CHAPTER I

INTRODUCTION

Genus *Pythium*, inhabits in aquatic and moist soil environment, is often responsible for serious diseases in plants (1). Based on its general morphology and lifestyle, this group was traditionally classified as fungi (2). However, reclassification was conducted, based on the genetic information. It is, therefore, placed in the Kingdom Stramenopila which is related to green algae and diatoms (3). Up to now, only one species of this Genus *Pythium*, *Pythium insidiosum* (*P. insidiosum*) has been reported as pathogen for mammals. The disease caused by this pathogen, is called "Pythiosis".

Pythiosis or Pythium insidiosi is an emerging/re-emerging infectious disease of human and mammals that are seen with increasing frequency in tropical, subtropical and temperate areas worldwide (4-6). Clinical manifestations of the pythiosis include cutaneous/subcutaneous (7), ocular (8-9), vascular and disseminated forms (10-11). Animal pythiosis has been found in several countries, such as Australia, Brazil, Costa Rica, Japan, Mali, New Zealand, New Guinea, South Korea, Venezuela, and the United State of America (USA) (4, 12-14), but so far there is no report from Thailand.

The first human pythiosis was reported from Thailand in 1985 (6). The review literature on the 18 year-epidemiology of human pythiosis in Thailand from 1985 to 2003 was reported. There were 100 cases, approximately from 9 tertiary care hospitals. The human pythiosis is seen in a nationwide scale with 59% of vascular

type, 33% ocular type and 5%, 3% of cutaneous/subcutaneous and disseminated type, respectively (15). These records indicated that the highest incidence of human pythiosis in the world is in Thailand, whereas only few cases were documented in other countries, such as in the state of Texas and Tennessee in USA, Australia, India, New Zealand, and Brazil (5, 10). Most of the vascular and disseminated pythiosis patients seriously suffered from the disease and often died in short period if the diagnosis and the appropriate treatment were not given in time (16). It is interesting that all the patients with vascular feature were associated with hematological disorders, such as thalassemia, leukemia and paroxysmal nocturnal hemoglobinuria (PNH) as an underlying disease (17-19). In contrast, ocular infected cases are usually found in apparently healthy individuals (8, 15). Similarly, the ocular pythiosis patients need early diagnosis and treatment to save their eye(s).

The clinical data and pathogenesis of *P. insidiosum* and the disease it causes are similar to other mycosis such as; basidiobolomycosis and conidiobolomycosis, leading to overlooking, misdiagnosis, delay and abuse treatment (20). Moreover, *P. insidiosum* is not fungi (aquatic parafungus); antifungal drugs are not effective in treatment of this disease (21-22). Therefore, early and accurate diagnosis is indispensable for complete cure of pythiosis. Diagnosis with high specificity and sensitivity such as serological diagnosis, classical diagnosis, and molecular technique have been reported (23-27). In addition to treatment of pythiosis, the combination of treatment; surgery, drugs: terbinafine/itraconazole, and immunotherapy using *P. insidiosum* antigen (PIA), is a common choice for vascular and ocular type (10, 28).

The information on this disease has been distributed to alert some physicians, resulting in more recognized cases. The data from Mycology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, indicated that at least 3 – 4 cases/ year of ocular and vascular human pythiosis are recorded. In fact, the numbers of cases are still underestimated. Even though this disease can be life-threatening, only limited information, such as pathogenesis, virulence factors, and treatments have been revealed. Fortunately, whole genome sequences of the closed related Oomycetous group, genus *Phytophthora* and genus *Hyaloperonospora* have been completed recently (29). Comparing to the data of *P. insidiosum*, mostly the studies have been focused on phylogenetic relationship, diagnosis, epidemiology, antigenic profiles, antimicrobial drug susceptibility test, and a few of immunotherapeutic treatment (15, 25-26, 30-36). This information is highly essential elements for this study.

There were the molecular epidemiological studies of *P. insidiosum* isolates from different regions of the world using internal transcribed spacer (ITS) and intergenic spacer (IGS) sequences. Three clades, I, II, and III based on the geographical differences were demonstrated. The clade I is composed of the isolates from USA, Central and South America, clade II is composed of *P. insidiosum* from Australia and Asia, and clade III was from USA and Thailand (26, 31).

It is already known that the ability of pathogenic microorganism to grow at 37°C is the prerequisite essential factor to cause the disease in hosts, whereas the failure to grow at 37°C is predictive indicator of attenuated virulence (37). This fact should be able to extend to *P. insidiosum*. For the life cycle, the natural habitat of

P. insidiosum is in aquatic reservoir with the temperature in the range of 25-30°C, and its parasitic phase in mammals at approximately 37°C. Moreover, previous studies demonstrated the significant rapid growth of *P. insidiosum* at 37°C than at 25°C in Cornmeal agar (CMA). They also found that the turgor pressure of the apices hyphae *in vitro* at 37°C was higher than at 25°C (38-39). Our preliminary experiment showed that the growth of the first isolate of *P. insidiosum* from human tissue biopsy at 37°C was significantly faster than when incubated at 27°C. If the virulence is related to the ability to grow at 37°C, then all clinical and some potentially virulent environmental *P. insidiosum* will have the ability to survive in the expected temperature in order to express possible characteristics.

With the advance in technology of molecularbiology, the methods to screen any candidate genes from the unknown sequence targets are available (40-41). These methods are Representational Differential Analysis (RDA), Subtractive Hybridization (SH) and Suppression Subtractive Hybridization (SSH) (42). Currently, suppression subtractive hybridization (SSH) has come to be widely employed to investigate at the transcriptomic level in both prokaryote and eukaryote (43-52).

A PCR – select cDNA subtraction technique, termed suppression subtractive hybridization (SSH), based on suppression PCR is simple and efficient for investigating a novel genes differentially expressed between two groups (53). Nowadays, the effects of temperature on the transcriptional response of fungi were examined by using two high – throughput method: SSH and cDNA microarrays (54-57). In comparison to cDNA microarrays technology, SSH method has the advantage that it does not require the special equipments and the main reason is this method can

solve the data limitation of *P. insidiosum*. Because of SSH method allows to find a novel genes without any prior analysis knowledge for selecting specific probes (58). While still disadvantage of this method is false positive can be found, the second approach is recommended.

Recently, real-time RT-PCR has become the most powerful tool for mRNA quantification. Most studies using RT-PCR are taken semi-quantitatively (fold-variation) and it is assume that the mRNAs of reference genes are stably expressed or that any changes that might occur are balanced (59). The real-time PCR system is based on the detection and quantification of a fluorescent reporter (60-61). There are 3 groups of fluorescence – monitoring systems; hydrolysis probes, hybridizing probes and DNA – binding agents (62-63). A double-stranded DNA-binding dye is more cost effective than other dyes, because it quantifies the amplicon production (including non-specific amplification and primer-dimer complex) by the use of a non-sequence-specific fluorescent intercalating agent (SYBR-green I or ethidium bromide) (64).

Based on the recent available data, with an emphasis on the ability to survive at different temperatures (27°C and 37°C), three distinct clades based on the geographical areas were reported but there is no data has been published on the study at the genetic levels. Therefore, the first step in *P. insidiosum* transcriptomic study is to gain the basic information to support another future research.

In this study, we identified the temperature sensitive genes at 37°C growth condition of *P. insidiosum* based on the molecular technique (i.e., SSH method) and the available databases in GenBank of *Phytophthora, Pythium* species. These tools enabled us to obtain SSH pooled cDNA library, genetic sequencing and candidate

temperature sensitive genes of *P. insidiosum*. Next, the quantitative gene expression of candidate genes from each clade of *P. insidiosum* (isolated from different sources) was determined by semi-quantitative PCR analysis and real time reverse transcriptase (RT)-PCR. Based on the results of this study, a worthy SSH pooled cDNA library, sequencing information and candidate temperature sensitive genes of *P. insidiosum* were obtained. We expect that this research is a one part of many pieces of jigsaw which will lead to better understand this organism and disease it causes.

Hypothesis

Genes expressed in *P. insidiosum* cultured at 37°C might be involved in its pathogenesis.

Objectives

1. To establish the subtracted cDNA libraries at 37°C and 27°C conditions
2. To identify and characterize genes which express at 37°C (pathogenic condition)

Conceptual frame work