CHAPTER V

DISCUSSION AND CONCLUSION

It is known that the transition from environmental reservoir into a human host is associated with temperature-regulated and virulence-related genes that contribute to pathogenesis of pathogenic organisms such as bacteria, fungi and protozoa. Some of the gene products encoded by bacterial temperature-regulated genes are considered to be true virulence factors such as *Mycobacterium tuberculosis* (206). In pathogenic fungi, the ability to grow at 37°C is a prerequisite for fungal pathogenesis, and failure to grow at 37°C is predictive of attenuated virulence, examples of which have been reported on diverse of human fungal pathogens such as *C. neoformans*, *A. fumigatus*, *S. schenckii* and *H. capsulatum* (207-210).

In fact, *P. insidiosum* has evolved both pathogenic at 37°C and saprophytic lifestyle of 27°C (97). In Mycology Unit, Faculty of Medicine, Chulalongkorn University, all clinical isolates grew better at 37°C than lower temperature, 27°C. This observation was supported with the reported by Ravishankar *et al* (38) and Supabandhu *et al* (143), that they revealed the ability of *P. insidiosum* to grow in optimal temperature ranging between 30-37°C and complete inhibition at 40°C, these were unique characteristics shared by the clinical and environmental *P. insidiosum.* The genetic factors that are required for growth at 37°C may be worth considering as one from various factors for pathogenesis of *P. insidiosum.* The aim of this study was characterized the genes effected by temperature growth at 37°C of *P. insidiosum.*

The clinical isolates from human pythiosis were identified by sporulation and PCR techniques based on ITS region. All isolates were positive in both techniques but the zoospore induction method requires the steps of subculturing and zoospore induction which are time-consuming and labour-intensive. The result of this step agreed with the previous reports that the PCR based method using ITS region, is a proper choice to develop and facilitate rapid identification of *P. insidiosum* (26, 111, 143). In Thailand, the first report of animal pythiosis was recently identified and reported by Banrunava, *et al.* (data unpublished), and included in this study (strain PAC2).

There are many high - throughput techniques that can be used to study transcriptome including DNA microarray, subtractive hybridization and suppression subtractive hybridization (SSH). Of these, DNA microarray method is certainly the most widely used but the least of sequence analysis information is a limitation. This drawback can resolve with SSH method because it does not require any prior analysis data of the expression profile as required in the probe selecting step in microarray (211). Moreover the SSH based on suppression PCR approach has been successfully used to identify genes response to temperature stress. It was showed the ability to detect novel genes in Maize, Poplar and *Festuca* spp (212-214). As the incubation temperature for clinical isolates in routine laboratory were 27°C. And the average temperature for 10 year period in Bangkok Metropolitan, Thailand (1997 - 2007) from Thai Metrological Department was around 26 – 30°C (215). Then, the temperatures used in the SSH experiment were 37°C and 27°C.

Six hundred and six inserted clones out of all white colonies (1,200 colonies) from both subtracted cDNA libraries were detected from SSH experiment. These

colonies were subjected to BLASTN and BLASTX analysis. After the analysis, it was found that more than 60% of subtracted cDNA libraries were similar to the available sequence databases in GenBank. Another 40% remaining were found as unknown sequences, possessing either up or down – regulated genes at 37°C condition. Due to the very few information of genome sequence and be overlooked on this organism, these unknown genes are very worth to study their functions.

Among the known genes obtained the data from GenBank, most of the inserted clones possessed rRNA sequences and mitochondrial sequences, after BLASTN analysis. In terms of BLASTX analysis, several genes were related to senescence-associated proteins, involved stress response mechanism. It was believed that these proteins compensated the ribosome assembly and maintained the translational process by binding to 5S RNA (216). At this point, the result of BLAST analysis focused on the mitochondria mechanism affected from temperature stress condition. These information were corresponded to the report by Schmitt *et al.* They revealed that mitochondrial proteins synthesis is a thermosensitive process (217). All the above data obtained from the SSH cDNA libraries at 27°C and 37°C conditions. It is remarkable to note that the optimum temperature for growth of *P. insidiosum* is 37°C. Then the stress temperature might be the lower one or the temperature of its natural habitat (39). Another four known functional groups of genes derived from temperature at 37°C condition were linked to protein carrier, cell cycle, signal transduction mechanism and proteolysis functions. Two encoded genes; phosphate carrier protein and inositol – 3 – phosphate synthase were the members in a family of mitochondria protein. The previous publication revealed that myo – inositol, an essential precursor for phospholipomannan, played an important role in *Candida.*

Phospholipomannan, a family of glycophosphatidylinositol (GPI)-anchored glycolipids located on the cell surface of *Candida,* is involved in the pathogenicity of this fungus. This compound binds to human macrophages and also stimulates the macrophage (218). Thus, phospholipomannan is considered a virulence factor in *Candida* and has been shown to possess immunomodulatory properties such as TNF-α induction (219). The third was gene encoding serine carboxypeptidase, one kind of proteases, plays a significant role in the molecular dialogue in the host-parasite relationship. This result agreed to the previous data of Ravishankar *et al* (39) that the extracellular proteinase was increased when *P. insidiosum* was cultured on protein rich – medium and incubated at 37°C (38-39), *in vitro*. They concluded that this protein is a one factor which help the hyphae to penetrate or invade into host tissue (39). It is surprised that the last one was similar to gag polymerase gene. As we know that this gene locates in viral DNA,

Even though SSH is one of the powerful tool to isolate the differentially expressed genes, the drawback of false positive gene can be found (220). To detect the true positive gene, another approach of the semi – quantitative PCR is performed (221). In this step, the specific primer pair was required. But with the *Rsa* I digestion step, the obtained cDNA fragments ranged from 200 to 1,000 bp (average ~300 bp) (53, 173). Thus, the incomplete cDNA fragment was obtained. This is one of the hindrance to design specific primers for all 4 genes from their sequences. More experiments to obtain the full length of the functional gene, such as RACE PCR and inverted PCR should be tried. Next, the function (s) should be determined by mutation, and small interfering RNA (siRNA).

Fortunately, there was a review literature explained that the virulence genes of the oomycetes, fungal phytopathogen in the same family of *P. insidiosum* mostly related to chitin and structural proteins (222). Based on these information and their genetic sequences in GenBank, three genes, cytochrome c oxidase subunit II (*COX* II), β- tubulin (*TUB*), chitin synthase subunit II (*CHI* II), were fetched. The specific primer of 2 genes; *TUB* and *CHI* II, were designed in this study whereas those of *COX* II was referred from Villa *et al* (194). To examine whether these genes was in the SSH cDNA libraries or not, PCR using these cDNA as template were carried out with the above specific primers.

To perform the quantitative expression level, real time RT – PCR, was selected as the confirmation method (223). The normalization strategies were the prioritized process in controlling the experiment and technical variation. To achieve quantitative analysis, endogenous housekeeping or reference gene is indispensable (224-225). As mentioned above, no such a system has been established for *P. insidiosum* gene expression assays. There have been several studies in eukaryote gene expression using glyceraldehydes 3 – phosphate dehydrogenase (*GAPDH*), β - actin (*ACTB*), RNA polymerase II (*RNAP*II), ribosomal protein L13 (L13) gene, β – tubulin (*TUB*), 18S and 28S rRNA as a housekeeping gene (226-230). From result of semi – quantitative PCR, 28rRNA showed the consistence expression in both temperature conditions, thus it can be used as a control. To use this control, there have been ambiguous supports (231-232). The reason why 28rRNA was not in popular due to its disable to annealed with oligo (dT) primer during cDNA synthesis (202). However, there was an evidence to support our system to use this control instead of housekeeping genes (225). All three candidate genes were subjected to investigate

their expression level by real time RT-PCR. The result showed that only *COX* II gene, encoded cytochrome oxidase subunit II, expressed at 37°C higher than at 27°C condition. *COX* II gene is a part of cytochrome oxidase complex, located in inner mitochondria membrane. Since the structure of this complex contain a pore, it makes the movement of protons from the mitochondria matrix to the intermembrane space, generating the proton gradient for ATP synthesis in fungi (233). In yeast, and higher plants, *COX* II is synthesized as a precursor protein on mitochondrial ribosomes (234-235). Another support was found the temperature dependent *COX* II expression in *Saccharomyces cerevisiae* temperature sensitive strain using mutation by changing Ala189 of the Cox2 protein to proline (236). Our study suggested that *COX* II expression in *P. insidiosum* is temperature dependent.

Another two genes, *TUB* and *CHI* II, they were located in nuclear DNA. Their expressions at both temperature were difference with non-significant ($p>0.05$). No evidence from the related organism of the expression of these genes was found. Thus, the expression of both genes was temperature independent. This result provides the novel supporting to apply *TUB* and CHI *II* as housekeeping genes in gene expression of *P. insidiosum.*

Regarding to the phylogeographic preferences of *P. insidiosum* as mentioned before, three clades, I, II, and III were separated (26, 31). Presently, there still no answer whether the expression of certain gene depends on the geographic distribution or not. Then, using isolates from all clades were determined. Indistinguishable expression of all three genes was detected at 27°C condition. Only *CHI II* showed the clade dependent at 37°C condition. The relationship between geographic preference and expression of *CHI* II gene is concerned and required to further study.

Recently, phylogenetic analysis of the environmental Thai strains of *P. insidiosum* using the ITS regions revealed that these strains shared phylogenetic features in common with the clinical isolates recovered from humans and animals (143). In all 3 clades, Schurko *et al* described clade C comprised of cryptic group of *P. insidiosum* (31). They believe that this group might represent new phylogenetic species of this pathogen. This notice needs to investigate phylogenetic relationship using other genes. Among genes encoding proteins, very few reports are available on the use of cytochrome c oxidase subunit II (*COX* II) and β - tubulin (*TUB*) gene for phylogenetic studies in Genus *Phytophthora* and *Pythium* (194). The *COX* II genes were applied and succeed to phylogenetic relationship study, whereas another gene remains to be examined. Unlike the ITS regions, *COX* II gene is a mitochondrial-encoded gene which is generally considered to be more variable than the genomic DNA. This gene was used to analyze phylogenetic trees in *Pythium* spp. and *Phytophthora* spp. (237-238).

Phylogenetic analyses using *COX* II and ITS regions placed the thirty-three isolates of *P. insidiosum* into three main groups: A_{TH}, B_{TH}, and C_{TH} cluster (Figures 42 and 43). In the *COX* II phylogenetic tree the two American isolates in the cluster A_{TH} (equine isolates, from Costa Rica and Texas) were separated from the other thirty-one *P. insidiosum* Thai isolates located in clusters B_{TH} and C_{TH}. Based on *P. insidiosum* ITS region Schurko *et al.* found three clades among mammalian isolates of *P. insidiosum* designated as clades I, II, and III (31). These clades have strong association with the geographical regions where the isolates were recovered. Recent phylogenetic analysis in Thailand using the ITS DNA sequences of environmental *P.*

insidiosum showed similar trees clustered in three strongly supported clades (A, B, and C) (143). These ITS analyses were similar to that reported by Schurko *et al* (31).

Our study support previous reports (Table 25). The MTPI04 and MTPI19 were placed in cluster A_{TH} in both *COX* II- and ITS- phylogenetic trees. Environmental isolates from Supabandhu *et al* (143) were comparable to B_{TH} and C_{TH} in this study and clade B and C of Supabandhu *et al* (143) and clade II and III of Schurko *et al.* (31, 143). The *COX* II gene analysis showed higher resolution than phylogenetic analysis using ITS DNA sequences (31, 143). Although an ITS region has been widely used in phylogenetic studies for over a decade, some newly proposed genes coding for metabolic proteins are becoming better candidates for studying microorganisms. Our results showed that *COX* II gene generate more resolved phylogenetic tree than ITS. Therefore, the use of this coding gene sequence should be used as a novel marker for the phylogenetic analysis of *P. insidiosum*. Moreover, our phylogenetic analysis using *COX* II gene placed *Pythium* spp. in a monophyletic group distinct from the isolates of *Basidiobolus meristosporus* (data not shown).

Table 25. Showing the cluster terminology obtained from the phylogenetic analysis of three studies including the present study. Clin: clinical source, env: environmental source

	This study (33 isolates)	Supabandhu *et al.*, 2008 (ITS, 59 isolates)	Schurko *et al.*, 2003 (ITS, 23 isolates)
***COX* II** (558-564 bp.)			
A_{TH}: (clin.:env. = 2:0) from Costa Rica & Texas	A_{TH}	A	I
B_{TH}: (clin.:env. = 6:15) all from Thailand	B_{TH}	B	II
C_{TH}: (clin.:env. = 6:4) all from Thailand	C_{TH}	C	III
ITS1-ITS2 Region (871-898 bp.)			
A_{TH}: (clin.:env. = 2:0) from Costa Rica & Texas	A_{TH}	A	I
B_{TH}: (clin.:env. = 6:15) all from Thailand	B_{TH}	B	II
C_{TH}: (clin.:env. = 6:4) all from Thailand	C_{TH}	C	III

In conclusion, this study showed that

1. SSH cDNA libraries from 27°C and 37°C were constructed.
2. Expression system for *P. insidiosum* system using real time RT-PCR with 28S rRNA as a control was constructed.
3. The expression of *COX* II depends on temperature, that is at 37°C is over at 27C condition.
4. *COX* II gene is the good candidate gene to reveal the genetic association of *P. insidiosum*. It also is an alternative gene of ITS, commonly used to the phylogenetic study.

Future work

1. To describe the relationship between the expression of *COX* II on the pathology of the disease that still requires more study about functions, targets and ultimately how the expression and translation are regulated.
2. The up – or – down regulated remaining genes from SSH cDNA libraries, such as serine carboxypeptidase, myo-inositol phosphate, phosphate carrier protein and unknown clones are required for further investigation on identification and characterization. The gain of these information will provide the understanding of pathogenesis in the genetic level. This is very useful in finding effective treatments and protection in the future.