and intestinal contents (Lane 4-6). Lane M: 100 bp DNA ladder; Lane 1, 4: PCR positive for scuticociliate using universal primer (1760 bp); Lane 2, 5: nested PCR positive for *P. dicentrarchi* (326 bp); Lane 3, 6: nested PCR negative for *U. marinum* (722 bp).

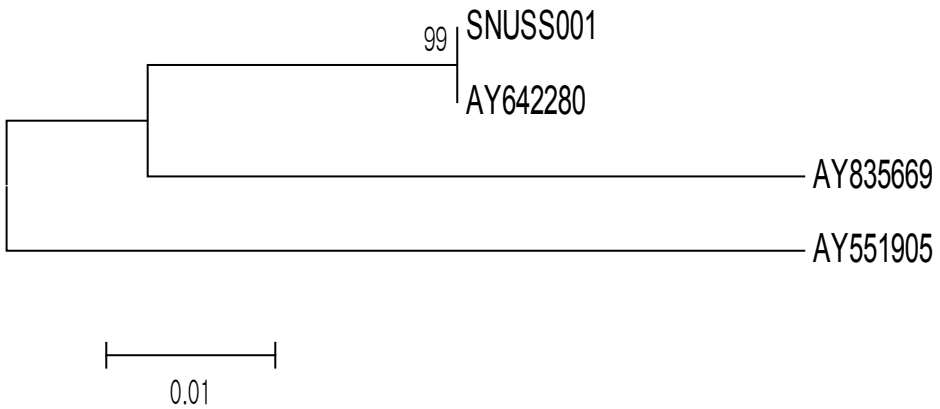

Figure 2.3. Phylogenetic tree based on the nucleotide sequence of small subunit ribosomal RNA of 3 scuticociliate species reported in Korea and 1 isolate (SNUSS001) from seahorses. Nucleotide sequences of known scuticociliates strains were obtained from GeneBank and their accession numbers are as follows: *P. dicentrarchi* (AY642280), *P. persalinus* (AY835669), and *U. marinum* (AY551905).

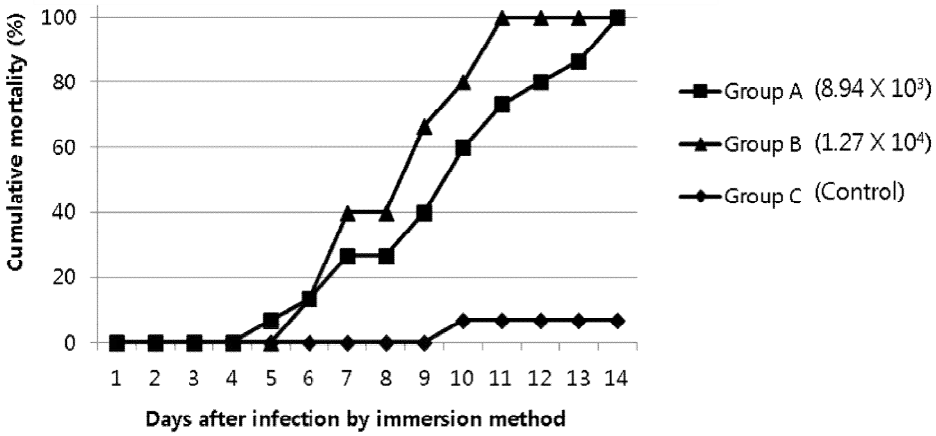

Figure 2.4. Mortality (%) of olive flounder *Paralichthys olivaceus* after immersion exposure to *P. dicentrarchi* SNUSS001. Group A and B were exposed to 8.94×10^3 and 1.27×10^4 of concentration, respectively.

Chapter III

Expression of cathepsin L-like cysteine protease from *Philasterides dicentrarchi*

Abstract

Philasterides dicentrarchi is a causative agent of scuticociliatosis in Olive flounder (*Paralichthys olivaceus*) aquaculture of Korea. In this study, a cDNA encoding cathepsin L-like cysteine protease (PdCtL) from a cDNA library of *P. dicentrarchi* (synonym *Miamiensis avidus*) was identified. To express the PdCtL recombinant protein in heterologous system, 10 codons were redesigned to conform to the standard eukaryotic genetic code using PCR-based site-directed mutagenesis. The synthetic *P. dicentrarchi* procathepsin L (proPdCtL) was expressed at high levels in *E. coli* Rosetta (DE3) pLysS with pPET21a vector, and successfully refolded, purified and activated into a functional and enzymatically active form.The optimal pH for protease activity was found to be 5. Like any typical cysteine protease, the enzyme was inhibited by E64 and leupeptin. Our results suggest that the biochemical characteristics of the recombinant ciliate proPdCtL protein are similar to that of the cathepsin L-like cysteine protease, and that the PCR-based site-direct mutated ciliate gene was successfully expressed in a biochemically active form.

Key words: *Philasterides dicentrarchi*, Site direct mutagenesis, Cathepsin L like protease, *E. coli* expression system, cystein proteases

3.1. Introduction

Several scuticociliate species belonging to the genera *Uronema*, *Miamiensis* and *Philasterides* have recognized as important opportunistic pathogens or environmental scavengers in marine fish. (2, 6, 11, 12, 14, 16, 30, 39, 43). These ciliates have been reported from farmed sea bass (*Dicentrarchus labrax*) in the Mediterranean (11), Olive flounder, *Paralichthys olivaceus* (20, 43) and bluefin tuna *Thunnus maccoyii* (30) and indo-pacific seahorse *Hippocampus kuda* (37). Among the ciliates, three scuticociliate species namely *Pseudocohnilembus persalinus*, *U. marinum* and *P. dicentrarchi* were responsible for scuticociliatosis in cultured olive flounder in Korea (23, 24). Especially, scuticociliatosis caused by *P. dicentrarachi* is recognized as one of the most important parasitological problems affecting cultured olive flounder fish farming industries in Korea (21) and other countries like Galicia (northwest Spain) and Norway (12, 16). The presence of *P. dicentrarachi* in fish tissues has been associated with various pathological changes, including bleeding cutaneous ulcers, dystrophic and necrotic effects in muscle, hypochromic anaemia, and encephalitis associated with softening or liquefaction of brain tissues (21).

Although little is currently known regarding the process by which the *P. dicentrarchi* invades host tissue, as well as the mechanisms by which this parasite evades the host's defense response, it has been theorized that cysteine protease might be involved in the invasion and pathogenicity characteristics of *P. dicentrarchi* (31, 32). However, they have not yet been completely cloned and characterized at the molecular level. Also, the genetic code of ciliates differs from other eukaryotes (28). The standard UAA and UAG stop codons specify glutamine

(Gln, Q) in Scuticociliatida and UGA is the sole stop codon (33). Therefore, the expression of ciliate gene in a heterologous protein expression systems remains rather limited (26).

The parasite cathepsin L-like cysteine protease family is important in parasite development and pathogenesis (8, 13). They also allow the parasite to evade the host's immune system (10) and are virulence factors in some pathogenic organisms (29). The cathepsin L-like cysteine protease of parasites have therefore gained attention as targets for development of new anti-parasitic drugs as well as anti-parasite vaccines (1, 10). In the present paper, cloning and enzymatic characterization of catehpsin L-like protease from *P. dicentrarchi* was described.

3.2. Materials and methods

3.2.1. *Parasites and total RNA isolation*

Ciliates were isolated from olive flounders *P. olivaceus* used the pathogenicity test. *P. dicentrarchi* were cultured in sterile artificial sea water (20‰ salinity) supplemented with 0.1% yeast extract and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Identification was done as previous reported elsewhere (37). Total RNA was extracted using an RNA extraction kit with TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions.

3.2.2. *cDNA synthesis and rapid amplification cDNA ends-PCR*

Gene specific primer (GSP) was designed based on partial region of cathepsin L-like proteinase sequences from *M. avidus* (GenBank accession no. DR981402). To obtain a fulllength copy of *P. dicecntrarchi* Cathepsin L (PdCtL) like protease, 3'

rapid amplification of cDNA ends (RACE) PCR was performed using a SMATerTM RACE cDNA Amplication Kit (Clontech) according to the manufacturer's instructions. The 3' RACE-PCR product was amplified by PCR using PdCtL-specific primer (Sense primer: GSP1, 5-ATG AAA GCC GCT TTA ATA TTA-3). New primers were designed in order to confirm the obtained sequence and to amplify the full-length PdCtL cDNA PCR (Sense primer: GSP2, 5-GAC AAT GTG GAT CTT GCT G-3; Antisense primer: GSP3, 5-CAG CAA GAT CCA CAT TGT C-3; Antisense primer: GSP4, 5- TGT TAC AAA TGA ATA TAT TTT GCT-3).

3.2.3. *Sequence and phylogenetic analysis*

The PCR amplification products were cloned into a plasmid vector using a TOPO cloner PCR cloning kit (Enzynomics, Korea), following the manufacturer's instructions. The cloned products were sequenced using universal primers (M13F: 5-GTA AAA CGA CGG CCA GT-3; M13RpUC: 5-CAG GAA ACA GCT ATG AC-3) and a terminator cycle sequencing kit (Applied Biosystems, USA). Electrophoresis of the sequencing reactions was performed using an automatic DNA analyzer (Applied Biosystems, USA).

Sequencing data were compared with the database using BLAST program. The position of the putative signal peptide cleavage site was predicted using the SignalP 3.0 (4). Alignment of the predicted amino acid sequences of PdCtL and protozoan cathepsin L-like proteases was carried out using the ClustalW (40) and was edited for presentation using the Boxshade 3.21 software. For the phylogenetic tree, amino acid sequences of the complete preproenzyme of PdCtL and other parasite

cathepsin L-like proteases were analyzed phylogenetically using MEGA4 software (38). The tree was constructed using a Neighbor-Joining method with the following settings: 1000 bootstrap replicates and 92,441 seed. Gaps were considered as pairwise deletion. The molecular mass and predicted pI of the deduced protein were determined by the Compute pI/Mw software (5).

3.2.4. *Site-direct mutagenesis of PdCtL*

For the over expression of PdCtL in *E. coli*, mutagenic oligonucleotides were used to convert 10 UAA stop codons in the coding sequence to CAA glutamine codons using the PCR based site directed mutagenesis **(Table 3.1)**. The mutagenesis was modified as previously described (1). First, GSP1-337-338R and GSP4-337-338F were utilized as two primer sets for the conversion of the stop codons at amino acid positions 337 and 338. The first step was carried out in a T-personal 48 thermocycler (Biometra) with the following parameters: an initial denaturation step of 94°C, 3 min; 35 serial cycles of a denaturation step of 94°C, 30 s, annealing at 55°C, 30 s extension at 72°C, 1 min; and final extension step of 72°C, 10 min with GSP1-337-338R and GSP4-337-338F primer sets using i-pfu DNApolymerase PCR preMix (Intronbio, Korea), respectively. The PCR products were purified with an Plus PCR Purification Kit (Nucleogen, Korea). One microlitre of purified PCR products were employed for efficient annealing and extension in the following step. The second step for annealing and extension was performed as follows: annealing at 60°C, 30 s, extension at 72°C, 50 s, and denaturation at 94°C, 30 s, for 20 cycles except for the primers. The resulting product was then used in the third step. The third step was performed for 35 cycles

of denaturation (94°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 50 s), with the GSP1-GSP4 primer set. In this manner, the stop codons 337 and 338 of the *P. dicentrarchi* cathepsin L were converted, and were then usable as the template for the next mutagenesis. The above method, from the first step to third step, was reiterated for the conversion of the remainder of the stop codons in serial order. Finally, this process generated a synthetic allele, which mutated scuticociliate preprocathepsin L.

3.2.5. *Expression, affinity purification and refolding of recombinant PdCtL*

The sequence encoding the predicted proPdCtL was amplified from the obtained plasmid clone by PCR using NdeI-proPdCtL-F (5-CGC CAT ATG ATC TTC ATG ATG AGC AAC AAC CAA AC-3) and XhoI-proPdCtL-R (5-CCG CTC GAG GTA GAG GGG GTA GGC AAC G-3) primers set to which NdeI and XhoI restriction sites had been added to assist cloning. The PCR product was cloned into pPET21aHISTag expression vector and designated pHIST-proPdCtL. The ligation product was used to transform chemically competent *Escherichia coli*, Rosetta (DE3) pLysS (Novagen, USA) and the protein expression was induced by 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 24 h at 37°C. The bacterial lysate was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the expressed fusion protein was identified by Coomassie blue staining and Western blot using anti-6Xhis tag antibody.

The inclusion bodies containing the recombinant protein were isolated from bacterial lysates by centrifugation, and solubilized with 50 mL of denaturation buffer (8 M urea, 20 mM Tris HCl, pH 8.0, 0.5 M NaCl, 5 mM imidazole and 1

mM β-mercaptoethanol) and stirred for 16 hours at room temperature. The recombinant protein present in the suspension was purified by metal-affinity chromatography in Chelating-Sepharose® Fast Flow resin (GE Healthcare, USA), previously charged with 300 mM NiSO4 and equilibrated with denaturation buffer. The column was washed with ten volumes of denaturation buffer (8 M urea, 20 mM Tris HCl, pH 8.0, 0.5 M NaCl, 5 mM imidazole and 1 mM β-mercaptoethanol). This procedure was followed by three additional washings: ten volumes of a buffer (20 mM Tris HCl, 0.5 M NaCl, pH 8.0) containing 6 M urea and 20 mM imidazole in the first wash; ten volumes of the same buffer containing 4 M urea and 40 mM imidazole in the second wash; and ten volumes of the same buffer containing 3 M urea and 60 mM imidazole in the third wash.

The recombinant protein was eluted with five volumes of the elution buffer containing 20 mM Tris HCl, 0.5 M Na Cl (pH 8.0), 3 M urea and 1 M imidazole. Several steps of dialysis were also performed: first against a solution containing 20 mM Tris HCl, 0.5 M NaCl (pH 8.0), 2 M urea, 0.1% glycine, 10 mM EDTA and 0.5 M imidazole; a second step, against a solution containing 20 mM Tris HCl, 0.5 M NaCl, (pH 8.0), 2 M urea, 0.1% glycine and 10 mM EDTA. The last dialysis was against phosphate-buffered saline solution (PBS), 0.1% glycine and 0 M urea. Purified protein samples were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of the purified fusion protein was determined using a BCA kit (Pierce, USA).

3.2.6. *Autoprocessing and assays of enzyme activity and inhibition*

The purified proPdCtL were incubated in 0.1 M sodium acetate buffer (pH 4)

containing 10 mM cysteine with 30 μg/mL of dextran sulphate for 30 minutes and then fluorometric assays of recombinant proPdCtL was carried out as previously described (1). In brief, hydrolysis of substrates containing the 7-amino-4-methyl coumarin (AMC) fluorophore was carried out in microtitre plate format using a Microplate Fluorometer (Packard Co., USA) (excitation wavelength: 355 nm and emission wavelength: 460 nm). The synthetic substrates carbobenzoxy-phenylalanyl-arginyl-AMC (Z-Phe-Arg-AMC) (Sigma) was used as substrates for activity assay, or for inhibitor assay of cathepsin L protease. Assays were performed at 37°C in a total volume of 100 μl [100 mM sodium acetate/pH 5 (or at other pH as required) and 0.2 mM DTT]. Substrates were added to a final concentration of 0.1 mM, or other concentration as required. The effects of various inhibitors (Pepstatin A, PMSF, EDTA, 1, 10-Phenanthroline, E64, Leupeptin) were evaluated by measuring the residual enzyme activity using Z-Phe-Arg-AMC as previously described (25). All assays were carried out in triplicate.

3.3. Results

3.3.1. Molecular cloning of a cDNA encoding a cysteine protease from P. dicentrarchi

The 1182 bp PdCtL cDNA contained an open reading frame of 1038 bp from the first ATG start codon through the TGA stop codon, flanked by 144 bp of the 3'-untranslated region (UTR). The PdCtL sequence was deposited in the GenBank database under the accession no. JQ673412. The PdCtL harbored a 16-residue putative signal peptide, a 111-residue propeptide, and the 218-residue mature enzyme. The molecular weights of the putative prepro-, pro and mature-forms of

PdCtL were as follows: 37,330, 35,761, and 22,942 Da, respectively

3.3.2. Characterization of the deduced amino acid sequence of PdCtL

The prodomain contained an ERFNIN like motif and a GNFD motif, both typical of cathepsin L-like cysteine proteinases **(Figure 3.3)**. The theoretical cleavage site of the prodomain between the asparatic acid at position 127 and the leucine at position 128 was determined by homology comparison. The proline residue (position 129) in the N-terminal domain of the catalytic region (position 2 in the mature protein), which may function to prevent N-terminal proteolysis, was conserved. PdCtL contains the characteristic cysteine, histidine, and asparagine active site residues. A phylogenetic analysis of 30 cathepsin L-like proteases of parasites has revealed that PdCtL is more closely related to those of ciliophora **(Figure 3.2)**. PdCtL exhibited a high degree of identity with *U. marinum* cathepsin L-like protease (41%).

3.3.3. Site-directed mutagenesis, heterologous expression and in vitro refolding of proPdCtL

In PdCtL contains 10 UAA universal stop condons, which code for Glutamine (Q) in *P. dicentrarchi*. The mutagenic oligonucleotides **(Table 3.1)** were used to convert 10 UAA stop codons in the coding sequence to CAA glutamine codons by PCR-based site-directed mutagenesis. The expected band of PdCtL protein (35.76 kDa) was observed, by 12% SDS- PAGE, in bacterial cell lysates from induced cultures **(Figure 3.4, lane 1, 2)**. Based on the western blotting, the recombinant proPdCtL was present in the pellet of bacterial cell lysates in an insoluble form as

inclusion bodies **(Figure 3.4, lane 3, 4)**. Recombinant proteins present in inclusion bodies were isolated, solubilized and purified **(Figure 3.4, lane 5)**. The purified recombinant protein yield was approximately 2.4 mg/ml and activated by sodium acetate buffer (pH 4) containing cysteine and dextran sulphate.

3.3.4. Enzymatic characterization of recombinant PdCtL

The purified recombinant PdCtL was shown to be enzymatically active against the substrate (Z-Phe-Arg-AMC) at pH 5 or 6.0 **(Figure 3.5)**. The proteolytic activity was completely inhibited by addition of the cysteine protease specific inhibitors E64 and leupeptin. The effect of inhibitors on the protease activity of PdCtL using Z-Phe-Arg-AMC as the substrate is shown in **Table 3.2.**

3.4. Discussion

The gene coding for a cathepsin L-like cysteine proteinase of *P. dicentrarchi* was cloned and sequenced. The deduced amino acid sequence of the cloned gene belongs to the C1 peptidase (papain superfamily) protease clan. Sequence comparison with other cathepsin L proteases suggests that PdCtL is a typical cathepsin L-like cysteine protease with a short putative N-terminal hydrophobic signal peptide, a pro-region domain, and a mature catalytic domain **(Figure 3.1)**. The mature enzyme contains the cysteine protease catalytic triad, Cys152, His290, and Asn310 as well as the sixe cysteine residues known to be involved in the formation of the disulphide bond (Cys149-190, Cys183-225, and Cys284-334). The Cys152 is embedded within a highly conserved peptide sequence, CGSCWAFS. The His290 is adjacent to small amino acid residues, such as glycine, and Asn310

is a portion of the Asn-Ser-Trp motif **(Figure 3.1)**. Also, two potential N-glycosylation sites were identified (positions 23 and 228), one of which was located in the proregion (Asn-Gln-Thr) and the other in the mature region (Asn-Gln-Ser). A potential N-glycosylation site is present in the PdCtL and other ciliates cathepsins L just prior to the putative proregion cleavage site or in the mature region (1).

It is usual for the papain superfamily cysteine proteases, the identity among these enzymes is lower in the proregion than in the mature domain. When the amino acid sequence was aligned with cathepsin L like cysteine proteinases of various ciliates, PdCtL was homologous to other cathepsins L in the proximity of the core catalytic triad residues but showed less homology in the proregion **(Figure 3.3)**. Cleavage of the PdCtL proprotein would result in a 218 amino acid mature protein with a molecular mass of approximately 23 kDa. Charateristic for the propeptide region of non-cathepsin B papain-like proteases is the ERFNIN motif that is suggested to play a role in the inhibition of proteolytic activity (22). This ERFNIN motif is found in cathepsin L of other ciliates (1, 22). The GNFD, which may be involved in pH-dependent intramolecular processing (42), is also found in the pro-region of PdCtL. This motifis also conserved although asparatic acid is substituted by alanine in PdCtL **(Figure 3.3)**. Phylogenetic analysis showed that PdCtL is more closely related to the cathepsin L of ciliates than to other parasites cathepsin L **(Figure 3.2)**. In conclusion, the molecular characteristics of the *P. dicentrarchi* protease sequence fully supported its classification as a cathepsin L-like protease.

An interesting feature of the ciliate is their use of alternative nuclear genetic

codes (28). Such codons result in premature polypeptide chain termination when corresponding transgenes are expressed as recombinant proteins in bacteria, yeast, or insect host cell lines. In a previous study, PCR-based site-directed mutagenesis could be used in the conversion of UAA and UAG triplets to CAA and CAG triplets, respectively (27). Likewise, PdCtL contains 10 UAA universal stop condons, which code for Glutamine (Q) in *P. dicentrarchi*. For the overexpression of PdCtL in *E. coli,* mutagenic oligonucleotides (Table 1) were used to convert 10 UAA stop codons in the coding sequence to CAA glutamine codon by PCR based site directed mutagenesis. Previous studies have indicated that the bacterial expression of the papain like cysteine proteases is significantly impaired in the presence of the hydrophobic presequence. The presequence is responsible for the targeting of the protein into specific cellular compartments including the lysosome-like digestive vacuoles, or to the cytoplasmic membrane, whereas the prosequence is required for appropriate folding and for the maintenance of the enzyme in an inactive conformation (15). Therefore, only the part of the gene for PdCtL that encodes for the proenzyme was cloned into the *E. coli* expression vector, pPET21a. The cell extracts from proPdCtL transformed *E. coli* cultures induced with IPTG, showed a major protein band of about 36 kDa in a SDS-PAGE analysis **(Figure 3.4, lane 2)** which is consistent with the predicted size for the fusion protein. The fusion proPdCtL protein was solubilized in 8M urea, and refolded as described above.

Previous studies have shown that recombinant cyteine proteases are able to undergo further maturation by autocatalytical cleavage which could be induced by changes of pH and added chemicals such as dextran sulphate (19, 36). Proteolytic activity of recombinant proPdCtLwas determined using fluorogenic substrate.

Using the synthetic substrate, Z-Phe-Arg-AMC, the recombinant proPdCtL showed optimal activity pH 5 **(Figure 3.5)**. Reagents known to specifically inhibit serine, aspartic, or metallo- proteases exerted little detectable influences on the activity of PdCtL. By way of contrast, the enzymatic activity of PdCtL was greatly reduced or blocked completely with all of the tested cysteine protease inhibitors, including E64 and leupeptin. Leupeptin was the most potent inhibitor of those tested, with 0.01mM of this inhibitor evidencing 100% inhibition. In the presence of 0.1mM E64, a cysteine protease-specific inhibitor, 98% inhibition was observed **(Table 3.2)**. These sensitivities were similar to those of the cathepsin L derived from *U. marinuum* L (1). These results support the conclusion that proPdCtL exhibits cysteine protease activity.

Although several attempts have been made to treat the disease with various chemotherapeutants (17, 34), due to the high virulence and endoparasitic habitat of the ciliate, there are at present no available chemotherapeutants especially for systemic infections. In addition some agents (such as niclosamide and oxyclozanide) which have showed effective *P. dicentrarchi* is toxic to fish (9) and formalin (saturated by 37% formaldehyde) is known carcinogen (3, 7). Thus, vaccination is an attractive alternative to chemotherapeutic treatments for effective prevention of this disease. Iglesias et al. (18) reported that immunization of turbot with *P. dicentrarchi* lysate plus adjuvant or with formalin fixed ciliates induced synthesis of agglutinating antibodies and conferred a degree of protection against challenge infection, suggesting the usefulness of vaccine. In addition, based on previous studies, the highest level of protection was achieved in animals immunized with the purified protein from *E. coli*, suggesting that even the inactive

form of the protein can provide significant epitopes that contribute for protection (35, 41). Therefore, the availability of synthetic PdCtL might facilitate studies of its potential as a candidate for the development of novel immunoprophylatic or chemotherapeutic modalities.

3.5. References

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Table 3.1. Oligonucleotide. primers used for site-direct mutagenesis of PdCtL

Primers	Mutation position	Sequences (5'-3')
24F 24R	Glutamine, 24	AGCAACAACCAAACTACTTTCTTG CAAGAAAGTAGTTTGGTTGTTGCT
44F 44R	Glutamine, 44	AACCTACGTTCAATGGAAATCC GGATTTCCATTGAACGTAGGTT
73F 73R	Glutamine, 73	CTATATTCAACAATTCAACTCTG CAGAGTTGAATTGTTGAATATAG
168F 169R	Glutamine, 168	TACGCCATTCAATCTGGATC GATCCAGATTGAATGGCGTA
203F 203R	Glutamine, 203	CCGCTTATGCTTACACCCAA TTGGGTGTAAGCATAAGCGG
239F 239R	Glutamine, 239	TCCGGACAAGTCAAAGTTGC GCAACTTTGACTTGTCCGGA
252-256F 252-256R	Glutamine, 252, 256	CATCCAAAAAGCTGTTCAAAAC GTTTTGAACAGCTTTTTGGATG
337-338F 337-338R	Glutamine, 337, 338	GGAGTTCAACAATACGTTGC GCAACGTATTGTTGAACTCC

The substituted amino acids and their positions are shown. Mutagenic bases which differed from the wild-type sequence are underlined and the codon sequences which yield the mutation in the amino acid sequence are Glutamine.

Table 3.2. Inhibition assay of proPdCtL by various inhibitors

Inhibitors	Specificity	Concentrartion (mM)	Enzyme activity (% control)*
E64	Cystein proteases	0.1	2±3
Leupeptin	Cystein proteases and trypsin like serine proteases	0.01	0±3
PMSF	Serineprotease	1	98±4
Pepstatin	Asparatic proteases	0.1	97±4
1,10-Phenanthroline	Metalloproteases	1	93±1
EDTA	Metalloproteases	1	95±4

*Enzyme activity was estimated using N-carbobenzoxy-phenylalanyl-arginyl-AMC (Z-Arg-Arg-AMC) as a substrate. The results are mean values from triplicate experiments±Standard deviation.

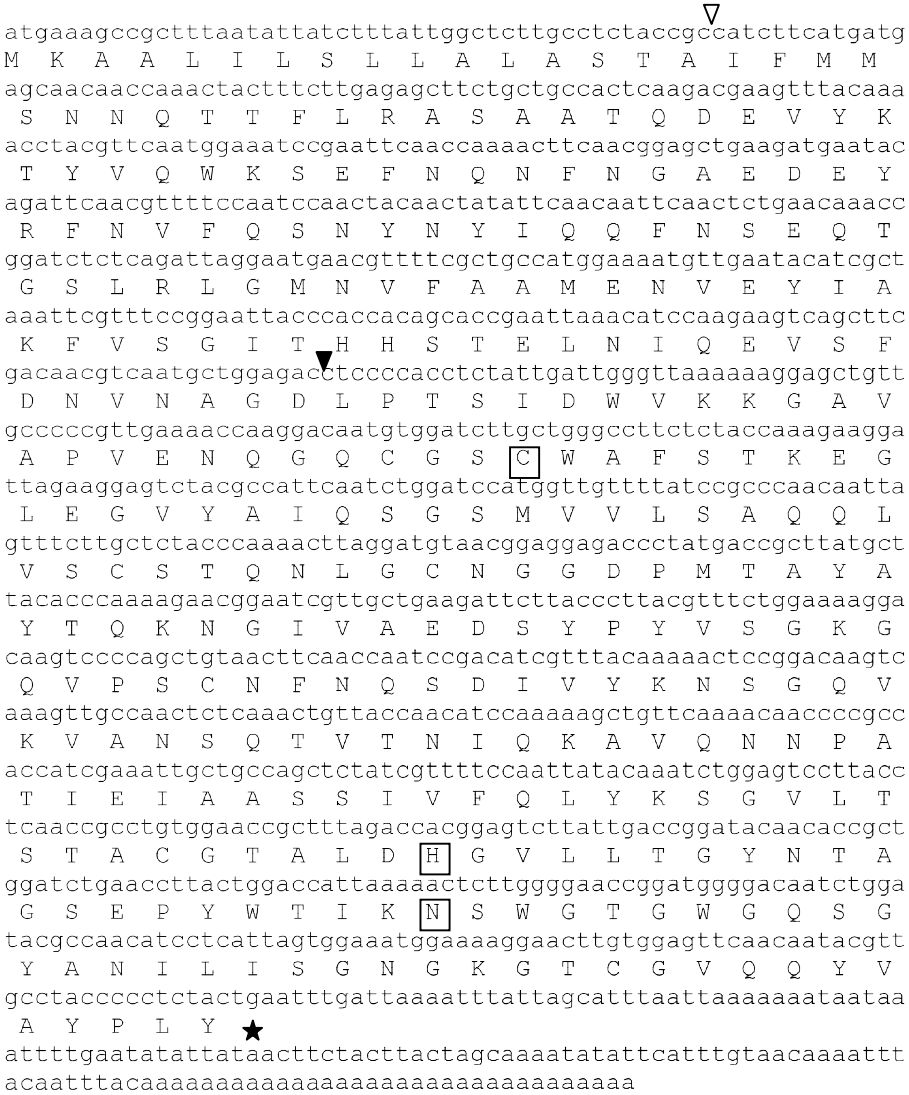

Figure 3.1. Nucleotide and deduced amino acid sequences of a cDNA clone encoding PdCtL (GenBank accession no. JQ673412). The open and shaded arrowheads in the amino acid sequences indicate the putative signal peptide and the pro-peptides of PdCtL, respectively. The active site cysteine, histidine and asparagines residues are shown in open boxes. The asterisk (★) at the end of the amino acid sequences indicates the stop codon. The Poly (A) tail is underlined. The sequence took into consideration that in *P. dicentrarchi*, as in several ciliates, TAA and TAG code for Gln.

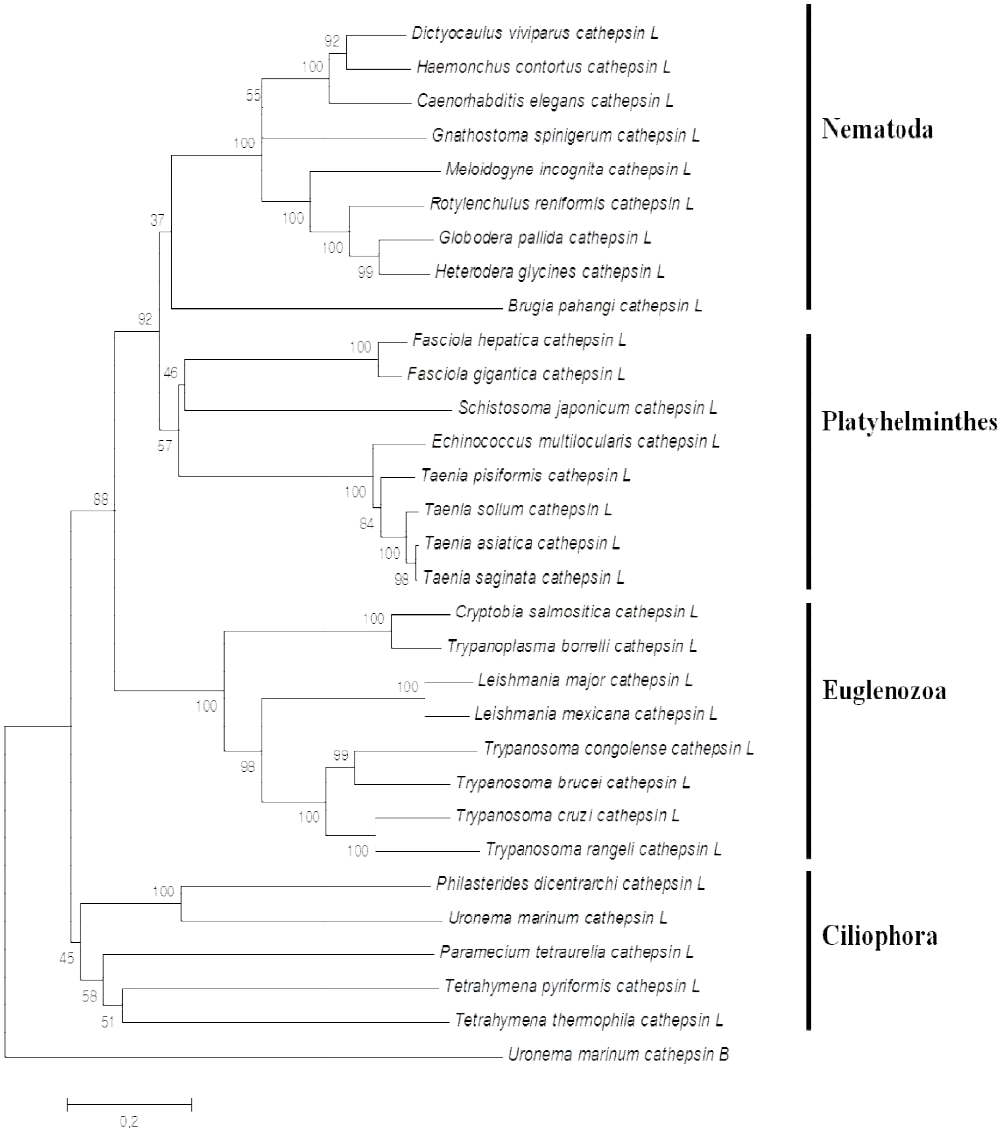

Figure 3.2. Phylogram of the predicted protein sequence of PdCtL and selected sequences of other eukaryote cathepsin L proteases. The neighbor-joining tree was constructed with the MEGA4 software. The scale bar represents 0.2 amino acid substitutions per site. The cathepsinL proteases aligned with *P. dicentrarchi* cathepsin L (JR673412) were from *Caenorhabditis elegans* cathepsin L (NP507199), *Dictyocaulus viviparus* cathepsin L (AAK77918), *Haemonchus contortus* cathepsin L (AAF86584), *Meloidogyne incognita* cathepsin L (CAD89795), *Rotylenchulus reniformis* cathepsin L (AAY45870), *Heterodera glycines* cathepsin L (CAA70693), *Globodera pallida* cathepsin L (AAY46196),

Gnathostoma spinigerum cathepsin L (ABY28387), *Uronema marinum* cathepsin L (AAX51228), *Taenia solium* cathepsin L (AAS00027), *Fasciola hepatica* cathepsin L (AAF76330), *Fasciola gigantica* cathepsin L (AAF44676), *Brugia pahangi* cathepsin L (O17473), *Brugia malayi* cathepsin L (BAD11762), *Tetrahymena pyriformis* cathepsin L (BAA31161), *Cryptobia salmositica* cathepsin L (AAU14993), *Leishmania major* cathepsin L (AAB48120), *Leishmania mexicana* cathepsin L (CAA78443), *Trypanosoma brucei* cathepsin L (CAA38238), *Trypanosoma congolense* cathepsin L (CAA81061), *Trypanosoma cruzi* cathepsin L (AAA30181), *Paramecium tetraurelia* cathepsin L (CAA62869), *Tetrahymena thermophila* cathepsin L (AAA30114), *Trypanosoma rangeli* cathepsin L (2117247A), *Trypanoplasma borrelli* cathepsin L (ABQ23398), *Uronema marinum* cathepsin B (AAR19103), *Taenia pisiformis* cathepsin L (AEG19548), *Taenia asiatica* cathepsin L (BAH03397), *Taenia saginata* cathepsin L (BAH03396), *Echinococcus multilocularis* cathepsin L (BAF02517), *Clonorchis sinensis* cathepsin L (GAA56666), *Schistosoma japonicum* cathepsin L (CAX72171).

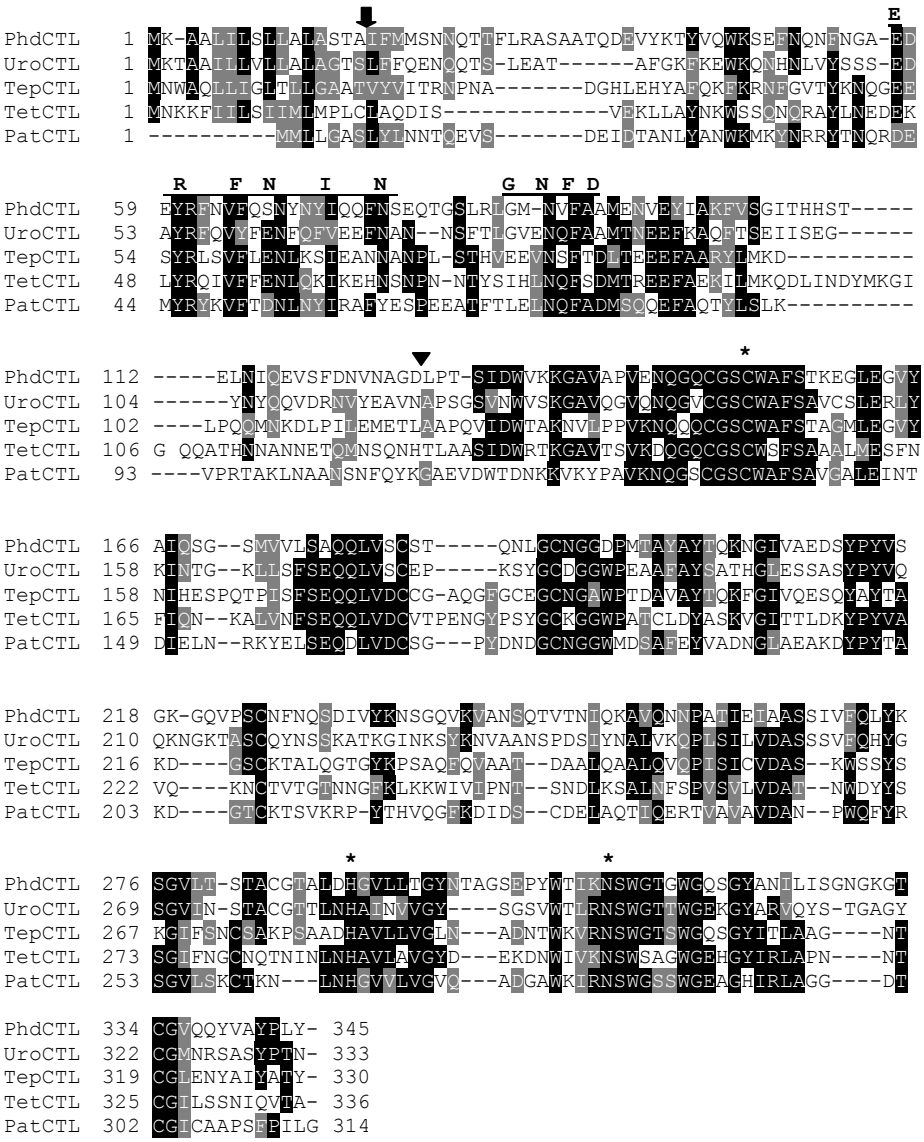

Figure 3.3. Alignment of the predicted amino acid sequence of *P. dicentrarchi* cathepsin L with those of other ciliates cathepsin L. Identical amino acid residues are darkly shaded, similar amino acids are lightly shaded, unrelated residues have a white background, and the amino acid numbers are shown on the left. The catalytic triad residues are marked with the asterisks, and the gaps (-) are introduced to maximize alignment. Closed arrow and arrowheads indicate putative cleavage sites for the signal sequence and propeptide of *P. dicentrarchi* cathepsin L, respectively. The ERFNIN and the intramolecular processing GNFD motifs are shown by underline. The cathepsin L proteases aligned with *P. dicentrarchi* (PhdCTL,

JR673412) are from *Uronema marinum* (UroCTL, AAX51228), *Tetrahymena pyriformis* (TepCTL, BAA31161), *Tetrahymena thermophila* (TetCTL, AAA30114), *Paramecium tetraurelia* (PatCTL, CAA62869).

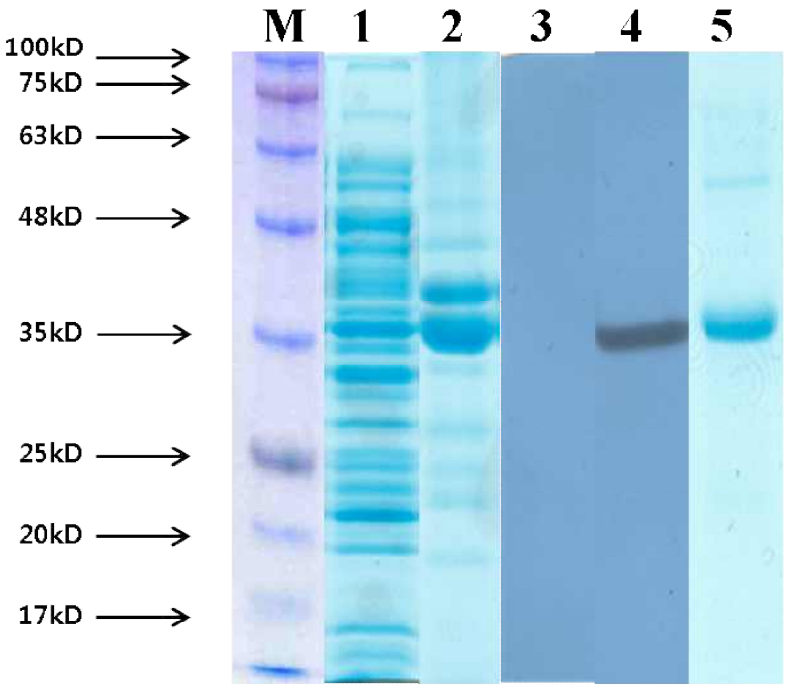

Figure 3.4. Analysis of the recombinant PdCtLexpressed from IPTG-induced *E. coli* Rosetta (DE3) pLysS by 12% SDS-PAGE. Bands were visualized by coomassie staining. Lane M: molecular mass protein markers; lane 1: supernatant after centrifugation (soluble fraction); lane 2: inclusion body pellet after centrifugation (insoluble fraction); lane 3: western blotting of the reactivity of mouse anti-his-proPdCtL in supernatant; lane 4: western blotting of the reactivity of mouse anti-his-proPdCtL in inclusion body pellet; lane 5: protein purified from insoluble fraction.

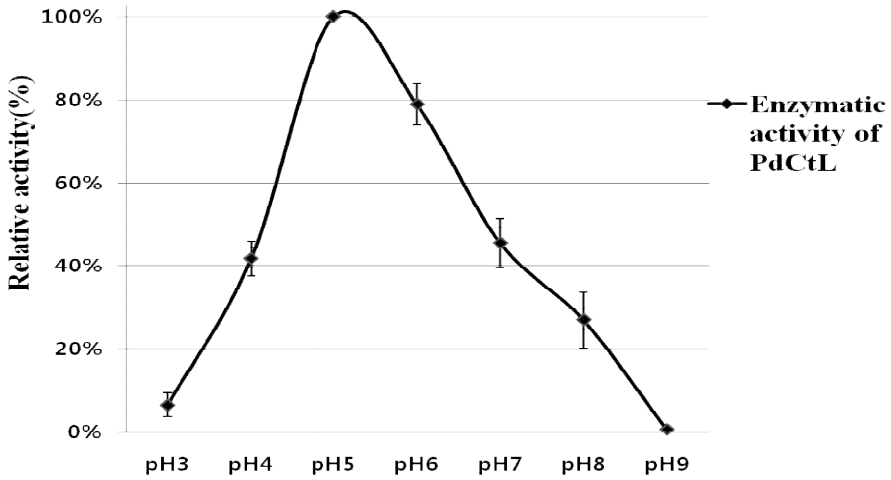

Figure 3.5. pH activity profile of recombinant PdCtL. The activity of PdCtL against Z-Phe-Arg-AMC substrate was measured at different pHs. The activities are shown relative to that at pH 5 being 100%. The results are mean values from triplicate experiments±Standard deviation.

GENERAL CONCLUSION

The infections of myxosporeans and scuticociliates have been known to hazardous disease in aquatic animals. In this study, the surgery was carried out for the treatment and the cysteine protease gene was expressed for the development of vaccine. In addition, the gene of parasites was analyzed to investigate the phylogenetic characteristics.

Koi carp infected *Thelohanellus kitauei* was treated by the surgery and the 18S rRNA gene of *T. kitauei* was amplified for the identification and phylogenetic analysis. The sequence of *T. kitauei* was compared with other *Thelohanellus* sp., *Myxobolus* sp. and *Heneguya* sp. The result revealed that infection site and host specificity (subfamily level) are reflected in the genetic relationships among *Thelohanellus* species. Also phylogenetic analysis indicated the co-speciation of the parasite with the host fish after host switch. Based on the phylogenetic analysis, the characteristics of *T. kitauei* derived from the ancient non specific organ tropism Myxobolidae that was able to infect Cyprinidae.

A number of ciliates were isolated from seahorses had been reared in aquarium. The ciliates were identified by morphological and molecular methods as *Philasterides dicentrarchi*. This pathogen was injected to olive flounder for the pathogenicity test. The result suggested the possibility of the cross-infection between seahorse and olive flounder. To identify the possibility vaccine candidate, the cathepsin L-like gene of *P. dicentrarchi* was cloned and redesigned for the expression in *E. coli* system. The cathepsin L-like gene was expressed and processed to functional active form. The recombinant protease exhibited enzymatic

activity at optimal pH and inhibited by cystein proteases. These results indicated that the biochemical characteristics of the recombinant cathepsin L like protease are similar to other cathepsin L-like cysteine protease, and that the mutated ciliate gene was successfully expressed in a biochemically active form.

Based on these results, the surgery could be considered as alternative treatment against *T. kitauei* infection and the recombinant protease will be used to develop the vaccine against *P. dicentrarchi* infection. Genetic informations of *T. kitauei* and *P. dicentrarhi* will give information to research about evolution, speciation and identification of the parasites.

국문초록

장포자충(*Thelohanellus kitauei*)과 고병원성 스쿠치카증(*Philasterides dicentrarchi*)의 치료, 예방 및 계통분류학적 특성에 관한 연구

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수의학에서 다루는 동물은 반려동물과 경제 산업동물로 나눌 수 있다. 이번 연구에서는 수생 반려-산업동물에서 발생하는 기생충성 질환의 치료방법, 원인체 분석, 역학적 추론 및 예방의학등을 다루고 있다.

첫째, 비단잉어에 감염한 장포자충의 치료와 계통분류학적 분석. 항문에 cyst 소견을 보이는 비단 잉어의 진단을 위하여 조직학적 생검이 실시 되었다. 그 결과 cyst내에 많은수의 포자를 관찰할 수 있었으며, 포자의 형태학적 특징 및 plasmodia의 발생 위치를 근거로 *Thelohanellus kitauei*에 의한 감염으로 진단하였다. 본 연구에서는 외과적 수술을 실시하여 cyst를 제거하였고, 수술후 진정과 2차 감염 예방을 위하여 저농도의 MS222와 항생제 처치를 시행하였다. 삼개월 후 수술 자리는 완전히 회복 되었으며 수술후 14개월간 임상적으로 정상적인 상태를 유지하였다. 이러한 수술학적 치료 성공은 외과적 수술을 통한 *T. kitauei* 감염 치료로

서 가능성을 보여주었다. 또한 fresh mount와 주사전자현미경 (SEM)을 이용하여 밝혀낸 형태학적 특징은 이후 *T. kitauei*의 동정을 위한 기초 자료로서의 활용할 수 있을 것이다. *T. kitauei*의 분자학적인 동정과 계통분류학적 특징 조사를 위하여 18S rRNA gene을 nested PCR을 통하여 증폭하였으며 다른 *Thelohanellus* 종, *Myxobolus* 종, 그리고 *Henneguya* 종의 18S rRNA gene과 비교하였다. 연구 결과 *T. kitauei*는 장에 감염하는 다른 *Thelohanellus* 종 (*T. hovorkai*)과 가장 근연의 관계임을 나타내었고, 계통분류학적 조사에서 *T. kitauei*는 잉어아과 어류에 감염하는 다른 *Thelohanellus* 종과 군을 이루었다. 이번 연구결과는 *Thelohanellus* 종들 사이에서 숙주특이성(아과 수준)과 감염부위에 대한 유전학적 관계가 있음을 제시하였다. 또한 *Myxobolus* 종과 *Henneguya* 종을 포함한 계통분류학적 분석 결과에서는 포자충과 (Myxobolidae)의 숙주 전환이 어류의 과 수준 (family level)에서 일어나고 이후 숙주와 기생충이 공진화되어 왔음을 나타내었다. 특히 잉어과에 감염하는 포자충과는 특이 감염부위 친화성 (SIT)과 비특이 감염부위 친화성 (NSIT)을 나타내는 것으로 나누어졌으며, *T. kitauei*의 감염부위 친화성은 고대의 잉어과에 감염하는 포자충 중에서 비특이 감염부위 친화성을 나타내는 포자충에서 기원하였음을 알 수 있었다.

둘째, 해마에 감염한 스쿠치카증의 원인체 동정 및 병원성 실험. 해마의 폐사가 지속되고 있는 수족관에서 해마 5마리의 배설물과 장내용물의 현미경학적 조사결과 많은 수의 스쿠치카섬모충이 발견되었다. 본 연구

에서는 wet mount, SEM, PCR 그리고 sequencing을 통하여 원인체가 *Philasterides dicentrarchi* 임을 동정하였고, 넙치에서의 병원성 검사를 통하여 교차 감염의 가능성을 제시하였다.

셋째, 스쿠치카증 백신 개발을 위한 선행 연구. *P. dicentrarchi* 는 한국의 넙치양식에 막대한 피해를 주는 스쿠치카증의 원인체로서 보고되어 왔으며, 효과적인 치료제의 부재로 인하여 백신이 개발이 시급한 실정이다. 백신 후보로서의 가능성을 확인하기 위하여 *P. dicentrarchi*의 cDNA library에서 cathepsin L- like cysein protease (PdCtL)을 동정하였고 10개의 TAA stop codon을 CAA 코돈으로 site direct mutation 시킨후 *E. coli* system에서 고농도로 발현시켰다. 발현된 재조합 단백질은 refolding 과정을 거친후 정제하였고, 활성화 과정 이후 효소로서의 기능이 측정되었다. 적정 pH의 존재와 cysteine protease inhibitor에 의해서 활성이 억제되는 점은 다른 생물에서 발현되는 cathepsin L- like cystein protease 와 생화학적으로 유사함을 나타내었고, 이는 proPdCtL에서 성공적으로 변이가 일어났으며 생화학적으로 활성이 있는 형태로의 재조합이 성공한것으로 사료된다.

이번 연구 결과들을 통하여 기생충성 질병 치료에 대한 인식을 전환할 수 있었으며, 기생충의 진화적 특성을 유전학적으로 규명하였고, 기생충의 숙주 교차 감염 가능성 제시하였으며 또 한편으로 기존의 기생충 백신 target과 다른 target 부위를 발굴하였다. 수생동물에서 발생하는 기생충성 질병에 대하여 수의학적 관점으로 접근, 분석하여 다양한 연구를 시행한 점은 이후 다른 질병 치료 및 예방에 다양한 관점을 제시할 수

있을 것으로 사료된다.

Key words: *Thelohanellus kitauei,* 외과수술, 계통분류학적 특성, *Philasterides dicentrarchi*, 교차 감염, 카뎁신 L like 시스테인 프로테아제

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2. Jee Eun Han, Ji Hyung Kim, Casiano Choresca Jr., **Sang Phil Shin**, Jin Woo Jun, Se Chang Park: Multilocus Sequence Typing of *Riemerella anatipestifer* Isolates from Ducklings with Tremor in South Korea. 2012 Korean Society of Veterinary Science Conference and General Meeting, Korea (Seoul) Oct., 2012.
3. Jee Eun Han, Ji Hyung Kim, Casiano Choresca Jr., **Sang Phil Shin**, Jin Woo Jun, Se Chang Park: Isolation of IncQ-type plasmid carrying the quinolone resistance (*qnrS2*) gene from *Aeromonas hydrophila*. 2012 Korean Society of Veterinary Science Conference and General Meeting, Korea (Seoul) Oct., 2012.
4. Jee Eun Han, Ji Hyung Kim, Casiano Choresca Jr., **Sang Phil Shin**, Jin Woo Jun, Se Chang Park: Two Small ColE-Type Plasmid in *Aeromonas* spp. Carrying Quinolone Resistance (*qnrS2*) Gene. 2012 Korean Society of Veterinary Science Conference and General Meeting, Korea (Seoul) Oct., 2012.
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6. Kim Mun Sup, **Sang Phil Shin**, Jee Eun Han, Ji Hyung Kim, Casiano Choresca Jr., Jin Woo Jun, Se Chang Park: Antimicrobial effect of hypochlorous acid on pathogenic microorganisms. 2012 Korean Society of Veterinary Science Conference and General Meeting, Korea (Seoul) Oct., 2012.

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2013 관악산 끝자락에서

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