CHAPTER IV

RESULTS

1. Isolation and Identification of *P. insidiosum* Thai isolates.

Eleven isolates from human cases and animal cases as shown in Table 7 (Materials and Methods; page 64) were examined for the zoospore production and verified by their DNA sequencing in ITS region. Another four isolates, one from human case and three from environmental sources, were provided by Prof. Nongnuch Vanittanakom, Chiang Mai University. All these four isolates were proved for both zoospore production and the genotypic characteristics in ITS region. The demography was shown in the Table 12. (Materials and Methods; page 95)

1.1. Zoospore production

Under zoospore production condition, eleven isolates of *P. insidiosum* developed filamentous sporangia and motile biflagellate zoospores of each isolate in an induction medium (Figure 22). Microscopically, the process took ~ 35 min for the formation of differentiated sporangia and (Figure 22A – 22C), then motile zoospores mechanically broke the sporangia's wall and released into the induction medium (Figure 22D). The bi-flagellate zoospores (Figure 22E) continued to swim before encystment and finally came to develop a germ tube (Figure 22F). Positive control in this study was *P. insidiosum* strain CBS 574.85 (MTPI19). However, the morphology of *P. insidiosum* was difficult to distinguish from other *Pythium* spp, eleven isolates of *P. insidiosum* were continued to test with DNA sequencing using ITS region.

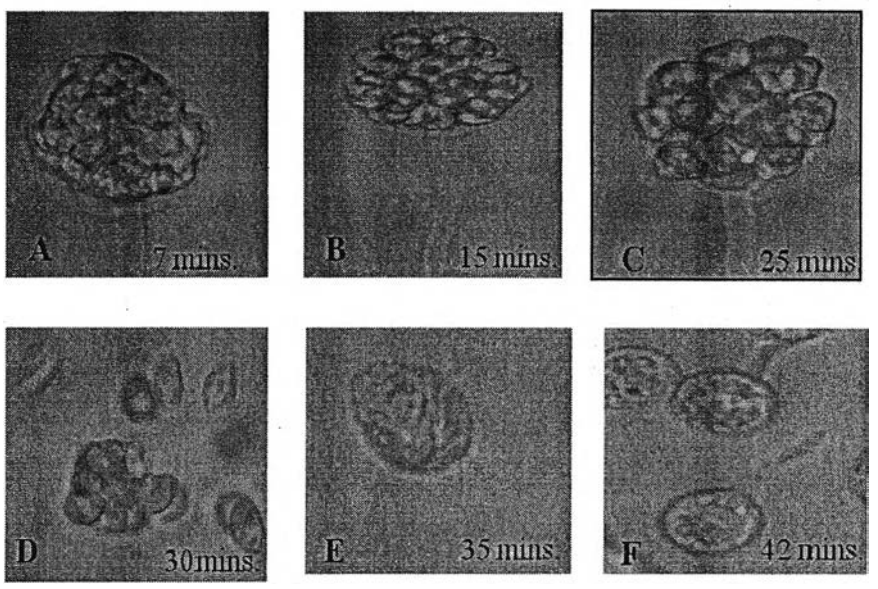

Figure 22. Morphology of *P. insidiosum* clinical isolate. (A to C) Zoospore production inside the zoosporangium at time intervals of 25 min. (D to F) Biflagella zoospore swam and then encyst in thirty minutes.

1.2 Verification of *P. insidiosum* Thai Strain by DNA sequencing in ITS region

The PCR amplification of the ITS region was performed in order to verify isolates of *P. insidiosum*. The genomic DNAs of 11 isolates (CBS 574.85 was the reference strain) were prepared as template for PCR amplification with ITS1 and ITS 4 primers, PCR product revealed approximately 900 bp in length. These products were sequenced and subjective to BLASTN analysis. The results showed 95-99% homology of these products to 28S rRNA of *P. insidiosum* strain CBS 574.85 in GenBank databases (data now shown).

2. Selection of Temperature Sensitive Genes

2.1. cDNA 37°C library by SSH technique

2.1.1 RNA qualification

The estimation of total RNA quality was essential step before processing to the gene expression experiment. To check the quality and quantity of RNA, standard measuring methods for RNA concentration with NanoDrop spectrophotometer via 260 nm absorbance and Gel electrophoresis technique were performed. The purity of the extracted RNA was ranged between 1.8 – 2.2 (Table 13) indicating the little or no protein contamination whereas the RNA concentration was varied between 7 – 40 µg/mL (Table 13). The average concentration was 23.25 µg/mL. We evaluated the total RNA integrity on 1% denature agarose gel electrophoresis and visualized the RNA bands on gel by UV transiluminator. By the theory, two bands of 28S and 18S rRNA from intact total RNA should be observed. The prepared RNA was also appeared 2 bands at the same sizes as expected with 28S and 18S rRNA (Figure 23).

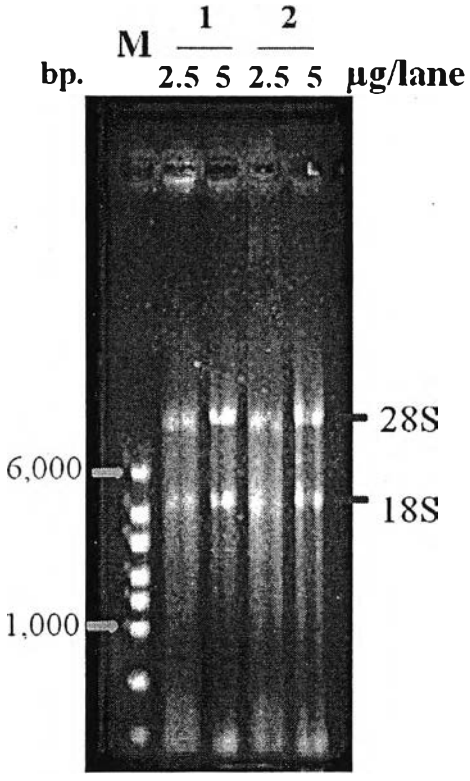

Figure 23. Denaturing agarose gel electrophoresis of total RNA from *P. insidiosum* strain PC7 which was cultured at 27°C and 37°C. M = RNA marker (0.2 – 6 kb, Fermentas), Lane 1 = total RNA isolated from temperature 27°C (conc. 2.5 & 5 µg/lane) and lane 2 = total RNA isolated from temperature 37°C (conc. 2.5 & 5 µg/lane).

Table 13. Quality and quantity of total RNA by Hot phenol extraction from 16 *P. insidiosum* isolates

Isolated Code	Temp. (°C)	Absorbance (nm)		Ratio (*A*260/*A*280)	Concentration (µg/mL)
		260	280		
MTPI 19	27	0.821	0.412	1.9934	32.83
	37	0.195	0.099	1.9738	7.8154
MTPI04	27	0.333	0.153	2.176	11.15
	37	0.323	0.148	2.1824	12.92
PAC2	27	0.857	0.474	1.8065	34.26
	37	0.708	0.38	1.862	28.33
PC1	27	0.982	0.49	2.0041	39.28
	37	0.901	0.45	2.0005	36
PC2	27	0.825	0.419	1.9694	33
	37	0.868	0.453	1.9171	34.72
PC5	27	0.701	0.34	2.0635	28.04
	37	0.55	0.28	1.9642	22.01
PC6	27	0.665	0.33	2.0151	26.58
	37	0.251	0.125	2.008	10.03
PC7	27	0.81	0.388	2.0876	32.4
	37	0.361	0.193	1.8769	14.44
PC10	27	0.73	0.374	1.9532	29.19
	37	0.94	0.475	1.9771	37.6
PC11	27	0.503	0.25	2.012	20.1
	37	0.565	0.29	1.9484	22.59
PCM1	27	0.343	0.16	2.143	13.75
	37	0.301	0.137	2.188	12.04
PMS1	27	0.654	0.328	1.9947	26.17
	37	0.466	0.247	1.8852	18.62
PR2	27	0.586	0.299	1.9627	23.45
	37	0.355	0.163	2.1779	14.214
PECM3	27	0.706	0.367	1.9224	28.24
	37	0.713	0.38	1.8735	28.5

Table 13. Quality and quantity of total RNA by Hot phenol extraction from 16 *P. insidiosum* isolates (cont.)

Isolated Code	Temp. (°C)	Absorbance (nm)		Ratio (*A*260/*A*280)	Concentration (µg/mL)
		260	280		
PECM8	27	0.348	0.159	2.1886	13.92
	37	0.308	0.142	2.169	12.31
PECM14	27	0.46	0.212	2.1698	18.4
	37	0.53	0.246	2.1545	21.2

2.1.2 Identification of Subtraction efficiency

To screen and select temperature inducible genes, SSH method was performed. Prior to the clone selection experiment, the efficiency of the subtraction result was identified. In principle, common cDNA in both conditions is subtracted and the cDNA in the selected condition, at 37°C remains (53). To check the efficiency of the subtraction, the 28S rRNA primers and *COX* II primers were performed. No PCR product in the region of 28S rRNA was detected. In contrast, the *COX* II PCR product was approximately 600 bp with specific primers in Table 10. The result showed that band was appeared after 30 amplification cycles when using the unsubstracted cDNA at 37°C condition as a template whereas bleary band appeared from the subtracted cDNA at 37°C condition as a template (Figure 24A). When the PCR cycle was increased to 40 cycles, PCR fragments (Figure 24B) were larger than 30 cycles. This result assumed that common cDNA homologous in both condition (27°C & 37°C) was eliminated by this procedure.

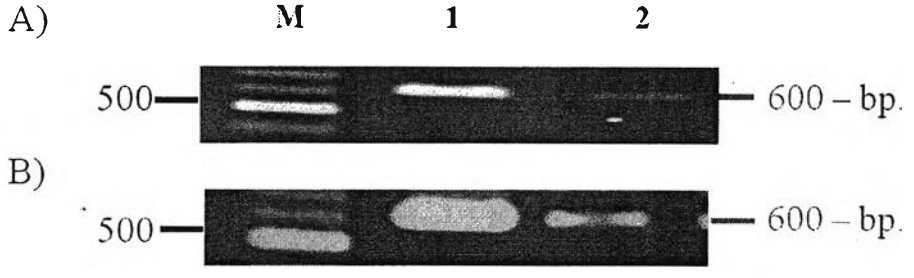

Figure 24. Efficiency of cDNA subtraction using specific *COX* II primers in PCR amplification. A): PCR product from 30 cycles-PCR amplification; B): PCR product from 40 cycles-PCR amplification. Lane M = 1 kb DNA ladder marker; lane 1 = unsubtracted cDNA at 37°C condition and lane 2 = subtracted cDNA at 37°C condition by cDNA at 27°C.condition The products were analyzed on a 1.5% agarose gel .

2.1.3 Inserted clones from Subtracted cDNA Libraries at 37°C and 27°C conditions

By suppression subtractive hybridization technique, the cDNA library with more differentially expressed sequences from *P. insidiosum* strain PC7 at 37°C condition was constructed (53). Higher frequency of the sequences was yielded because of an additional PCR amplification. The PCR products were cloned into pGEM-T easy vectors and transformed into *E. coli* JM109. To screen the bacterial colonies that possessed inserted cDNA in the cloning vectors, blue-white spots were indicated. The transformation efficiency was 95%, approximately by the protocol of pGEM-T easy kit. After transformation, the number of 1,000 and 200 bacterial colonies were all white colonies which indicated the inserted cDNA, from 37°C and 27°C conditions, in order, were selected. Only 456 colonies and 150

colonies contained 100-1,000 bp inserted cDNA fragments from 37°C and 27°C conditions, respectively (Figure 25). All these 606 positive clones represented the candidate population of cDNA fragments, specific for any function at both temperatures. To analyze the nucleotide and functional sequences, these inserted cDNA were sequenced and compared to available genome database in GenBank using BLAST & ExPASy – PROSITE programs.

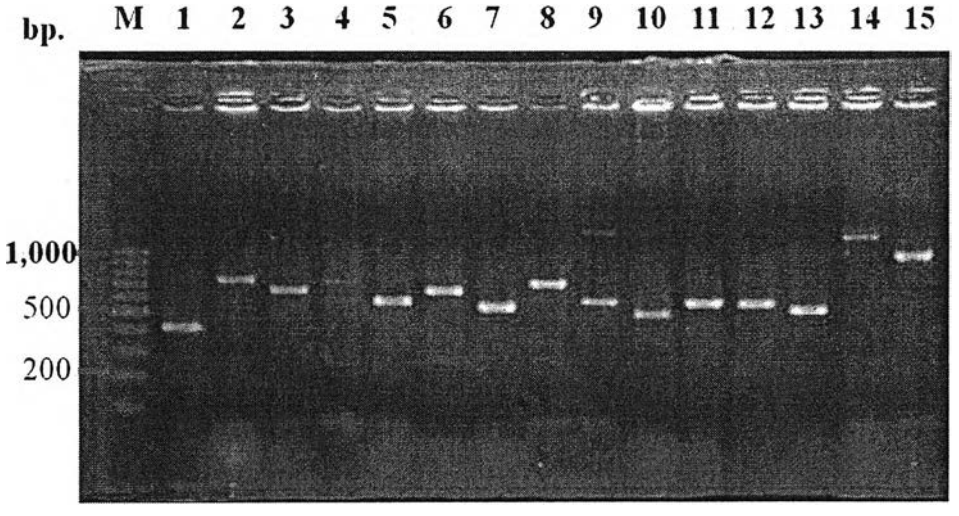

Figure 25. PCR products of some positive clones after performing the colony PCR using M13pUC primers Lanes 1-15 were 15 positive PCR products from the subtracted white colonies. M: 100 base pair DNA ladder marker.

2.1.4 Sequence Analysis

The six hundred and six clones selected for differential expression experiment, were sequenced using universal M13pUC primers.

Table 14. Summary of sequence analysis of subtracted cDNA libraries at 37°C and 27°C condition using BLAST & ExPASy – PROSITE programs.

	Number of Clones (%)		
	27°C (n=150)	**37°C** (n=456)	**Total** (n=606)
BLASTN			
Homology	137 (91)	300 (65.8)	437 (72.1)
Non homology	13 (9)	156 (34.2)	169 (27.9)
BLASTX			
Known protein	129 (86)	307 (67.3)	436 (71.9)
Unknown protein*	21 (14)	140 (32.7)	170 (28.1)
BLASTN/BLASTX			
+/+	122 (81.3)	289 (63.4)	411 (67.8)
+/-**	14 (9.3)	9 (2)	23 (3.8)
-***/+	6 (4.1)	18 (4)	24 (4)
-/-	8 (5.3)	140 (30.6)	148 (24.4)

Note +/+ = known gene and protein, +/- = known gene but unknown protein
-/+ = unknown gene but known protein, -/- = unknown gene and protein

* E value > cut off (E value <2e-04)
** Unclassified function
*** No matching genes

By nucleotide analysis, the numbers of 437 out of 606 clones (72.1%), a total clones of both SSH cDNA libraries, were homologous with the data sequences in GenBank databases whereas 169 (27.9 %) showed no significant similarity and could not be identified (Table 14). These 437 clones encoded for 10 different

genes, which are summarized in Table 15. After the analysis of the known sequences, it was found that these clones were homology to the related organisms. The number of 274 clones (62.7%) are homology to *Pythium* ribosomal gene; 142 clones (32.6%) of them are homology to *Phytophthora* mitochondrial gene, and 16 clones (3.7%) are related to the uncultured bacterium partial 16S rRNA. The major of harvested clones in this study belonged to the redundant genes, such as rRNA gene. However, the best hits with tRNA (*Ph. infestans*), NADH dehydrogenase subunit 5 (*Ph. lateralis*), mRNA (*Ph. caposi*), and myo-inositol-phosphate synthase gene of Malaria mosquito (*Anopheles gambiae*) were found with the frequency of 1 clone (0.2%) in each gene. The list of homologous comparison was reported in Table 15.

Four hundred fifty six inserted clones from SSH cDNA library at 37°C condition were detected by colony PCR. With BLASTN analysis, it was found that three – hundred clones (65.8%) were homologous to database in GenBank and the left was non-homologous (Table 14). In similarity sequences, they could be classified into 7 different genes which were shown in detail in Figure 27. Comparing the number of selected clones at both temperature conditions, only one-third clones, 150 clones, were shown at 27°C condition. Most of these clones harbored the sequences which (137/150 clones, 91%) were homology to known genes (Table 14, Figure 26).

Table 15. List of the nucleotide similarities of SSH cDNA libraries sequences from *P. insidiosum* strain PC7 at 27°C & 37°C cultivation conditions

Temp. (°C)	Genes	Genus and species	GenBank Accessions
27	Mitochondrion	*Ph. infestans*	U17009
	28S ribosomal RNA	*P. megasperma*	X75631
	Mitochondrion	*Ph. ramorum*	EU427470
	Mitochondrion	*Ph. infestans*	AY898627
	Mitochondrion	*Ph. sojae*	DQ832717
	Partial 16S ribosomal RNA	Uncultured bacterium	FM956611
	16S ribosomal RNA gene	Uncultured bacterium	EF662800
	Partial 16S ribosomal RNA	Uncultured bacterium	AB075125
	tRNA-Pro (trnP(ugg)) and tRNA-Met (trnM(cau)) genes	*Ph. infestans*	EF366732
	NADH dehydrogenase subunit 5	*Ph. lateralis*	AY423337
	18S ribosomal RNA gene	*P. insidiosum*	AF442497
37	Mitochondrion	*Ph. sojae*	DQ832717
	mRNA sequence	*Ph. caposi*	BT032625
	Myo-inositol-phosphate synthase	*Anopheles gambiae*	XM320685
	Internal transcribed spacer 1, 5.8S ribosomal RNA	*P. insidiosum*	AY598637
	28S ribosomal RNA	*P. megasperma*	X75631
	Mitochondrion	*Ph. ramorum*	EU427470
	28S rRNA	*Pythium* spp.	AB254193

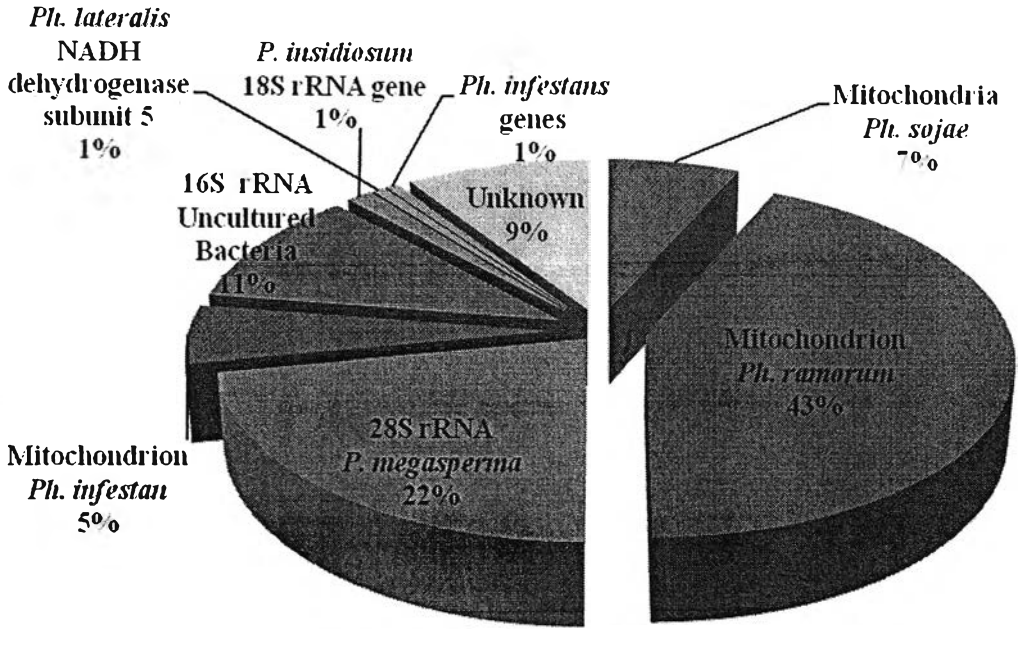

Figure 26. Nucleotides analysis of subtracted 150 up-regulated cDNA clones of *P. insidiousum* strain PC7 at 27°C cultivation condition with global distribution of BLASTN.

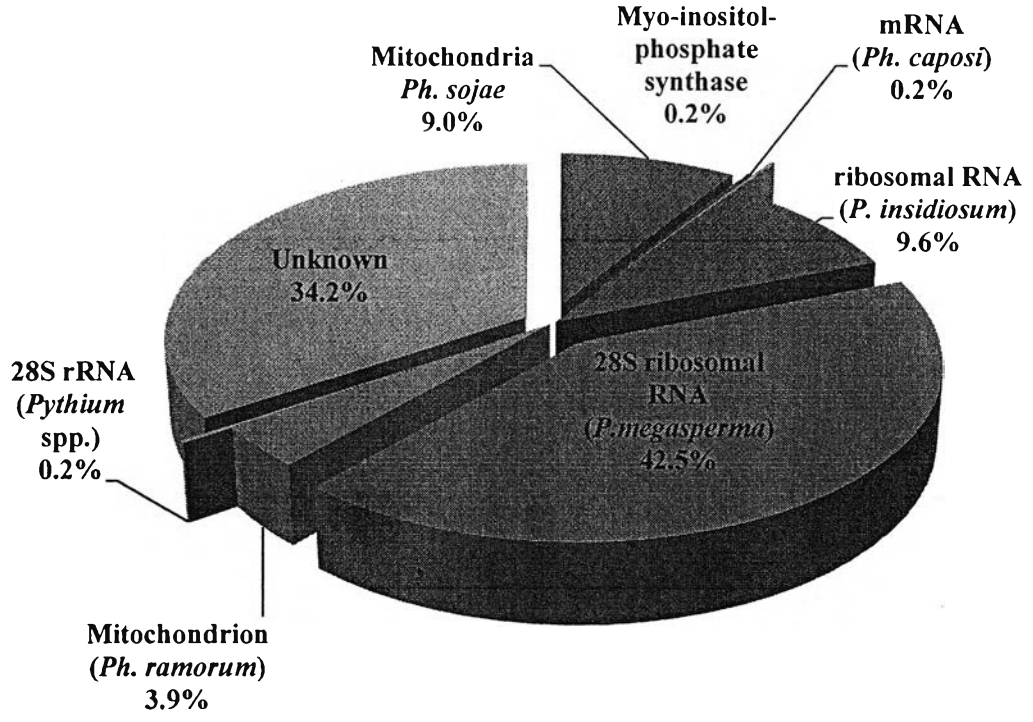

Figure 27. Nucleotides analysis of subtracted 456 up-regulated cDNA clones of *P. insidiousum* strain PC7 at 37°C cultivation condition with global distribution of BLASTN.

Not only the nucleotide sequences analysis was considered, the translation and functional annotation were examined also. The ExPASy – PROSITE program in 6 frame standard, then BLASTX and Swiss – Prot program were used to match 606 translated cDNA sequences with available databases by a cutoff with Expected (*E*) value < 2e-04 (200). The numbers of 436 out of 606 clones (71.9%) could be identified to known predicted proteins in GenBank databases whereas 170 clones (28.1 %) were unknown proteins (Table 14). Among the analysis of protein identity of each library, the 67.3 % (307/456) and 86% (129/150) of SSH cDNA at 37°C and 27°C condition libraries (Figure28 and Figure 29, respectively) could be matched with known predicted protein in present public database whereas the minor group of each library (at 37°C condition and 27°C condition) were unknown protein (Table 14). The lists of public proteins with GenBank accession numbers were shown in Table 16.

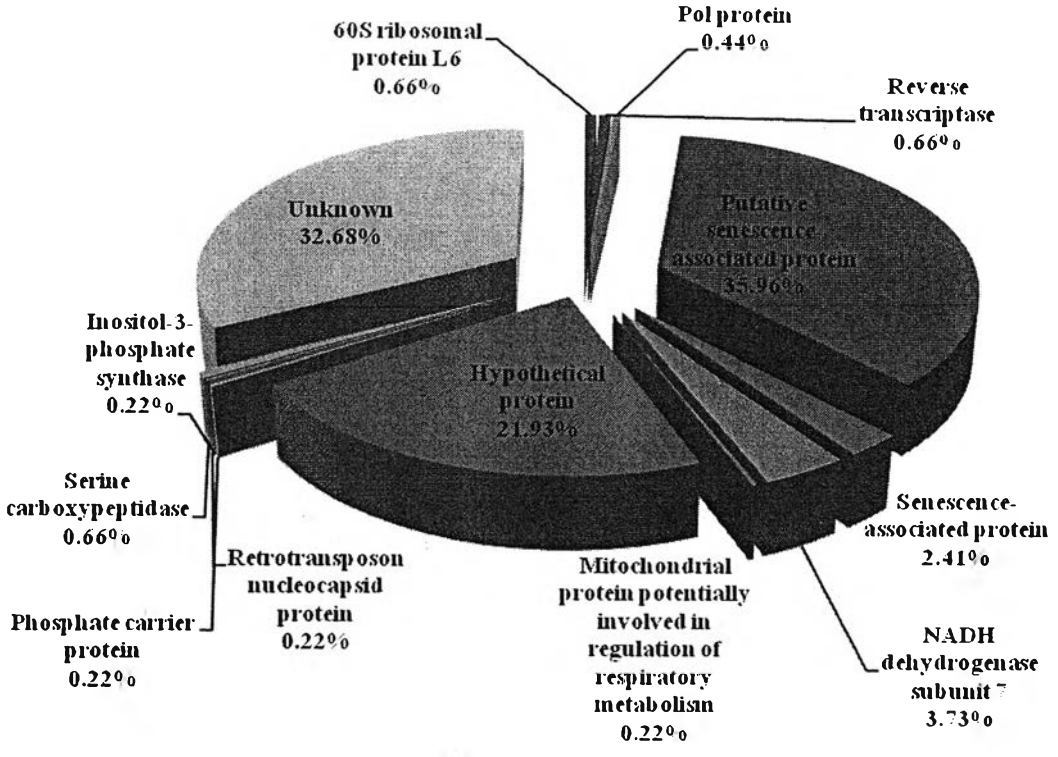

Figure 28. Translational analysis of subtracted cDNA library at 37°C condition of *P. insidiousum* strain PC7.

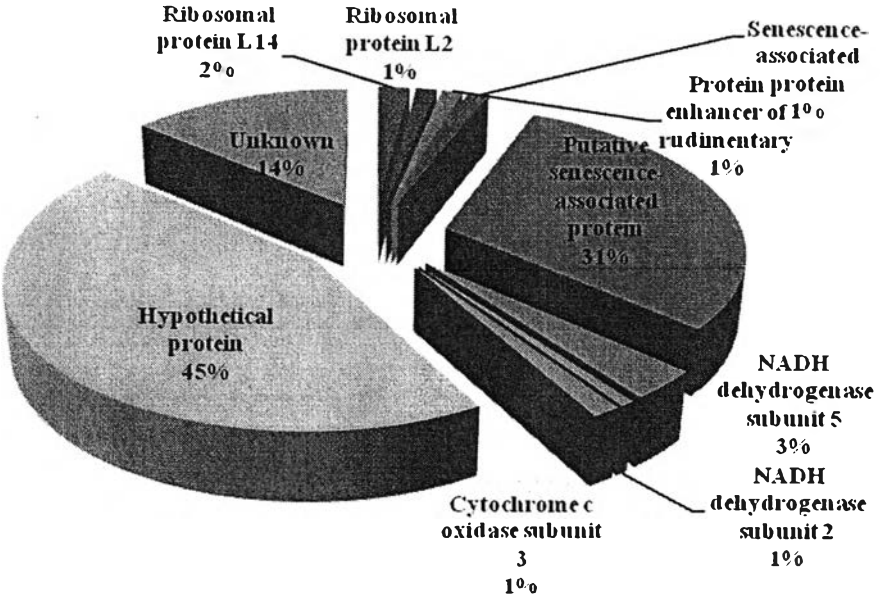

Figure 29. Translational analysis of subtracted cDNA library at 27°C condition of *P. insidiousum* strain PC7

Table 16. SSH cDNA libraries (27°C & 37°C conditions) with significant protein match in The National Center Biotechnology Information (NCBI) protein databases.

Temp. (°C)	Predicted proteins	Genus and species	GenBank Accessions	*E* - value
	Protein synthesis			
27	Ribosomal protein L14	*Ph. ramorum*	YP001165335	5.00E-21
	Ribosomal protein L2	*Ph. sojae*	YP001165413	2E-49 to 4E-16
37	60S ribosomal protein L6	*Ph. infestans*	EEY65770	2E-23 to 2E-19
	DNA replication			
27	Protein enhancer of rudimentary	*Ph. infestans*	EEY55861	2.00E-16
37	Reverse transcriptase	*Ph.ramorum*	ABG66535	2E-32 to 7E-7
	Pol protein	*Ph. infestans*	AAV92918	1E-27 to 3E-15
	Stress Response			
27	Senescence-associated protein	*Picea abies*	ACA04850	5.00E-55
	Putative senescence-associated protein	*Pisum sativum*	BAB33421	9E-45 to 3E-9
37	Putative senescence-associated protein	*Pisum sativum*	BAB33421	9E-45 to 3E-9
	Senescence-associated protein	*Picea abies*	ACA04850	1E-52 to 5E-5
	Cellular respiration			
27	NADH dehydrogenase subunit 5	*Ph. sojae*	YP001165399	2E-66 to 3E-22
	NADH dehydrogenase subunit 2	*Ph. ramorum*	YP001165351	3.00E-18
	Cytochrome c oxidase subunit 3	*Ph. ramorum*	YP001165347	4E-23 to4E-22
37	NADH dehydrogenase subunit 7	*Heterosigma akashiwo*	BAI70606	7.00E-07
	Mitochondrial protein potentially involved in regulation of respiratory metabolism	*Saccharomyces cerevisiae*	NP690845	2.00E-12

Table 16. SSH cDNA libraries (27°C & 37°C conditions) with significant protein matches in The National Center Biotechnology Information (NCBI) protein databases (cont.)

Temp. (°C)	Predicted proteins	Genus and species	GenBank Accessions	*E* - value
	Hypothetical protein (unknown function)			
27	Predicted protein	*Populus trichocarpa*	XP002337573	8.00E-05
	Conserved hypothetical protein	*Escherichia* spp.	ZP04532941	
	Hypothetical protein	*Curvibacter* putative symbiont of *Hydra magnipapillata*	CBA31938	3.00E-11
	Similar to predicted protein, partial	*Hydra magnipapillata*	XP002168570	6E-34 to 9E-33
	Conserved hypothetical protein	*Clostridium butyricum*	ZP04529120	7.00E-12
	Putative puroindoline b protein	*Triticum aestivum*	CAQ43070	1.00E-08
	Hypothetical protein	*Arabidopsis thaliana*	BAF01964	1.00E-55
	Unknown	*Medicago truncatula*	ACJ85262	9.00E-38
	Conserved hypothetical protein	Onion yellows phytoplasma	BAD04335	9.00E-26
	Conserved hypothetical protein	*Bacteroides* spp.	ZP06090969	8.00E-10
37	Predicted protein	*Hydra magnipapillata*	XP002168570	3E-45 to 2E-26
	Hypothetical protein	*Nematostella vectensis*	XP001618200	4.00E-15
	Conserved hypothetical protein	*Escherichia* spp.	ZP04532941	2.00E-05
	Putative puroindoline b protein	*Triticum aestivum*	CAQ43070	1.00E-08
	Hypothetical protein	*Providencia stuartii*	ZP02997467	9.00E-18
	Hypothetical protein	*Xanthomonas campestris*	ZP06487381	2.00E-09
	Hypothetical protein	*Dunaliella viridis*	ABG38270	3E-18 to 5E-18
	Hypothetical protein	*Curvibacter* putative symbiont of *Hydra magnipapillata*	CBA31938	3.00E-11

Table 16. SSH cDNA libraries (27°C & 37°C conditions) with significant protein match in The National Center Biotechnology Information (NCBI) protein databases (cont.)

Temp. (°C)	Predicted proteins	Genus and species	GenBank Accessions	*E* - value
	Hypothetical protein	*Ajellomyces capsulatus* NAm1	XP001535830	1.00E-15
	Hypothetical protein	*Campylobacter jejuni*	ZP06374539	4.00E-07
37	**Protein carrier**			
	Phosphate carrier protein	*Ph. infestans*	EEY66591	4.00E-46
37	**Signal transduction**			
	Inositol-3-phosphate synthase	*Ph. infestans*	EEY61403	7.00E-55
	Cell Cycle			
37	Retrotransposon nucleocapsid protein	*C. neoformans*	XP571377	3.00E-11
	Proteolysis			
37	Serine carboxypeptidase	*Ph. infestans*	EEY53505	1.00E-21

Both subtracted cDNA libraries (27°C & 37°C conditions) were annotated for protein function by using MIPS (Munich Information Center for Protein Sequences) and Swiss – Prot programs. These clones were grouped by the functional categories, according to their putative identification in Computer-assisted DNA and amino acid comparisons against available databases in GenBank. They were placed into 9 functional categories; [1] protein synthesis, [2] DNA replication, [3] stress response, [4] cellular respiratory, [5] protein carrier, [6] signal transduction, [7] proteolysis, [8] Cell cycle and [9] unknown function (Table 16). The nine functional groups were found in subtracted cDNA at 37°C condition whereas 4 groups i.e., signal transduction, proteolysis, cell cycle and protein carrier, could not be found in 27°C condition library.

The four-hundred and eleven (67.8%) clones from both SSH cDNA libraries (37°C & 27°C conditions) could be identified and predicted protein (+/+) which represented 289 (63.4%) and 122 (81.3%) clones, respectively in each libraries (Table 14). Simultaneously, 24.4 % (148/606 clones) of no homologous both nucleotide and amino acid databases (-/-) were found about 30.6% (140/456 clones) in subtracted cDNA library at 37°C condition and 5.3% (8/150 clones) in subtracted cDNA library at 27°C condition (Table 14). The 5 groups of functional protein which found in SSH cDNA libraries (37°C & 27°C conditions) were unknown function (unknown and hypothetical proteins) (54.6%& 59%), cellular respiration (3.95%&5%), DNA replication (1.1%&1%), protein synthesis (0.7%&4%) and stress response (38.4%&31%) (Figure 30 and Figure 31). Interestingly, four functional groups could be found only in subtracted cDNA library at 37°C condition (i.e. protein carrier (phosphate carrier protein,

Ph. infestans EEY66591), cell cycle (retrotransposon nucleocapsid protein, *C. neoformans* XP571377), proteolysis (Serine carboxypeptidase, *Ph. infestans* EEY66591) and signal transduction (inositol-3-phosphate synthase carboxypeptidase, *Ph. infestans* EEY61403)). In both subtracted cDNA libraries (at 37°C and 27°C conditions) in Table 14, there are 23 clones (3.8%) were rRNA or mitochondria genes whereas translation analysis was shown *E* value > 2e-4. Also twenty – four clones (4%) could not identified genes but the translation analysis results were represented probable proteins such as serine carboxypeptidase, hypothetical proteins, pol protein, reverse transcriptase, 60S rRNA and retrotransposon neucleocapsis protein (Table 16). For unknown group (-/-), 148 clones (24.4%) in both SSH libraries, demonstrated 30.6 % (140/456) up – regulated and 5.3 % (8/150) down – regulated SSH cDNA fragments at 37°C condition (Table 14).

2.2 GenBank database

The alternative approach for finding the genes related to growth promotion and/or virulent related genes in *P. insidiosum* was originated from previous molecular studies of other related species and genus; such as *Pythium* species and *Phytophthora* species. The interested genes (at least 9 genes) could not get from SSH cDNA libraries by PCR amplification method, except three genes, cytochrome c oxidase subunit II (*COX* II), β- tubulin (*TUB*), and chitin synthase subunit II (*CHI* II) gene. Only *COX* II's primers were referred from Villa *et al.* (194) whereas the others were designed in this study. To prove these genes were indeed up – regulated at 37°C, quantitative gene expression analysis was performed.

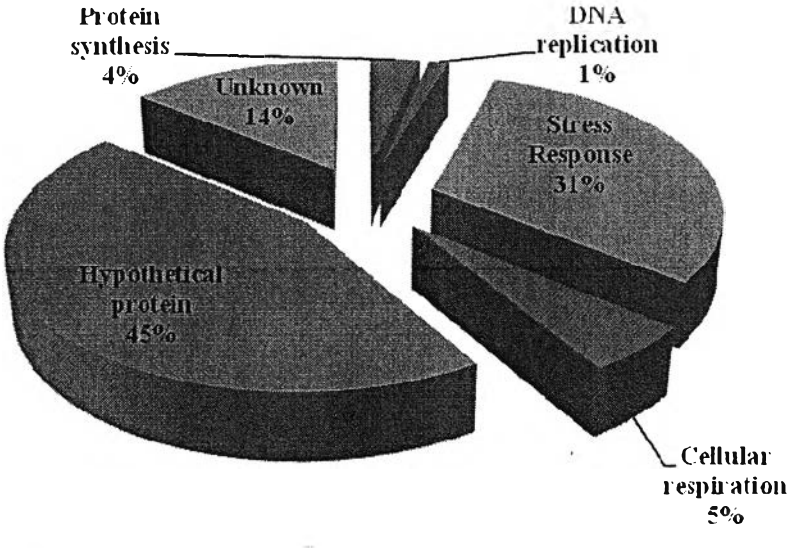

Figure 30. The functional percentage distribution of the genes up-regulated in *P. insidiosum* strain PC7 was grown at 27°C condition (150 genes in total).

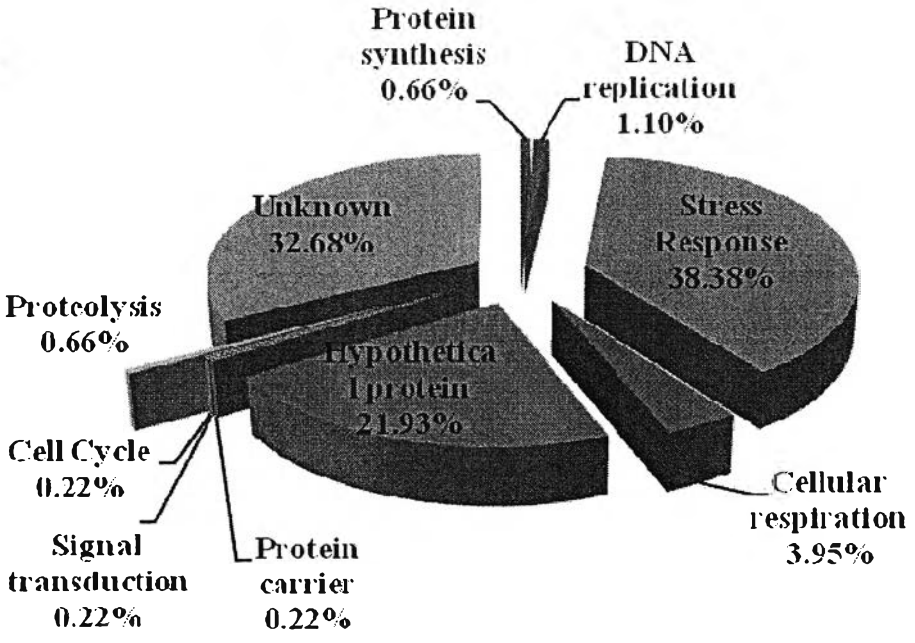

Figure 31. The functional distribution percentage of the genes up- regulated in *P. insidiosum* strain PC7 was grown at 37°C condition (456 genes in total)

3. Differential expression screening

3.1 Validation of reference gene for reference control in gene expression, semi – quantitative reverse transcriptase (RT) - PCR procedure

The transcriptional quantification level of each gene could be measured by semi – quantitative RT – PCR method. This method is well known that relative quantification is easier to perform and the expression level of the target genes is normalized, preferentially by using a reference gene. The reference (housekeeping) genes have been described in the previous literature and are used at different frequencies (201-205). In this study, the rRNA PCR product (Figure 32A) of both temperature (27°C & 37°C) conditions revealed stable intensity of band at 30 amplification cycles when semi – quantitative RT - PCR was performed (Figure 32 B). This result indicated that rRNA was suitable for a reference control for further gene expression procedure.

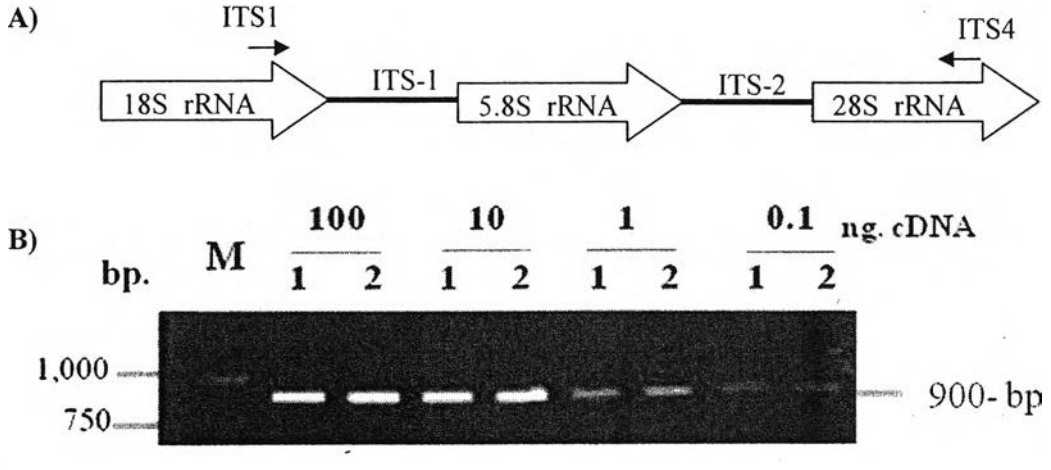

Figure 32. A : Annealing site for the ITS1 and ITS4 primers; B : The 900-bp band represented rRNA PCR product from serial concentration of cDNA templates (100 ng. – 0.1 ng) at 30 amplification cycles. Lane M = 1 kb DNA ladder. No.1 = 27°C cDNA template, and No.2 = 37°C cDNA template.

3.1 Semi – quantitative PCR amplification

Three genes (*COX* II, *TUB*, and *CHI* II) were chosen to measure in this study under the requirements; candidate genes were (i) virulence factor in other pathogenic microorganisms, (ii) classified as temperature responsive genes by semi – quantitative RT-PCR, or (iii) identified in the 37°C library. There are 2 specific primers had just designed and completed the PCR condition, as described in the Materials and Methods part. The total RNA from *P. insidiosum* strain PC7, grew at 27°C & 37°C conditions, were converted to cDNA. The four specific primers were tested with genomic DNA and cDNA of *P. insidiosum* strain MTPI19 and PC7 using conventional PCR amplification. After agarose gel electrophoresis, the size of β – tubulin, cytochrome c oxidase subunit II and chitin synthase subunit II PCR product were 600, 600 and 500 - bp, respectively (Figure 33). All of them were sequenced and compared with the databases in GenBank. The result of BLASTN for *TUB* gene, the amplicon was homologous with partially β – tubulin of *Pythium* spp. such as *P. catenulatum* (DQ071303), *P. zingiberum* (DQ071349) and *P. myriotylum* (DQ071323). While the homology of *COX* II and *CHI* II – PCR product was 99% with *P. insidiosum* (AF196597) and *P. insidiosum* (DQ116416), respectively.

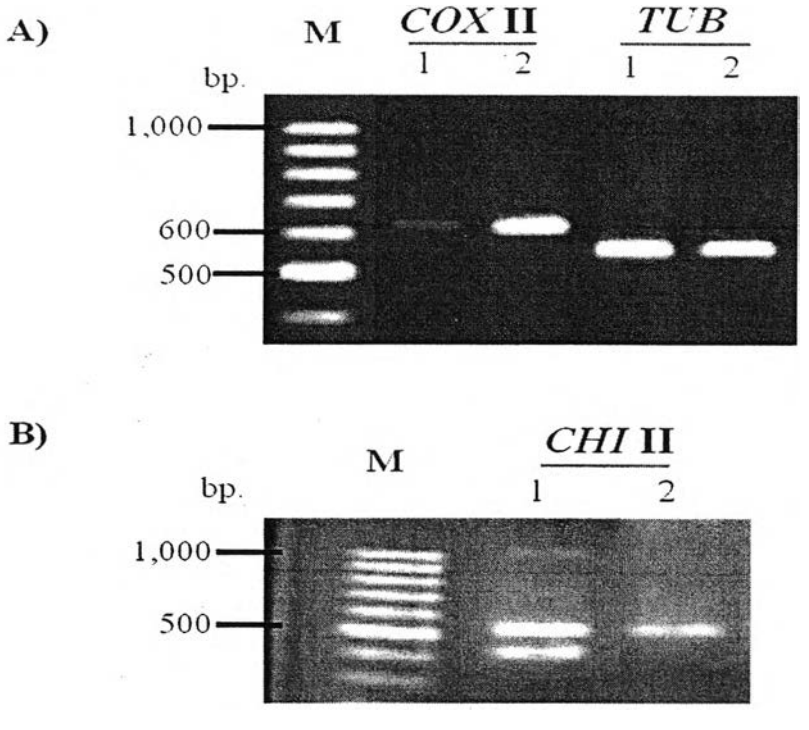

Figure 33. Comparing PCR product size between cDNA and genomicDNA (gDNA) of *P. insidiosum* templates by agarose gel electrophoresis. A) The 600-bp band represented *COX* II and *TUB* PCR product. Lane M = 100 bp. DNA ladder marker. No1 = cDNA template and No.2 = genomic (g)DNA template., B) The 500-bp band of *CHI* II PCR product. Lane M = 100 bp. DNA ladder marker. No1 = gDNA template and No.2 = cDNA template.

The semi – quantitative PCR demonstrated the expression level of each candidate genes that at the equal concentration of cDNA templates, *COX* II PCR product size approximately 600-bp was appeared from 100 pg at 27°C condition cDNA template whereas the intensity of band at 37°C condition cDNA template still appeared at 50 pg cDNA concentration (Figure 34).

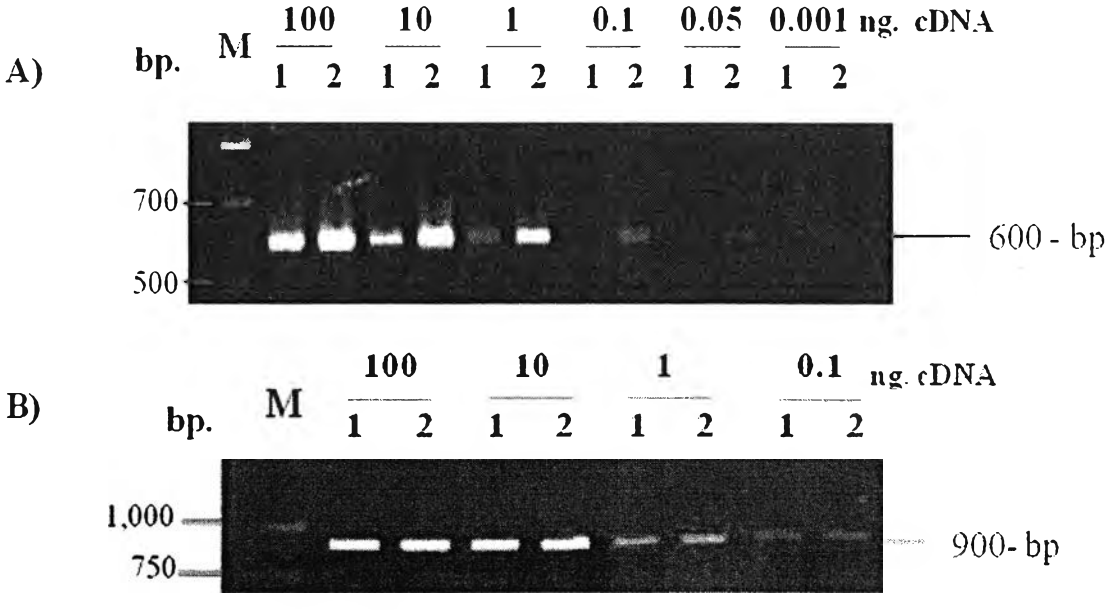

Figure 34. The semi–quantitative PCR of *COX* II and 28S rRNA at 30 amplification cycles using agarose gel electrophoresis analysis. A) 600-bp band represented *COX* II PCR products and B) the 900-bp band represented amplicon of internal cDNA control, from serial dilution cDNA template (100 ng – 0.01 ng). Lane M = 1 kb DNA ladder marker. No.1 = 27°C condition cDNA template, and No. 2 = 37°C condition cDNA template.

Similarity result to that obtained from *COX* II, the minimum cDNA concentration at 27°C condition cDNA template was 1 ng whereas 600-bp band size of *TUB* gene were appeared till 100 pg at 37°C condition cDNA concentration (Figure 35). The semi - quantitative expression analysis of *COX* II and *TUB* gene were performed with 28S rRNA, as a reference control.

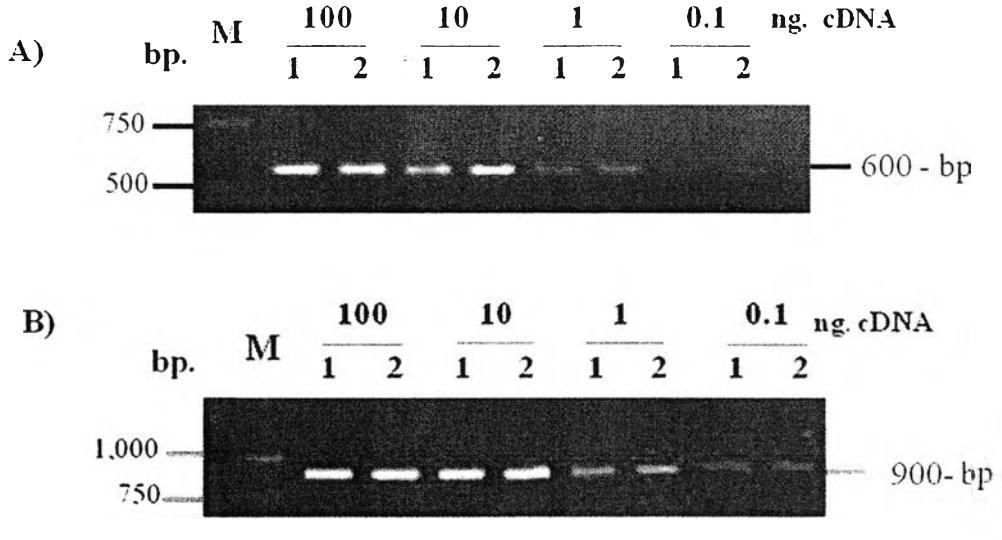

Figure 35. The semi – quantitative PCR of *TUB* gene and rRNA at 35 amplification cycles using agarose gel electrophoresis. A) 600-bp band represented *TUB* PCR products and B) 900-bp band represented 28S rRNA PCR product internal cDNA control, from serial dilution cDNA template (100 ng – 0.1 ng). Lane M = 1 kb DNA ladder marker. No.1 = 27°C condition cDNA template, and No. 2 = 37°C condition cDNA template.

3.3 Temporal expression of candidate genes by Real time reverse transcriptase - PCR (Real time RT – PCR)

Real time RT - PCR was conducted to confirm the presence of candidate genes represented their differential mRNA expression. The sixteen strains of *P. insidiosum* were a representative of 3 phylogeographic preferences clades, based on ITS and IGS. They were determined transcriptional quantification of three genes (*COX* II, *TUB*, and *CHI* II) level using quantitative real time RT - PCR procedure

The two specific primer pairs designed in this study fulfill all the requirements for PCR amplification. Net primer software analysis did not reveal

primer hairpins and dimers. The specificity of each primer pairs were checked using cDNA prepared from *P. insidiosum* strain PC7 at 27°C and 37°C conditions as a template. The real time RT – PCR products generated with each primer pairs and were confirmed by running on 1.5% agarose gel electrophoresis. A single band was observed in each lane with an appropriated size, indicating that the primers for each gene successfully amplified the target gene from cDNA samples (Figure 36). These PCR products were sequenced and confirmed to be specific product. Moreover, the PCR products of *COX* II and rRNA were submitted to GenBank databases. The lists of GenBank accession number were given in Table 17.

Furthermore, melting curve or dissociation curve analysis of each candidate genes were performed to ascertain whether minor nonspecific products were accumulated. The Melting curve is used to monitor the melting temperature (*T*m) of the amplicon. The analysis of the *T*m is dependent on the G + C content, sequence length and compositional variation in the nucleotide bases. A melting curve analysis of post amplification revealed that *COX* II and *TUB* specific primer pairs produced a single peak (Figure 37B & 37C) in the dissociation curve, indicating that no nonspecific products were amplified. Hence, it was established that *TUB* specific primers specifically amplify β – tubulin gene of *P. insidiosum* without producing any significant amount of nonspecific products. There are 2 peaks were generated from 28S rRNA and *CHI* II specific primers (Figure 37A & 37D) in melting curve analysis but the left peak give a peak with lower melting temperature. These might suggested the primer – dimer artifact because a single band of each primer pairs were revealed on 1.5% agrose gel electrophoresis whereas the negative control did not appeared

(Figure 36). In addition to confirming the PCR products of real time PCR, these products were sequenced and analyzed with GenBank databases.

Table 17. GenBank accession numbers of sixteen isolates *P. insidiosum* isolates.

Isolated Code	GenBank Accession	
	ITS	*COX*II
MTPI 19	ATCC58643	GQ451569
MTPI04	GQ475490	GQ451571
PAC2	FJ917396	GQ451572
PC1	GU812440	GU479367
PC2	FJ917390	GQ451589
PC5	FJ917389	GQ451590
PC6	GQ260125	GQ451574
PC7	GQ260120	GQ451588
PC10	FJ917395	GQ451575
PC11	-	GU479368
PCM1	FJ917393	GQ451587
PMS1	GQ260123	GQ451586
PR2	GQ260124	GQ451585
PECM3	EF016903*	GQ451577
PECM8	EF016891*	GQ451581
PECM14	EF016867*	GQ451597

* Supabandhu J, Fisher MC, Mendoza L, Vanittanakom N. Isolation and identification of the human pathogen *Pythium insidiosum* from environmental samples collected in Thai agricultural areas. Med Mycol2008 Feb;46(1):41-52.(143)

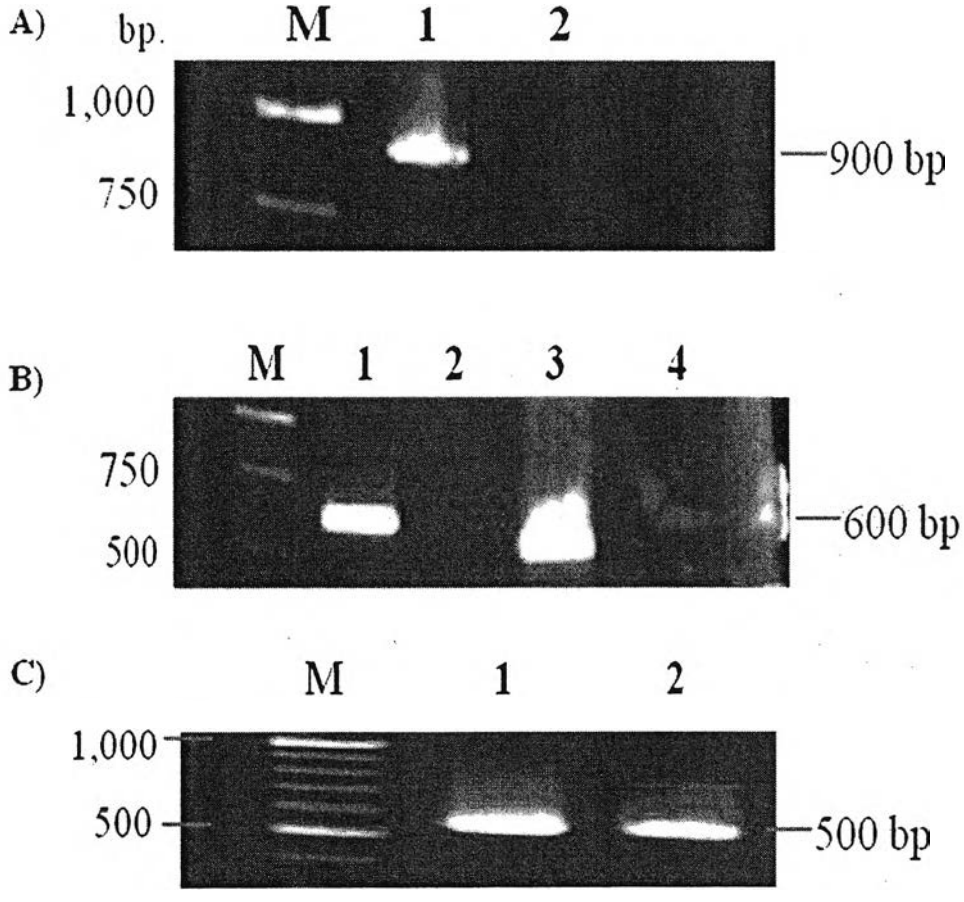

Figure 36. Real time RT – PCR products of 3 candidate genes were checked by 1.5% agarose gel electrophoresis (100V). A; Lane M = 1 kb ladder marker, 1 = 900-bp product of 28S rRNA, and 2 = water (negative control). B; Lane: M = 1 kb Ladder marker, 1 = 600 – bp PCR product of *COX* II gene, 2 = water (negative control), 3 = *TUB* gene PCR product 600 bp, and 4 = water (negative control). C; Lane M = 1 kb ladder marker, 1 = *CHI* II gene PCR product 500 - bp, and 2 = gDNA of *P. insidiosum* (positive control).

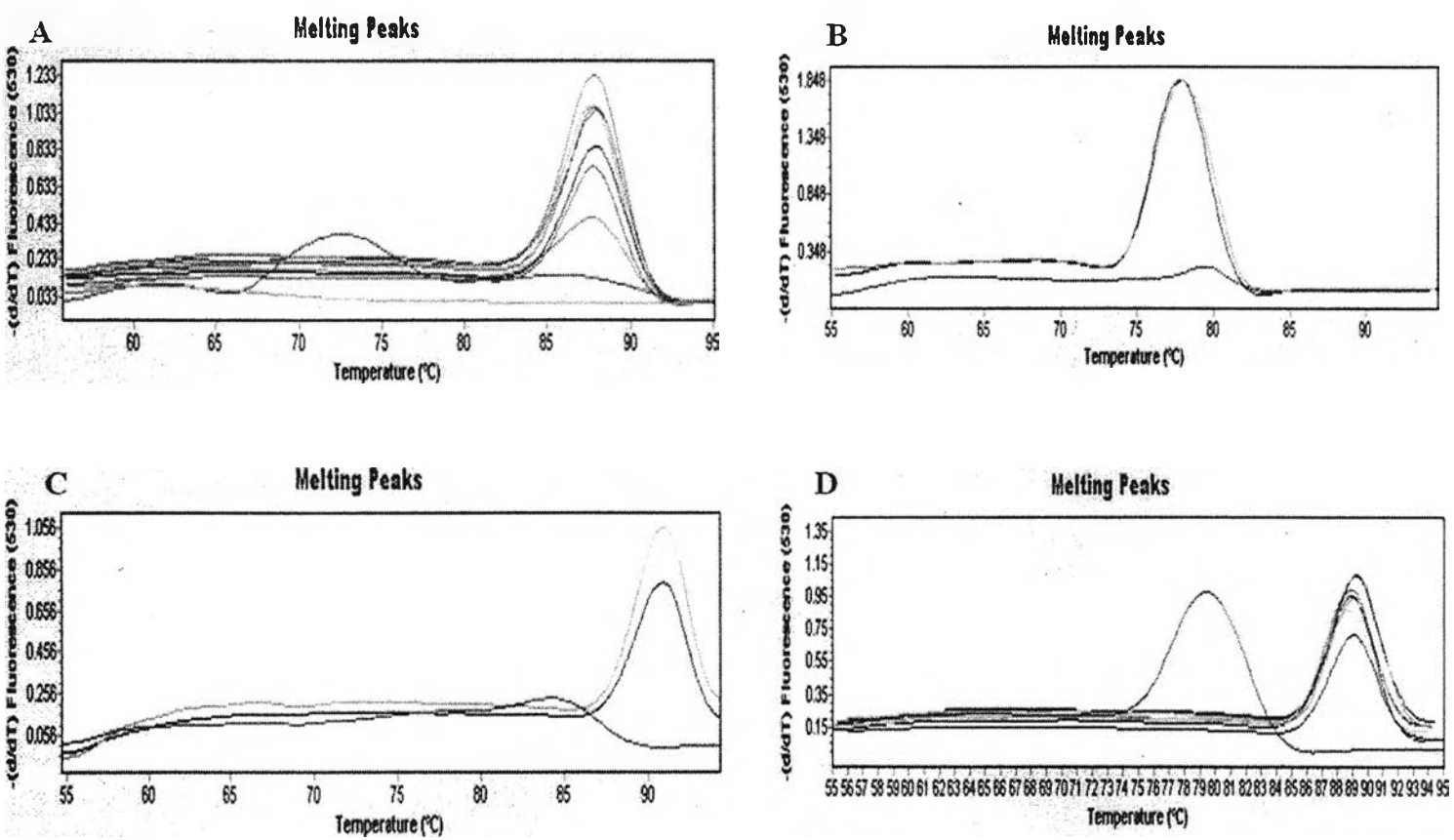

Figure 37. Melting peak analysis of each gene amplification using genes specific primer by real time RT – PCR. A; 28S rRNA, B; *COX* II gene, C; *TUB* gene, and D ; *CHI* II gene.

The fragments of rRNA, *COX* II, *TUB*, and *CHI* II real time RT - PCR products demonstrated the *T*m value, were approximately 88.4°C, 78.7°C, 90.2°C and 88.9°C, respectively (Table 18).

Table 18. The average melting temperature of each genes with genes specific primer pair (n = 32).

Genes	rRNA	*COX* II	*TUB*	*CHI* II
***T*m (°C)**	88.4±0.09	78.7±0.09	90.2±0.3	88.9±0.4

To measure the level of candidate genes expression, the real time RT – PCR was performed with 32 cDNA samples of 16 strain *P. insidiosum* at 27°C and 37°C conditions. The quantification strategy is the principal marker in gene quantification. In this study, relative quantitative strategy is performed in real time RT – PCR. The relative quantification measures the relative change in mRNA expression levels. This strategy is based on the expression levels of a target gene versus a reference/endogenous gene to normalization.

3.3.1 Identification and validation of endogenous control gene

The normalization of a reference control in relative quantification experimental design is required. Because of endogenous control expression levels should also have the least RNA level variation when tester is subjected to different experimental conditions. The result from semi - quantitative PCR analysis represented that the 28S rRNA was the best choice to be an endogenous control. Therefore, all 32 DNA – free total RNAs (at 27°C and 37°C growth conditions of

16 isolates *P. insidiosum*) were used as a template. The amplification process was revealed in amplification curve image which the cycle number was shown along the X – axis and the arbitrary fluorescence units (actually these are fold increase over background fluorescence) are shown on the Y-axis (Figure 38).

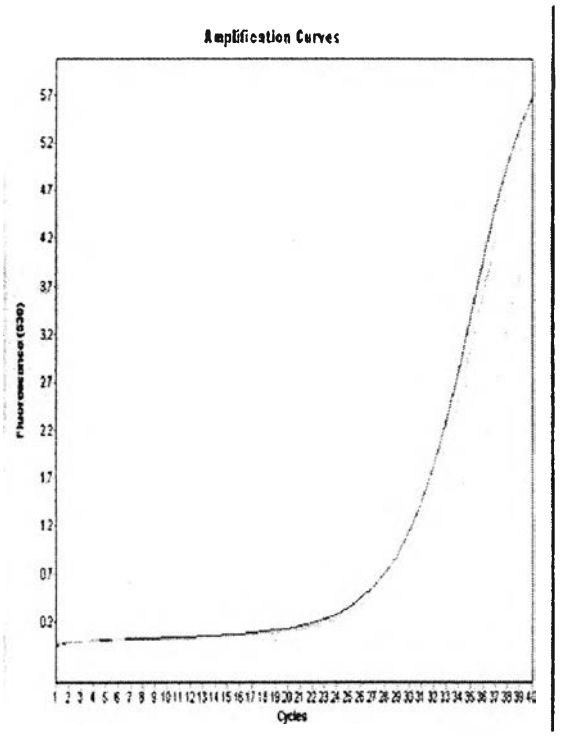

Figure 38. The amplification curve of 28S rRNA. Blue line and grey line were cDNA at 27°C & 37°C conditions, respectively. The red line was no cDNA template (water = negative control), then these products were checked band size on 1.5% agarose gel electrophoresis.

The statically calculation, a Graphad Prism 5 program, was used to analyze the data which repeated at least three times of real time amplification system. Following the Table 19, the average cycle threshold (C_T) value of rRNA at 27°C condition and 37°C condition were 26.68 and 26.27, respectively, and

mean ± standard deviation (SD) were 26.68 ± 3.95 and 27.67 ± 3.6. The student's *t* test indicated that the cycle threshold of 28S rRNA between 27°C and 37°C conditions were not different with $p = 0.3201$ (significant = $p < 0.05$). This result was summarized and presented in Table 19 and Figure 39. Thus the data suggested that rRNA was a reference control, and used to normalize differences in the amount of cDNA that was loaded into real time RT – PCR experiments.

Table 19. Data analysis and validation of endogenous control (28S rRNA gene) from 16 strains of *P. insidiosum* growth temperature at 27°C and 37°C conditions.

Subjects	Temperature (°C)	
	27	**37**
Minimum (C_T)	19.07	21.56
Maximum (C_T)	35	35
Mean (C_T)	26.68	26.27
Std. Deviation	3.95	3.6
Std. Error	1.01	1
Paired *t* test (p <0.05)		
P value	0.3201	
P value summary	Not significant	

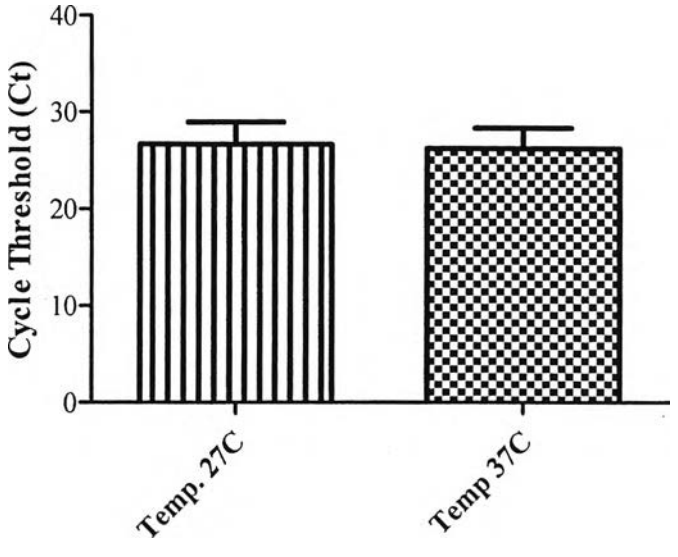

Figure 39. Data analysis of the expression of 28S rRNA (endogenous control) under different temperature (27°C & 37°C) conditions. The cycle threshold was represented in average ± SD value of the 16 isolates. Y axis ; cycle threshold (C_T), vertical box ; 27°C condition, table box; 37°C condition and T bar; the standard deviation (SD).

3.3.2 Quantitative analysis of *COX* II, *TUB* and *CHI* II gene expression using Real time RT-PCR on individual samples

The transcription profiling using real time RT – PCR assays was then conducted with these 3 candidate genes (*COX* II, *TUB* and *CHI* II), in different temperature conditions. The transcripts of 3 candidate genes were analyzed in the individual *P. insidiosum* strain samples by direct comparison of their cycle threshold (C_T), assuming equal C_T for equal transcript number since all RT – PCR reactions were performed with equal quantity of total RNA. In order to ensure the precision and reproducibility, PCR was repeated for three times for all the samples to obtain the C_T value. The PCR products which were amplified were bound to

SYBR Green I and contributed to the signal generated which could be seen by amplification curve (sigmoidal plots).

The results showed that *TUB* and *CHI* II genes were ranked according to their gene expression stability measure in both temperature conditions. In Table 20, the C_T value at 27°C condition was ranged from 14 – 32 (mean ± SD = 21.6 ± 4.4) while C_T value at 37°C condition was span from 14 – 27 (mean ± SD = 19.4 ± 3.4) of *TUB* gene. While *CHI* II gene was exhibited the similar results with *TUB* gene that both temperature (27°C and 37°C) conditions were a constant expression, mean ± SD = 24.6 ± 7.1 and 22.3 ± 5.9, respectively (Table 20). In contrast, only *COX* II gene was also highly expressed (Table 20) as indicated by C_T values at 37°C condition that ranged from 11 – 19 cycles (mean ± SD = 14.8± 2), but it exhibited rather high dispersion over the growth at 27°C condition as indicated by span from 10 – 35 cycles (mean ± SD = 19.7 ± 8).

Table 20. Summary data of cycle threshold (C_T) from 16 strains *P. insidiosum* (Table 7) growth at 27°C and 37°C conditions

	***COX* II**		***TUB***		***CHI* II**	
	27°C	37°C	27°C	37°C	27°C	37°C
Minimum	10.16	11.83	14.96	14.87	15.53	14.64
Maximum	35	18.66	32.13	27.82	37.8	32.56
Mean	19.7	14.8	21.6	19.4	24.56	22.3
SD	8	2.4	4.4	3.4	7.1	5.9

The advantage of using reference genes for normalization was further analyzed comparing expression results after normalization. The C_T values of three candidate genes provided from real time RT – PCR instrumentation. The changed in expression of these target genes normalized to rRNA was monitored. The data were analyzed using equation, where $\Delta\Delta C_T = (C_{T,\ 27°C} - C_{T,\ 28S\ rRNA}) - (C_{T,\ 37°C} - C_{T,28S\ rRNA})$. The mean value, standard derivation (SD), and coefficient of variation (CV) are then determined from the triplicate experiments. The schematic presentation of the quantitative real time RT - PCR and the relative quantification data of gene expression of 37°C condition were illustrated in Table 21.

Table 21. The fold change of the candidate temperature sensitive genes (*COX* II, *TUB* and *CHI* II gene) relative to the endogenous control gene (28S rRNA) at two different temperature conditions (27°C and 37°C).

Gene	Mean fold change in gene expression		SD of C_T		*p* value ($p<0.05$)*
	27°C	37°C	27°C	37°C	
COX II	0.004375	2.52	5.581	4.41	0.0347*
TUB	0.000625	-0.21	4.278	2.9	0.4103
CHI II	0.000625	0.05813	4.877	4.56	0.4752

* The data was significantly at $p<0.05$.

For comparison in each strain, the result showed no significant difference in all 3 candidate genes expression (data not shown).

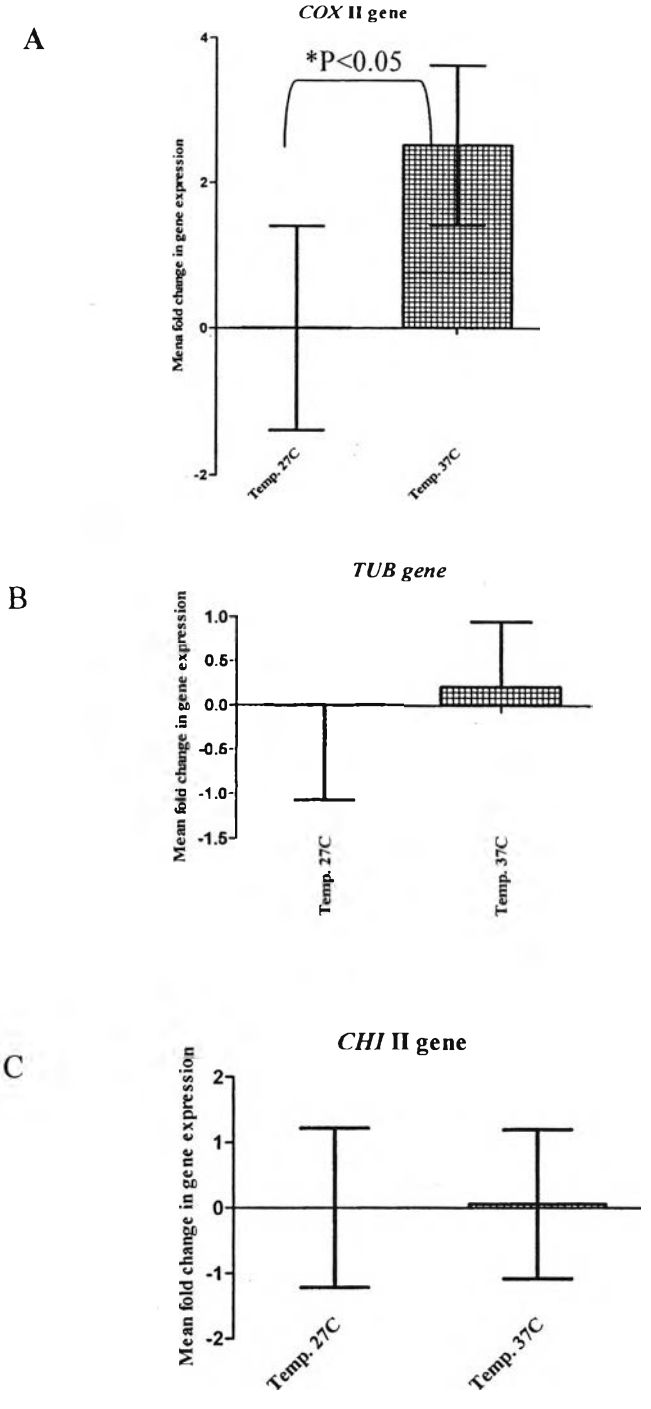

Figure 40. The relative expression of gene encoded: cytochrome oxidase II, β - tubulin, and chitin synthase II (*COX* II (A), *TUB* (B) and, *CHI* II (C) gene, respectively) at 27°C condition and 37°C condition. Boxes represent mean of fold change. Lower and upper boundaries of the box indicate the standard error (SEM).

According to the Table 21, we were able to verify the relevance of the increasing or decreasing in mRNA level, which changed in the different temperature conditions. Only candidate effecter mRNA for *COX* II gene was significantly up – regulated during growth of *P. insidiosum* at high temperature (37°C) (p = 0.0347, p <0.05). This result corresponded with semi – quantitative PCR in Figure 34 and Figure 35. Whereas the expression level of *TUB* gene seemed to be down – regulated at 37°C condition but no statistically significance was found between 27°C condition and 37°C condition (p = 0.4103). Similarity, the *CHI* II gene expression was not significantly different during growth at 37°C condition either or 27°C condition (p = 0.4752). The average fold change ± SD of *COX* II, *TUB* and *CHI* II gene at temperature 27°C and 37°C conditions were shown in Figure 40.

3.3.3 Analysis of the relationship between *COX* II, *TUB* and *CHI* II gene expression and 3 phylogeographic regions of *P. insidiosum*

As mentioned previously in Chapter I and II (Introduction and Review literature), that *P. insidiosum* isolates from worldwide are classified into 3 clades based on ITS and IGS studies. The relationship between temperature sensitive genes expression within the clades were investigated in this study. The result demonstrated in the Table 22 that no difference of target genes expression within each clade were found (p>0.05). On the other hand, the consensus between gene expression and 3 clades were examined. The only *CHI* II gene was remarkable that this gene was up – regulated in clade 2 and 3 higher significantly (p<0.05)than clade 1, when cultivated at 37°C condition (Figure 41). But at 27°C condition, no differences of *CHI* II gene expression were found in all 3 clades.

Table 22. Summary of relationship analysis within phylogeographic prevalence (in each clade) and between 3 clades.

Subjects	P value ($p < 0.05$)*		
	COX II	*CHI* II	*TUB*
Clade 1 : 27°C & 37°C	0.186	0.133	0.465
Clade 2 : 27°C & 37°C	0.117	0.135	0.417
Clade 3 : 27°C & 37°C	0.201	0.082	0.221
Clade 1 & clade 2 (37°C)	0.414	0.0282*	0.294
Clade 1 & clade 3 (37°C)	0.253	0.0345*	0.192
Clade 2 & clade 3 (37°C)	0.309	0.133	0.11

* The data is different significant.

Whereas no relationship between the expression level of *COX* II and *TUB* gene and each clade were found $p>0.05$ (Figure 41). Moreover, the relationship between gene expression level and the original source of *P. insidiosum* isolates were analyzed in this study. Fortunately, we had *P. insidiosum* strains from clinical isolates and environmental isolates. The result of analysis was shown in Table 23 which demonstrated that the gene expression level from both different original sources at 37°C condition were not different.

Table 23. The relationship analysis between different of original sources of *P. insidiosum* isolates.

	P value ($p < 0.05$)*		
	COX II	*CHI* II	*TUB*
Environment & Clinical isolates (27°C)	0.401	0.192	0.400
Environment & Clinical isolates (37°C)	0.147	0.278	0.056

* The data is different significant.

4 Phylogenetic relationship of 33 strains of *P. insidiosum*

4.1 Phylogenetic tree based on *COX* II gene

Thirty-three isolates of *P. insidiosum*, three other *Pythium* spp, one of *Ph. sojae* and *L. giganteum* as out groups were analyzed in this study. The size of *COX* II amplified products ranged from 558-568 bp, approximately. The nucleotide sequences were confirmed as *COX* II mtDNA of *P. insidiosum* against the data in GenBank and they appear to encode functional product when translate into amino acid using BLASTX. The ratio of conserve region to polymorphic region was 3:2 (356 bp and 262 bp, respectively). The transition, transversion, deletion and insertion were detected. The percentage of this polymorphism was 11.1% in AT-rich partial *COX* II sequences.

The *COX* II - phylogenetic tree was generated using Neighbor – Joining (NJ) analysis (Figure 42). Its pairwise sequence distance ranged from 0.0000 to 0.0608. Based on 10,000 replicates of bootstrap supporting, the tree of *COX* II revealed three major clusters among these 33 *P. insidiosum* isolates: A_{TH}, B_{TH}, and

CT_H clusters (Figure 42). Cluster B_{TH} and CT_H included major isolates, (93.9 %), from both human and environment sources whereas the left derived from equines from USA (MTPI19 and MTPI04 isolates) were grouped together as cluster A_{TH}. The two Thai clusters, B_{TH} and CT_H, could be divided into three and two sister groups, respectively, with bootstrap support values over 50% as indicated in Figure 42 (named as $B1_{TH}$, $B2_{TH}$, $B3_{TH}$, $C1_{TH}$, and $C2_{TH}$, respectively).

4.2 Phylogenetic tree based on ITS region

The ITS amplified regions of 12 *P. insidiosum* and three other *Pythium* species were sequenced in this study whereas the other 18 ITS sequences were fetched from GenBank (Table 24) (143). The PCR products of *P. insidiosum* isolates varied highly in size from 871-898 bp whereas slightly shorter of 825-836 bp. PCR fragment was found in other species of *Pythium*. Sequence variation among the aligned ITS sequences of *P. insidiosum* was due mostly to transitional substitutions (94.2 %), but 5.8% insertions and deletions were also found. Large insertions and deletions mainly occurred when the other three *Pythium* spp., *Ph. sojae,* and *L. giganteum* were added to alignment. The amount of 303 bp conserved bases and 636 bp variable nucleotide sequences were detected. The variation of ITS region was 47.72 %.

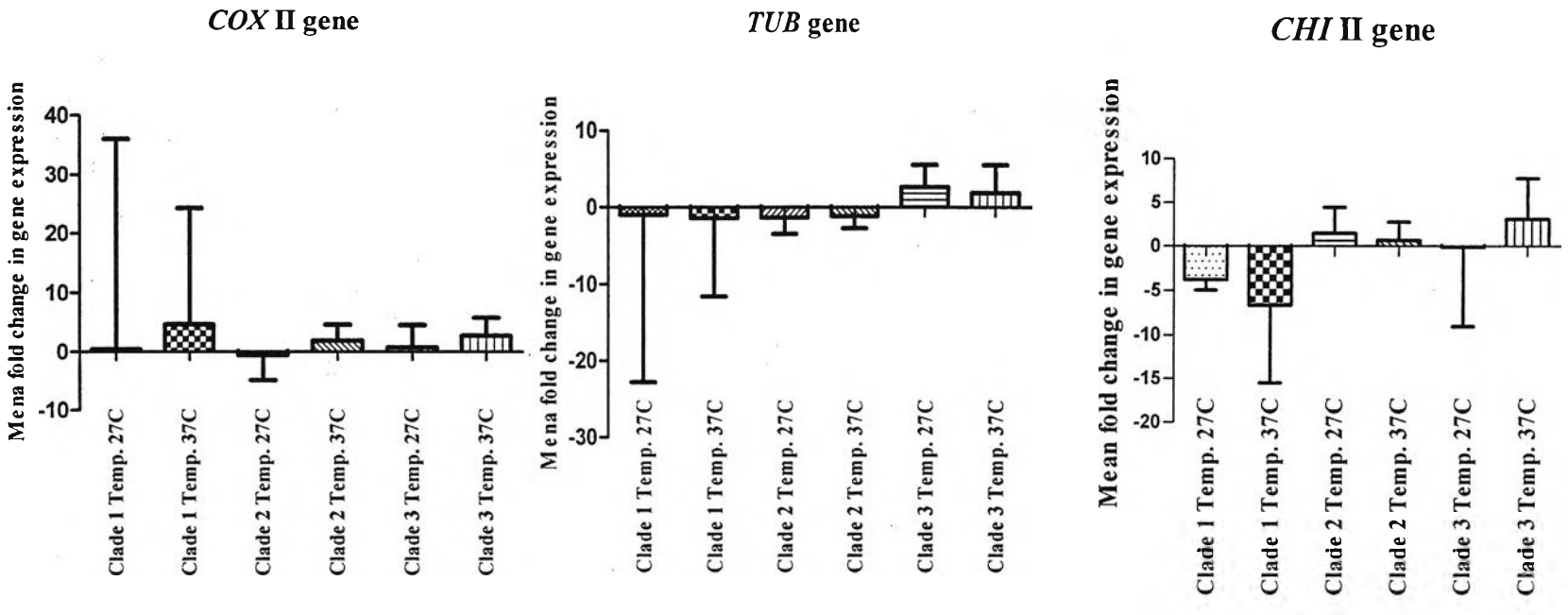

Figure 41. The relationship analysis gene expression of 3 candidate genes of *P. insidiosum* between in 3 clades at 27°C & 37°C condition. Boxes represent mean of fold change. Lower and upper boundaries of the box indicate the standard error (SEM).

The pairwise sequence distances of the ITS regions among *P. insidiosum* isolates ranged from 0.0000 to 0.2832. The phylogenetic relationship among *P. insidiosum* isolates from human and environment sources compared with those of other *Pythium* spp, *Ph. sojae* and *L. giganteum* showed that the clinical and environmental *P. insidiosum* isolates were grouped into two strongly supported clusters (clusters B_{TH} and C_{TH}, Figure 43) values (87% and 98% bootstrap values, respectively).

The groupings of *P. insidiosum* isolates in the ITS tree were topologically similar to those of the *COX* II tree. Considering the two NJ trees (Figures 43 and 42), all *P. insidiosum* isolates were phylogenetically clustered together as a monophyletic group and placed distantly from other *Pythium* spp. The results showed that both *COX* II and ITS DNA sequences used for phylogenetic analyses placed *P. insidiosum* in three major clusters with very high bootstrap supports. Likewise, the clusters B_{TH} and C_{TH} from both *COX* II and ITS region were composed of Thai strains including 12 cases from human (ten disseminated pythiosis, two ocular pythiosis) and 19 from environment. Moreover, both *COX* II and ITS trees grouped MTPI19 and MTPI04 isolates into the cluster A_{TH}. These two isolates were recovered from equine pythiosis in the Americas (Costa Rica and Texas, respectively). Within the cluster B_{TH} of the *COX* II tree revealed several subclusters with much higher resolution than those observed in the ITS tree (Figures 42 and 43).

Table 24. GenBank accession numbers of 33 *P. insidiosum* isolates.

Isolates	GenBank Accession Numbers	
	ITS	*COX* II
MTPI04	GQ475490	GQ451571
MTPI12	GQ475491	GQ451570
MTPI19	ATCC58643	GQ451569
PC2	GQ260125	GQ451589
PC3	GQ260122	GQ451573
PC5	GQ260120	GQ451590
PC6	FJ917389	GQ451574
PC7	GQ260124	GQ451588
PC10	FJ917395	GQ451575
PCM1	FJ917393	GQ451587
PCM2	GQ260121	GQ451591
PEC1	FJ917392	GQ451576
PECM3	EF016903*	GQ451577
PECM5	EF016896*	GQ451578
PECM6	EF016878*	GQ451579
PECM7	EF016897*	GQ451580
PECM8	EF016891*	GQ451581
PECM9	EF016898*	GQ451602
PECM10	EF016899*	GQ451603
PECM11	EF016906*	GQ451582
PECM12	EF016909*	GQ451596
PECM13	EF016890*	GQ451594
PECM14	EF016867*	GQ451597
PECM15	EF016868*	GQ451598
PECM16	EF016869*	GQ451599
PECM17	EF016873*	GQ451600
PECM18	EF016874*	GQ451601
PECM20	EF016888*	GQ451595
PECM21	EF016911*	GQ451593
PECM22	EF016885*	GQ451592
PMS1	GQ260123	GQ451586
PMR2	GQ260118	GQ451583
PMR3	GQ260119	GQ451584
ECU1	FJ917394	GQ451568

* Supabandhu J, Fisher MC, Mendoza L, Vanittanakom N. Isolation and identification of the human pathogen *Pythium insidiosum* from environmental samples collected in Thai agricultural areas. Med Mycol2008 Feb;46(1):41-52.(143)

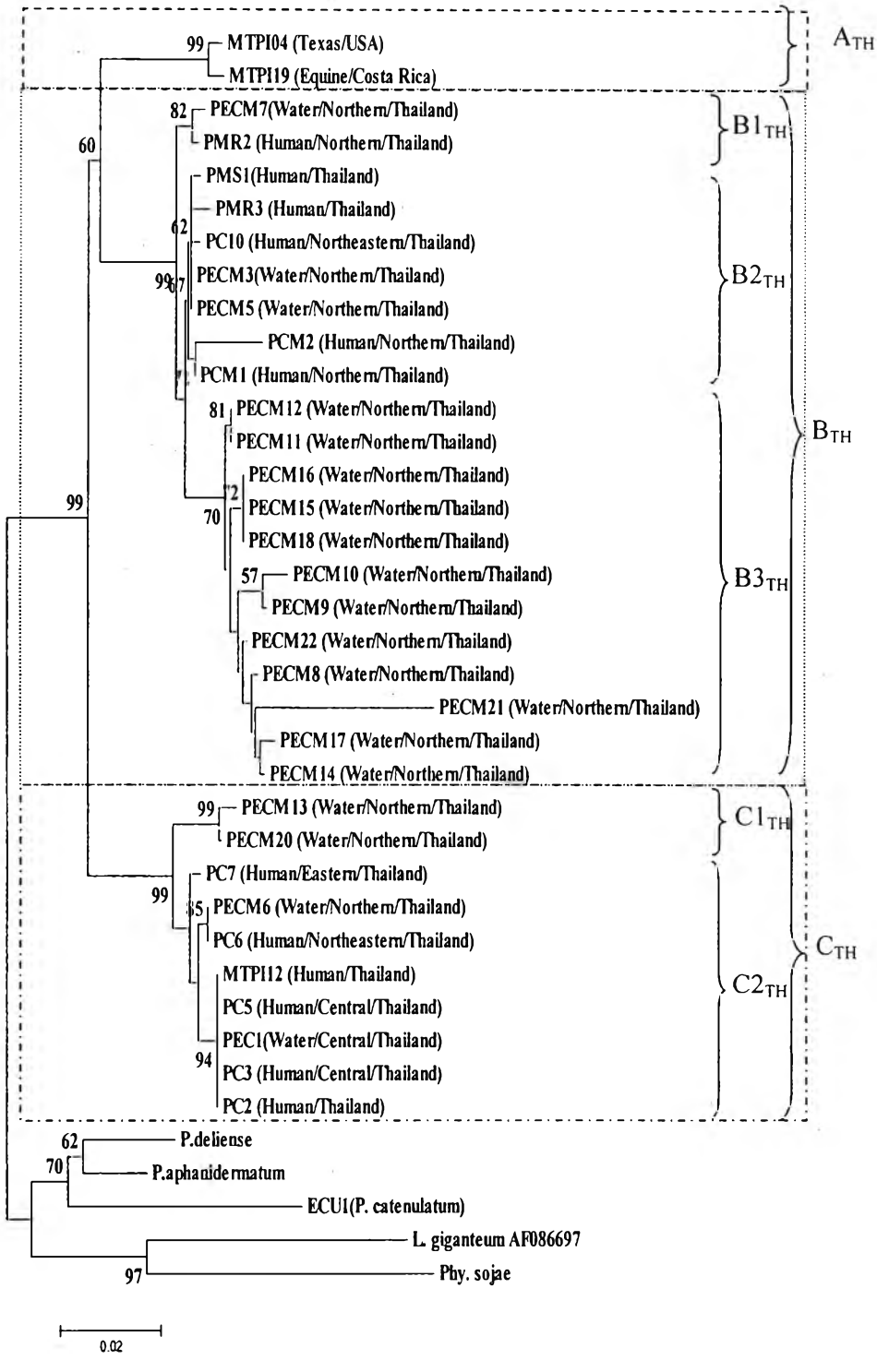

Figure 42 Neighbor Joining (NJ) tree based on cytochrome oxidase II (*COX*II). Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown. Phylogenetic analyses were conducted in MEGA4.

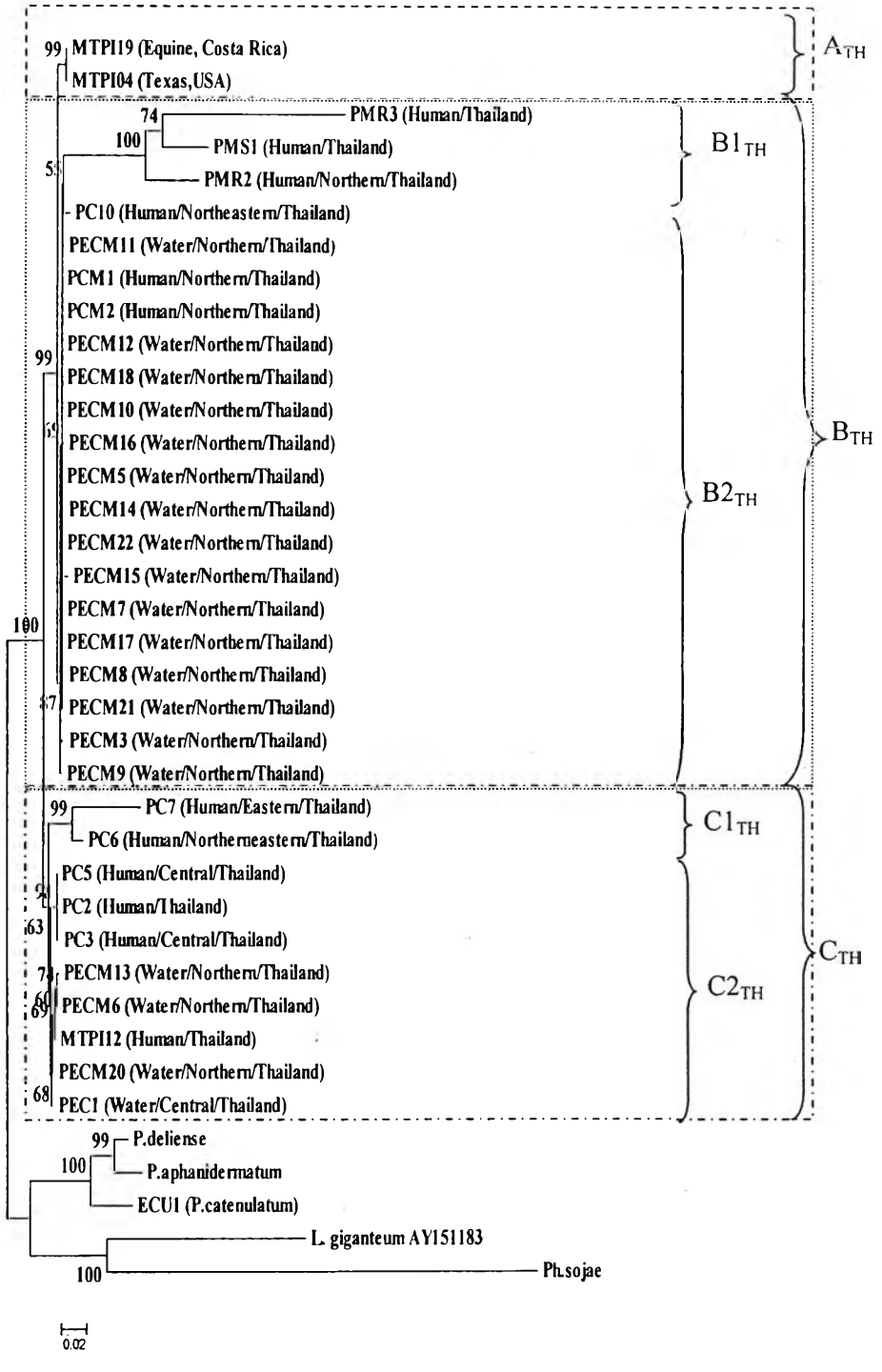

Figure 43 Neighbor Joining (NJ) tree based on ITS region. Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown. Phylogenetic analyses were conducted in MEGA4.