CHAPTER III

MATERIALS AND METHODS

1. Microorganisms and identification

1.1. Microorganisms

Sixteen isolates of *P. insidiosum* from clinical (human and animal) and environmental sources were used in this study as listed (Table 7). All of these aquatic fungus like organisms were isolated from the proven cases of pythiosis, and confirmed by Western blot analysis. They were tested for their ability of zoospore production. To maintain these *P. insidiosum* isolates, Sabouraud dextrose agar (SDA, Cat. 64494, Bio-Rad, USA), and potato dextrose agar (PDA, Cat. CM0139, Oxoid, England) were used and all of them were incubated at 37°C. To prepare these isolates for gene characterization at 27°C, the submerged colorless to white-cream fungal-like colonies growing from SDA at 37°C were subcultured on SDA and incubated for 7 days at 27°C. The subculturing process at 27°C was repeated at least 2–3rounds.

Each of these active cultures was cultivated in flask containing 300 mL of Sabouraud dextrose broth (SDB, Cat. 238230, Difco, USA), incubated at 37°C or 27°C, depending on the original incubation temperature and shake at 155 rpm. for 5 days in order to extract DNA and RNA which were used for further analysis.

1.2. Identification

1.2.1. Zoospore production (Modified from Mendoza , 1993 (97, 130))

Sporangia and motile biflagella–type zoospore production were performed in an induction medium (See in Appendix A). In detail, small pieces of *P. insidiosum* from 4 - 5 days old on SDA (Figure 16A) was inoculated on Petri-dish containing boiled Savanna Grass (*Axonopus compresus Beauv*), and the plates were incubated at 37°C for 2 days (Figure 16B). After that, the parasitized grass blades were transferred to another Petri-dish containing induction medium (Figure 16C) and the plates were incubated 2 hr at 37°C in dark. The culture on these plates were activated under light for zoospore production for approximately 30 min. To examine the zoospores and zoosporangiums, drop of the induction media together with the parasitized glass blade were observed under light microscope (Figure 16D).

Table 7. Hosts/sources and geographical origin where *P. insidiosum* were isolated.

Isolate code	Host	Geographic origin
MTPI 19	Equine	Costa Rica
MTPI 04	Equine	USA
PAC2	Canine	Central part, Thailand
PC1	(vascular pythiosis) Human	Northwestern, Thailand
PC2	(vascular pythiosis) Human	Central part, Thailand
PC5	(ocular pythiosis) Human	Central part, Thailand
PC6	(ocular pythiosis) Human	Northeastern, Thailand
PC7	(vascular pythiosis) Human	Eastern, Thailand
PC10	(vascular pythiosis) Human	Northeastern, Thailand
PC11	(vascular pythiosis) Human	Northern, Thailand
PCM1	(vascular pythiosis) Human	Northern, Thailand
PMS1	(vascular pythiosis) Human	Northern, Thailand
PR2	(vascular pythiosis) Human	Central part, Thailand
PECM3	Irrigation area [a]	Northern, Thailand
PECM8	Field reservoir [a]	Northern, Thailand
PECM14	Household reservoir [a]	Northern, Thailand

[a]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 Mycol 2008 Feb;46(1):41-52 (143).

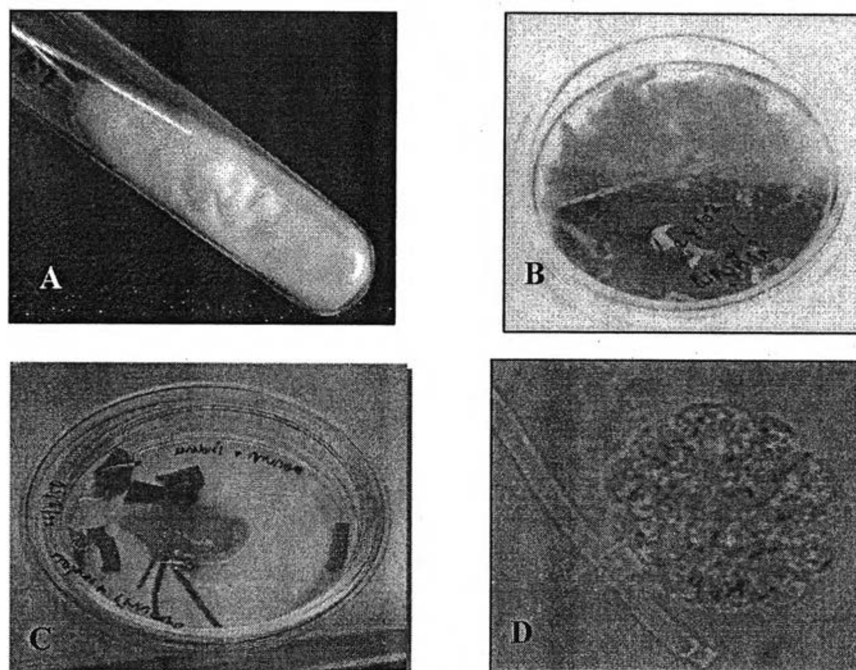

Figure 16. Zoosporogenesis of *P. insidiosum.* (A-C) Five day cultured - isolate was transferred to boil grass blades. After 2 days, the parasitized grass blades were transferred to induction medium; (D) Zoospore production inside the zoosporangium.

1.2.2 DNA extraction (Phenol extraction method)

Prior to DNA extraction, the 5 day old mycelial of *P. insidiosum* were treated with Thimerosal solution (Cat.T2299, Sigma, USA) to a final concentration of 0.02% (wt/v) for 30 min. The mycelial masses were filtered through the PES membrane filter with 0.22 μm pore size (Cat. 431118, Corning, USA) and ground in liquid nitrogen to produce 100% ruptured cells. DNA from filaments of each isolate was extracted using phenol – chloroform extraction method (188). The mycelial masses powder were extracted by adding 500 μl phenol–chloroform–isoamyl alcohol (25:24:1) and discharged phenol with twice of an equal volume of

chloroform – isoamyl alcohol (24:1). To precipitate DNA, three volumes of cold ethanol were added and incubated at -20°C overnight. After that, the suspension was centrifuged at 14,000 rpm for 15 min, and the supernatant was discarded. DNA pellets were air dried and resuspended in sterile distilled water. For quality and quantity of DNA, the absorbance at wavelength 260 and 280 nm was measured with a Bio - Rad SmartSpec 3000 Spectrophotometer (Bio – Rad, USA). DNA concentration was calculated using the following formula:

DNA concentration (ng/µl) = OD260 x dilution factor x 50

Purity of DNA was confirmed by electrophoresis and the ratio of *A*260/*A*280 at 1.8 – 2.0 is generally considered a high – quality DNA sample. DNA was aliquoted into small sterile tubes for storage (at -20°C) until use.

1.2.3 PCR amplification and nucleotide sequencing for identification of *P. insidiosum*

Amplification of the internal transcribed spacer (ITS) region of *P. insidiosum* was performed by the method of White *et al* (189). The forward primer, ITS1 and the reverse primer, ITS2 (Table 10), were used to amplify an 800-bp fragment of the ITS region. PCR reaction was performed in a 30 µL total volume consisting of 1× *Taq* DNA polymerase buffer (100 mM Tris-HCl at pH 8.3, 500 mM KCl, 20 mM $MgCl_2$, Enhancer solution), 0.2 mM concentration of each dNTP, 10 pmol of each primer, 1 U of *iTaq*^{tm} polymerase (iNtRON Biotechnology, Korea) and 100 ng of DNA template. Positive control was PCR reaction using 100 ng DNA of *P. insidiosum* strain CBS 574.85 (MTPI19). Negative control was PCR reaction using sterile distilled water without DNA template. The amplification

protocol was the following: initial denaturation step at 94°C for 3 min; 35 cycles of 94°C for 40 s, 55°C for 30 s, 72°C for 1min; and a final extension step at 72°C for 10 min. PCR amplification was carried out using ThermoHybrid PCR thermocycle (Ashford, Middlesex, UK).

The fragment of PCR products were sequenced by 'Macrogen'(Korea) using ABI PRISM® 377 DNA Sequencher by using a ITS1 and ITS2 primer; and the ABI BigDye kit. Each reaction was performed in a 20 μL total volume containing approximately 300 ng of template DNA, 1 μL of 3.2 μM ITS1 and ITS2 primers, 4 μL of ready reaction premix and 2 μL of BigDye sequencing buffer as described by the manufacturer. After thermocycling for 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extending at 60°C for 4 min, the extension products were purified by precipitation with ethanol acetate solution followed by washing with 70% ethanol. The samples were subsequently loaded on an ABI PRISM 377 DNA sequencher. In order to identify *P. insidiosum* isolates, each sequence was subjected to database search for similarity with available databases by BLAST web-based sequence analytical program.

2. Selection of Genes with Temperature-dependent Expression

Because there is the limitation genetic information of *P. insidiosum* which was available, it is necessary to find the sensitive and effective methods to identity the genes of this microorganism. In this study, two techniques were performed. The first method was to screen gene expression using cDNA library by Suppression Subtractive Hybridization method (SSH) and the second method was to use the primers available

from the database in GenBank of microorganisms closely related to *P. insidiosum* for specific gene amplification.

2.1. Suppression Subtractive Hybridization (SSH)

2.1.1 RNA extraction

The 5 day old of 37°C grown of *P. insidiosum* strain PC7 was inoculated in 300 ml. of SDB (Cat. 238230, Difco, USA) and incubated at 37°C in shaking incubator with shaking at 155 rpm for 5 days. The mycelial masses were then filtered using PES membrane filter with 0.22 μm pore size (Cat. 431118, Corning, USA) and ground in liquid nitrogen.

To extract RNA, hot phenol extraction method, as described by Köhrer and Domdey (190) was used. Briefly, 2 g of mycelial mass powder was mixed in RNA extraction solution (10 volumes of sodium acetate buffer (see Appendix A), 1/10 volume of 10% SDS (see Appendix A) and 1.2 volumes of saturated hot phenol (65°C) (see Appendix A)) in 50 mL conical tube for 5 min. During the mixing process, this suspension was vortexed for 30 s and alternated by 65°C incubation for 30 s, and centrifuged for 15 min at 3,500xg. The lower layer was discarded whereas the upper layer was re-extracted again by adding RNA extraction solution. At this step, the upper layer was transferred to a new sterile tube and mixed with 1 volume of Chloropane (see Appendix A) for 2 min. by vortexing, and centrifuged for 15 min, at 3,500xg. To remove phenol, the upper layer was transferred to a new sterile tube and added an equal volume of chloroform: isoamyl alcohol (24:1), and centrifuged for 15 min, at 3,500xg. This step was repeated and the upper layer was used as total RNA. The precipitation

step was performed twice, at -20°C and -70°C. For the first precipitation step, the precipitated RNA was prepared by mixing total RNA suspension with RNA precipitate solution (1/10 volume of 3 M sodium acetate (see Appendix A) and 3 volumes of cold isopropanol) and kept at -20°C at least 2 hr, This pellet RNA was harvested by centrifuging the cold suspension for 20 min at 4,000xg. This precipitated RNA was resuspended in RNase-free water and then the second precipitation process was performed as described above but the suspension was incubated at -70°C for at least 2 hr. Lastly, the pellets were washed in 75% cold ethanol and resuspended again in sterile RNase – free water.

The quality and quantity of total RNA were measured by using denatured gel electrophoresis and NanoDrop spectrophotometer (NanoDrop 1000, ThermoCycler, USA) with absorbance at 260 and 280 nm (*A*260/*A*280). The expected ratio should be at 1.8 – 2.0 for high quality nucleic acid. RNA concentration was calculated based on the optical density (OD) at 260 nm using Beer – Lambert Law as follows (191):

$$\mathbf{A = \varepsilon C\, l}$$

A = absorbance at particular wavelength;

ε = the extinction coefficient (for RNA is 0.027 $(mg/mL)^{-1}$ cm^{-1};

C = concentration of nucleic acid; and

l = path length of the spectrophotometer cuvette (typically 1 cm.).

Total RNA was aliquoted into small sterile tubes, stored at -70°C until use. Similarly, total RNA of *P. insidiosum* strain PC7 grown at 27°C was extracted as described above.

2.1.2 Determination of RNA quality

To check the quality of RNA, denatured agarose gel electrophoresis (1% formaldehyde gel) was used. All the equipments and the reagents were treated to remove RNase. For example, glass wares were treated in the hot air oven at 150°C for 2 hr. and water for RNA experiment was treated with 0.001% final concentration of Diethyl pyrocarbonate (DEPC; Cat. DB 0154, Bio Basic Inc., CA). One percent of agarose (Cat. 9012-36-6, USB, USA) in DEPC treated water was melted in microwave and cooled down to 55°C in a water bath, followed by adding of 5 x gel running buffer (final concentration is 1x gel running buffer) (see Appendix A) and 37% (12.3 M) formaldehyde (final concentration is 2.2 M) in fume hood. After that, the denatured agarose gel (FA gel) was poured into gel supporter which was cleaned in 1% H_2O_2 (Cat. 8.222287.1000, Merck, Germany) (see Appendix A), and the gel was allowed to solidify at room temperature (RT). After this step, the FA gel was placed in the electrophoresis tank, and 1 x gel running buffer to cover the gel was added. Prior to electrophoresis, the FA gel was equilibrated in 1x gel running buffer for 5 min at 5V/cm.

RNA sample preparation was performed by adding 1–5 µg of total RNA in 1x gel running buffer (2M formaldehyde and 0.5 volume of formamide). This suspension was denatured by heating at 65°C, 15 min and immediately placed on ice to cool and briefly centrifuged, followed by adding 1/10 volume of loading buffer (see Appendix A). Next, RNA samples were loaded in the equilibrated FA gel and electrophoresis was carried out at the constant voltage of 3 – 4 V/cm, in the

fume hood. RiboRuler™ High Range RNA Ladder (Cat. SM 1823, Fermentas, CA) was used as a standard marker.

2.1.3 mRNA purification

Protocol for mRNA purification was adopted from the Quickprep *Micro* mRNA purification kit (Cat. 27-9255-01, GE healthcare, UK) (192). The steps were as follows (Figure 17).

I. Preparation step (Figure 17A)

1. Working Oligo (dT) – cellulose was prepared by aliquoting 1 mL of stock Oligo (dT) – Cellulose resin into a new eppendorf tube.
2. Five micrograms of total RNA from isolates grown at 2 different temperatures (from 2.1.2) were mixed with 0.8 ml elution buffer.

II. Binding step (Figure 17B)

The tubes from step 1 & 2 were centrifuged at 14,000 rpm. for 1 min, and the supernatant in Working Oligo (dT) – cellulose tube was removed and instead added the RNA-supernatant from step I-2.

III. Washing step (Figure 17C)

This tube (total RNA + Oligo (dT) – cellulose) was centrifuged at 14,000 rpm. for 1 min, and removed the upper layer. The pellets were washed 5 times by High Salt Buffer (see Appendix A) and 2 times of Low Salt Buffer (see Appendix A). Each washing step was accomplished by a process of resuspension and centrifugation.

IV. Transfering to MicroSpin™ Column step (Figure 17D)

After the final washing step with Low Salt Buffer, the pelleted Oligo(dT) - Cellulose was brought up in a 0.3 ml of Low Salt Buffer. The slurry was transferred to a MicroSpin™ Column placed within a sterile microcentrifuge tubes, and the column was washed with Low Salt Buffer and centrifuged to separate the resin bind-RNA and the buffer.

V. Elution step (Figure 17E)

Finally, the mRNA was eluted with prewarmed (65°C) Elution Buffer. This purified mRNA required to keep cool or on ice for experiment or kept at -70°C until use.

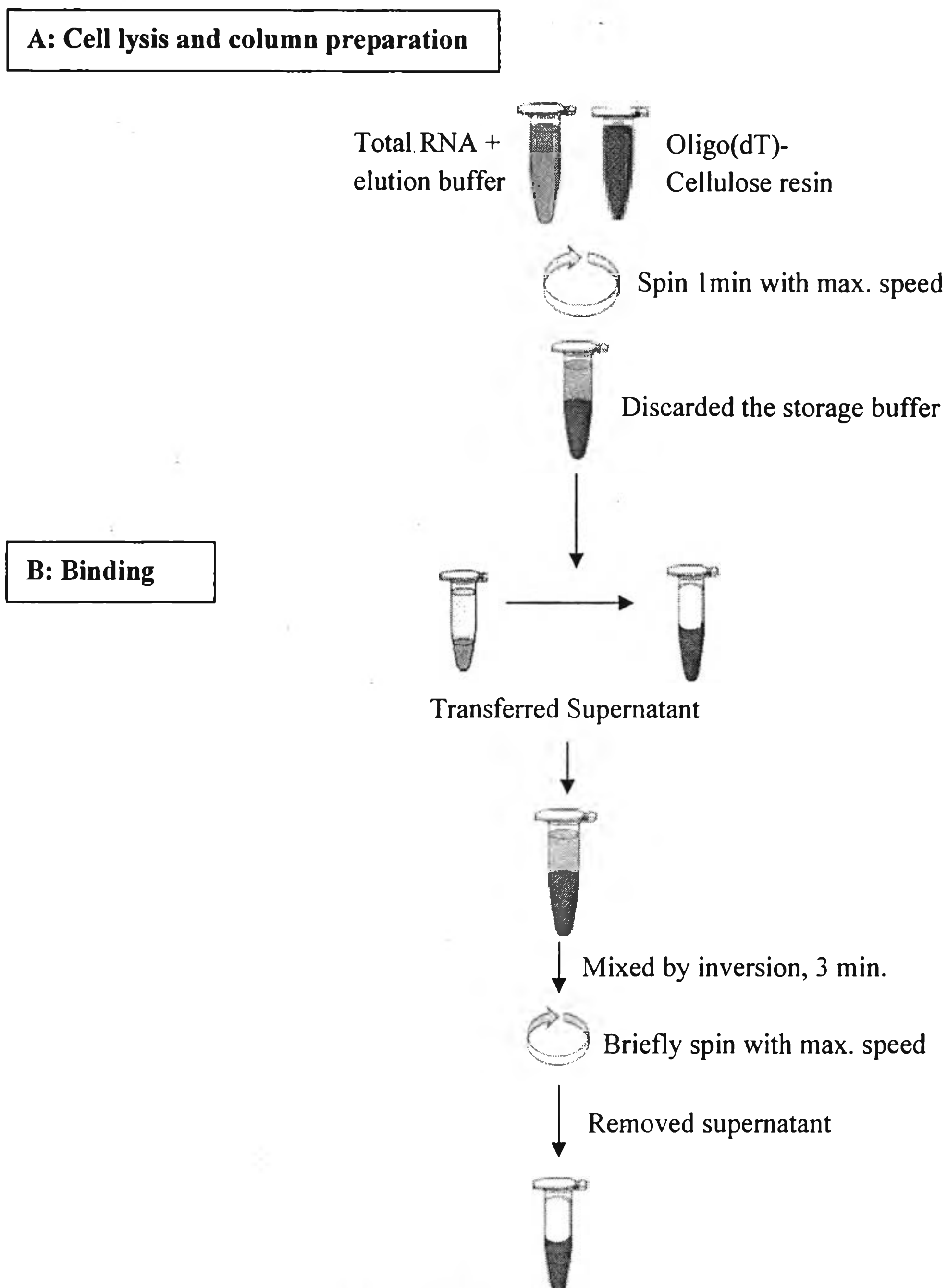

Figure 17. Scheme of the QuickPrep *Micro* mRNA Purification procedure (Part 1) (Quickprep *Micro* mRNA purification kit, GE healthcare).

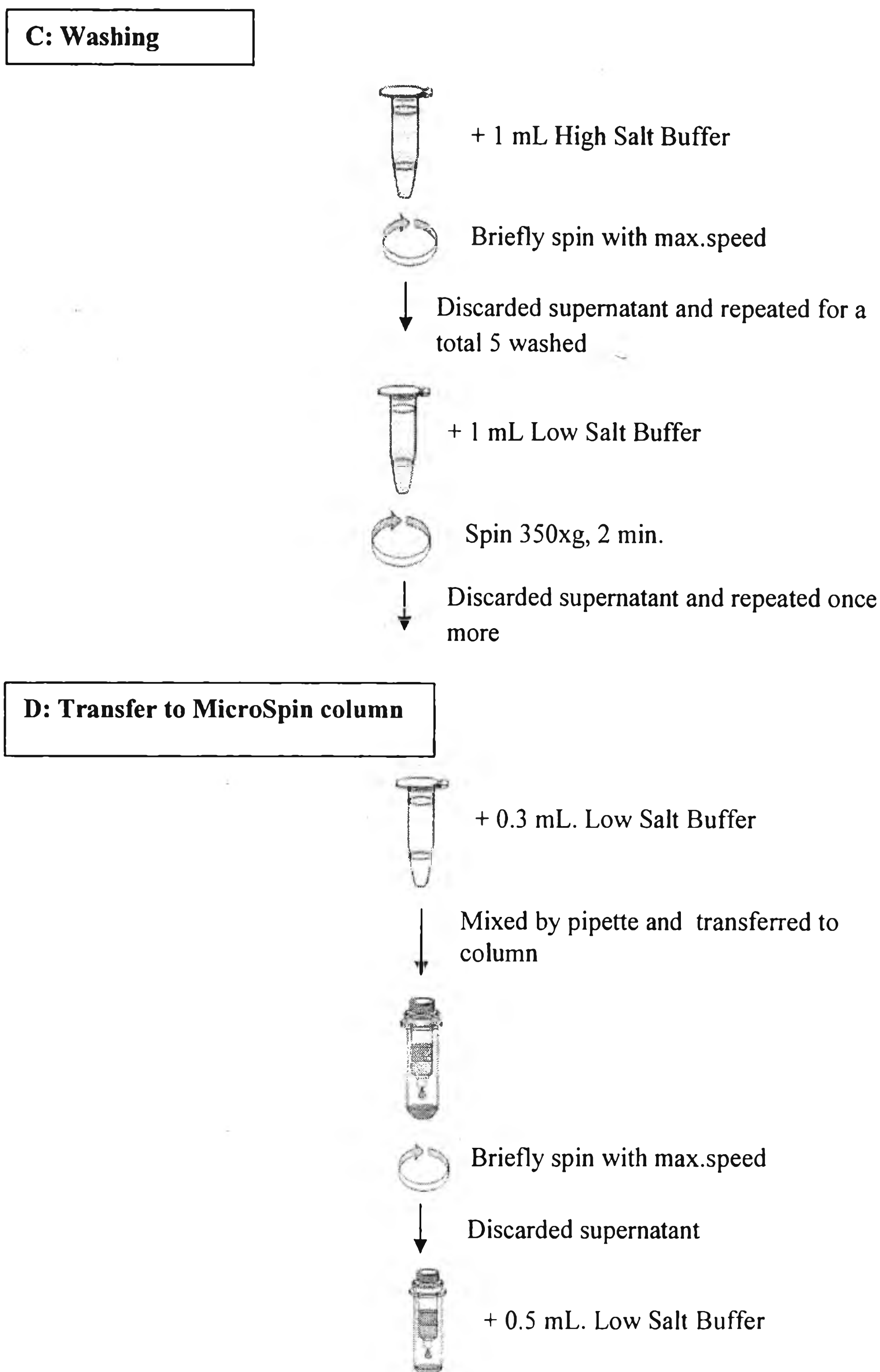

Figure 17. Scheme of the QuickPrep *Micro* mRNA Purification procedure (Part 2) (Quickprep *Micro* mRNA purification kit, GE healthcare).

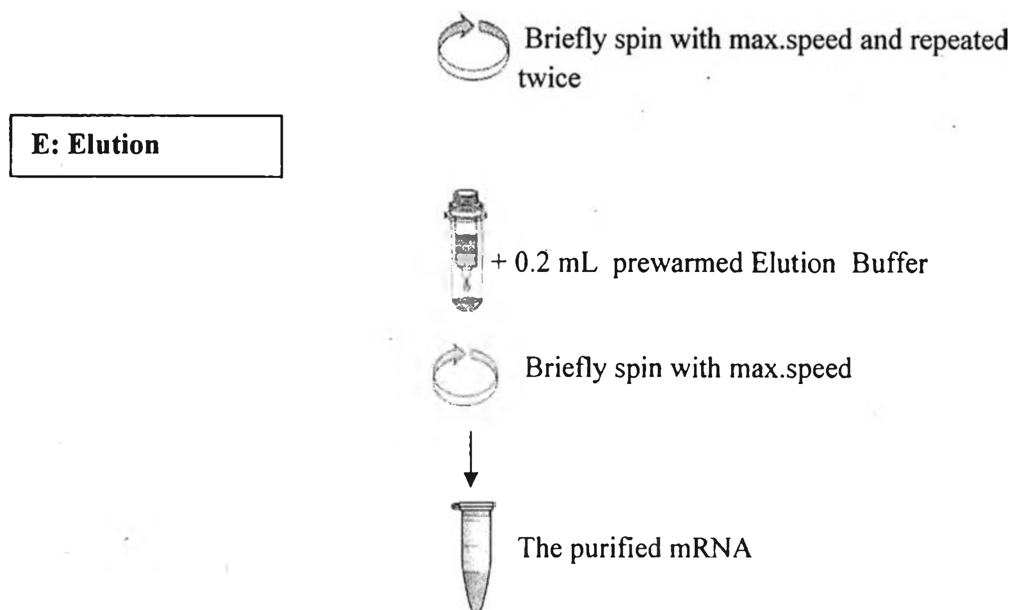

Figure 17. Scheme of the QuickPrep *Micro* mRNA Purification procedure (Part 3) (Quickprep *Micro* mRNA purification kit, GE healthcare).

2.1.4 Suppression subtractive hybridization (SSH)

The SSH method was performed with a PCR – Select cDNA subtraction kit (Cat. 637401, Clonetech, USA). The principle of this method is to screen cDNA library by suppression and subtraction of 2 populations of mRNA. The two populations mRNA in this study were mRNA from *P. insidiosum* strain PC7 grown at 27°C and 37°C. The properties of the required expression cDNA were up - regulated expression set at 37°C. The protocol was as followed (Figure 18):

- Step 1: Second strand cDNA synthesis

The second strand cDNA was synthesized using 2 μg of purified mRNA from each group with Super SMART PCR cDNA Synthesis Kit (Cat.635000, Clonetech, USA). According to the supplier's protocol, 2 μg of purified mRNA was mixed with 1 mM oligo $(dT)_{18}$ primer at the final volume 5 μl in 0.2 ml PCR tube, then incubated at 70°C, 2 min in Thermal cycler. The tube was cooled down by placing on ice for 2 min, added PCR mixture (1x First Strand buffer, 1 mM dNTP, Mix, and 20 U of AMV Reverse Transcriptase (20 units/μL)) and incubated at 42°C for 1.30 hr in Thermal cycler. Next, the first-strand synthesis reaction tube was placed on ice and added PCR mixture (1x Second Strand buffer, 0.2 mM dNTP Mix, 1x Second-Strand Enzyme Cocktail). The final volume was 80 μL, adjusted by RNase free – water, and then incubated 16°C, 2 hr in Thermal Cycler. After 2 hr, (6 U) of T4 DNA Polymerase was added and continue incubated for 30 min. To terminate second – strand synthesis, 20x EDTA/Glycogen 4 μL was mixed and purified cDNA using phenol – chloroform – isoamyl alcohol process.

- Step 2: *Rsa* I digestion

The second strand cDNA from both groups were digested with *Rsa* I to obtain shorter blunt – end fragment. The double strand (ds) cDNA 43.5 μl was filled into 1x *Rsa* I restriction buffer and 0.3 U of *Rsa* I, then incubated at 37°C for 1.30 hr. To terminate the reaction, 20x

EDTA/Glycogen 2.5 µL was mixed and purified dscDNA fragments by phenol – chloroform – isoamyl alcohol process.

- Step 3: Adaptor ligation

The one microlitre dscDNA fragments from group 37°C (dscDNA 37°C) were diluted with 5 µL of H_2O and divided into 2 separate tubes, each diluted dscDNA 37°C was pipette 2 µL, then added into 6 µL master mix tube (1x ligation buffer and 40 U T4 DNA Ligase). One tube (T1) was ligated with 1 µM Adaptor 1 (Table 8), and the second tube (T2) was ligated with 1 µM Adaptor 2R (Table 8). Both tubes were incubated at 16°C overnight, thus stopped ligation by adding 1 µL of EDTA/Glycogen (Appendix A) and purified ligating dscDNA 37°C by phenol–chloroform–isoamyl alcohol process.

- Step 4: First hybridization

To further enrich for differentially expressed genes, 1.5 µL of each ligation dscDNA 37°C was mixed with 1.5 µL of dscDNA fragments from group 27°C (excess dscDNA 27°C) and 1 x Hybridization buffer (prewarmed to room temperature for at least 15–20 min). The first hybridization was done at 98°C for 1.30 min in a thermal cycler, and followed with 68°C for 8 hr.

- Step 5: Second hybridization

One microliter of Mixture (1 µl of excess dscDNA 27°C, 1 x Hybridization buffer and 2 µL of H_2O) was denatured at 98°C for 1.30 min, and then the two tubes from the first hybridization were pooled and loaded into freshly denatured Mixture. The second hybridization reaction was

incubated overnight at 68°C, after that added 100 μL of dilution buffer and followed with heating at 68°C for 7 min. The hybridization product could kept at -20°C until used or progressed the next step.

- Step 6: Fill end & PCR amplification

To selectively amplify differentially expressed cDNA, this PCR amplification step was performed. The dilution subtracted cDNA was pipette 1 μL into the primary PCR Master Mix tube (total volume 25 μL contained 1x PCR reaction buffer, 1 mM dNTP Mix, 0.4 μM PCR Primer 1(Table 8) and 1x Advantage cDNA Polymerase Mix). The condition for the first PCR was as follows : 5 min at 75°C (fill end), 25 s at 94°C, and 30 cycles of 10 s at 94°C, 30 s at 66°C and 1.30 min at 72°C. The primary PCR product was diluted to 1:10 with H_2O for the nested PCR.

The aliqouting 1 μL of diluted primary PCR product was filled in the secondary PCR Master Mix tube (total volume 25 μL contained 1x PCR reaction buffer, 1 mM dNTP Mix, 0.4 μM Nested PCR Primer 1 (Table 8), 0.4 μM Nested PCR Primer 2R (Table 8) and 1x Advantage cDNA Polymerase Mix). The conditions for this secondary nested PCR were 12 cycles of 10 s at 94°C, 30 s at 68°C, and 1.30 min at 72°C. The PCR product was stored at -20°C until used.

Table 8. The oligonucleotides used for SSH method

Oligonucleotides	Length (bp)	Sequences
Adapter 1	53	5'-CTAATACGACTCACTATAGGGCTCGAGCG-3' 3'-GCCGCCGGGCAGGTGGCCCGTCCA-5'
Adapter 2R	52	5'-CTAATACGACTCACTATAGGGCAGCGTGG-3' 3'-TCGCGGCCGAGGTGCCGGCTCCA-5'
PCR primer 1	22	5'-CTAATACGACTCACTATAGGGC-3'
Nested primer 1	22	5'-TCGAGCGGCCGCCCGGGCAGGT-3'
Nested primer 2R	20	5'-AGCGTGGTCGCGGCCGAGGT-3'

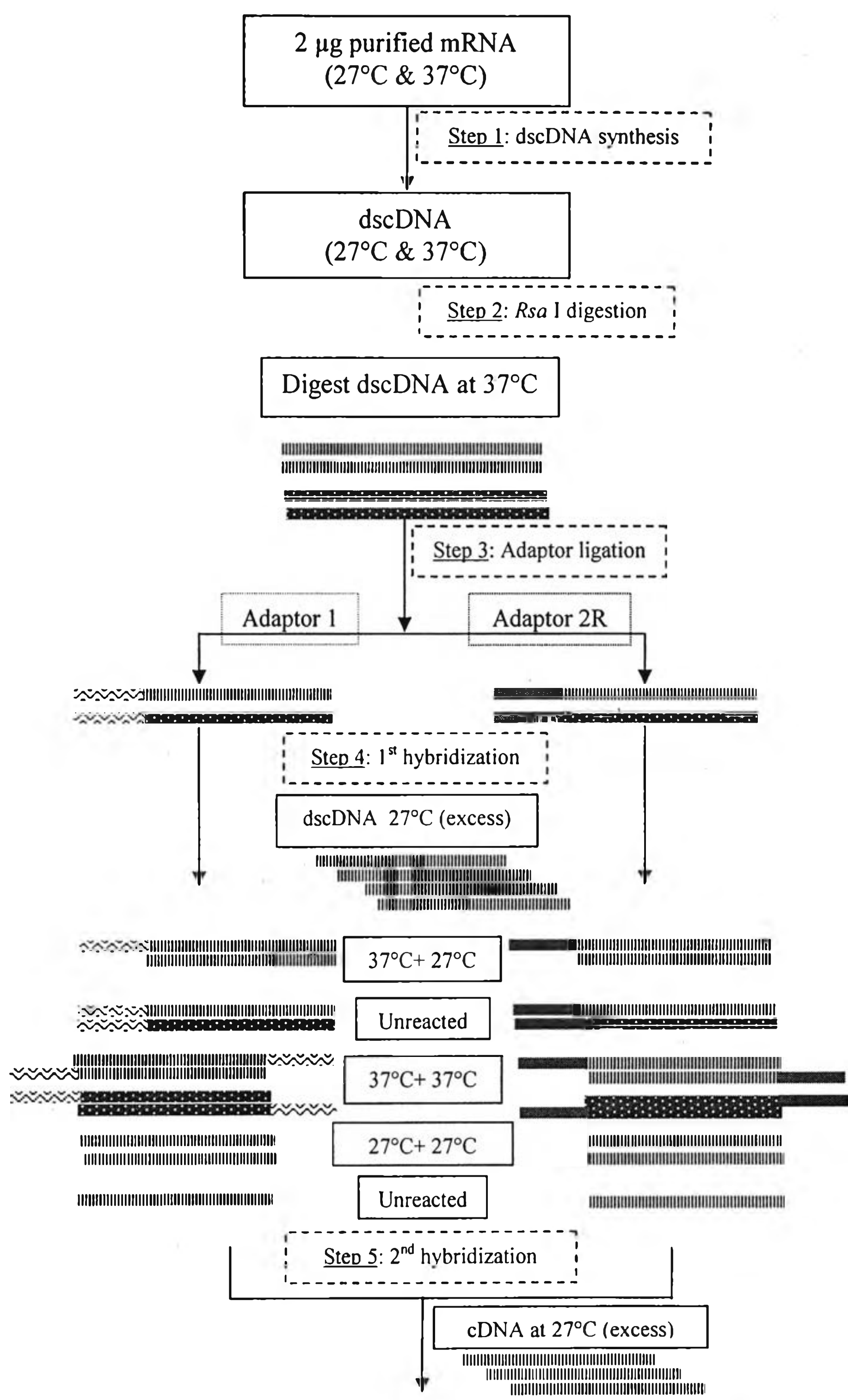

Figure 18. Diagram of PCR – select subtraction technique. & ; Adaptor 1 and Adaptor 2R, & ; Represents the *Rsa*I digested dscDNA (27°C & 37°C). (Part 1)

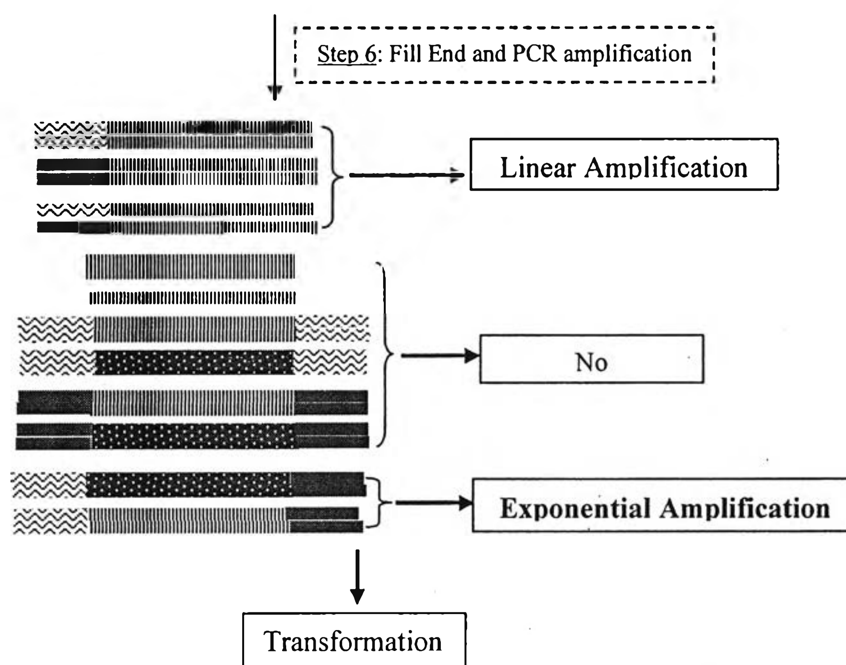

Figure 18. Diagram of PCR – select subtraction technique. & ; Adaptor 1 and Adaptor 2R & ; Represents the *Rsa*I digested dscDNA (27°C & 37°C). (Part 2)

To obtain successful SSH results was to effectively subtract common cDNA in both temperature (27°C cDNA and 37°C cDNA). The 28S rRNA primers and *COX* II primers were used to amplify PCR product with unsubtracted 37°C cDNA and the subtracted 37°C cDNA as a template.

2.1.5 The construction of SSH cDNA library

2.1.5.1 Competent cell preparation

To prepare the competent cells, *Escherichia coli* (*E. coli*) strain JM 109, were prepared by following the protocol of Z – competent *E. coli* Transformation kit (Cat.T3001, Zymo Research, CA). In brief, *E. coli* strain JM 109 was cultured in 0.5 mL of Luria-Bertani (LB) (see Appendix A) at 37°C, by

shaking at 100 rpm for overnight. The fresh overnight cultured *E. coli* strain JM 109 was transferred to 50 mL of SOC in 500 mL flask, and swirled vigorously at 25°C until OD_{600} reaches around 0.2–0.3. The cultured flask was placed on ice for 10 min, and centrifuged at 2,500xg at 4°C for 6 min for collecting cell pellets. The competent cells were washed twice with 5 mL of 1x wash buffer (see Appendix A) and centrifuged at 2,500xg at 4°C for 6 min. Finally, the competent cells were aliquoted 80 µL to sterile eppendorf tubes on ice and kept at -70°C until use.

2.1.5.2 pGEM® – T easy vector transformation

The subtracted cDNA which was enriched with differentially expressed cDNA obtained by secondary PCR were used for library construction. Five microlitre of the secondary PCR products was used for cloning with pGEM® – T easy vector (Figure 19) systems (Cat. A1380, Promega, USA) according to manufacturer's instructions. The ligation reaction (1X Rapid Ligation Buffer, T4 DNA Ligase, 50 ng pGEM®-T or pGEM®-T Easy Vector, and 3 Weiss units T4 DNA Ligase) was mixed with 5 µL of the secondary PCR products, and incubated for 1 hr at RT.

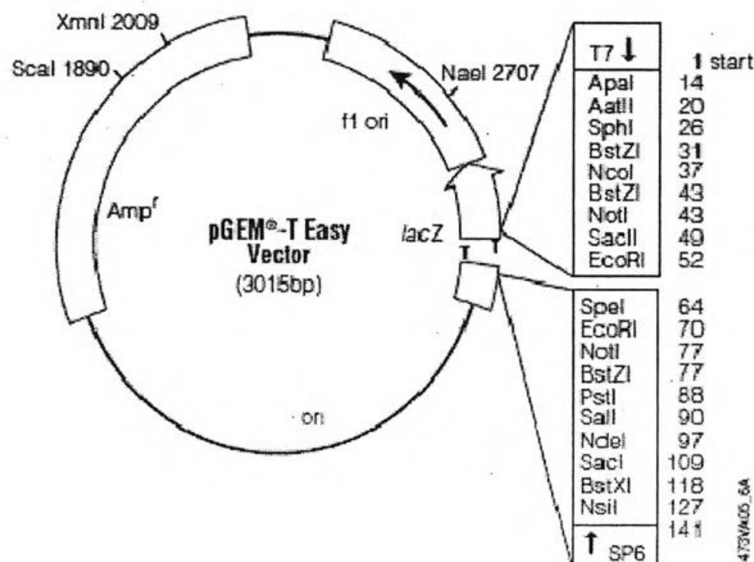

Figure 19. pGEM® – T Vector Map and sequence reference points.

For vector transformation, the vial of frozen competent cells was placed on ice, and added 5 µL sample to thawed competent cells and gently mixed. The tube was incubated on ice for 15 min, and for mRNA purification fifty µL of the culture were spreaded on LB agar containing 100 µg/mL amplicillin (see in Appendix A), 0.8 µg/mL X-Gal (Cat.V3941, Promega, USA) (see in Appendix A), and 0.5 mM IPTG (Cat.V3951, Promega, USA) (see in Appendix A) plate. Bacteria should allowed to grow at 37°C for 1 day.

2.1.5.3 Screening cDNA inserted by PCR amplification

The characteristics of the PCR products cloned into the vectors significantly affected the ratio of blue : white colonies. Usually clones containing PCR products produce white colonies, but blue colonies could result from PCR fragments that are cloned in – frame with the *lacZ* gene.

The white colony was picked up and subjected to PCR reaction, and the reaction was carried out in a 50 μL volume with 20 ng of each primers (M13pUC F/R) (Table 9), 200 μM dNTPs, 1x Taq DNA polymerase buffer (100 mM Tris-HCl at pH 8.3, 500 mM KCl, 20 mM $MgCl_2$, Enhancer solution) and 0.5 U of *iTaq*^{tm} polymerase (iNtRON Biotechnology, Korea). For amplification of the insertion clones, the thermal cycling conditions were as follows: an initial round of denaturation step at 94°C for 2 min, then 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final extension step of 72°C for 10 min. PCR products were resolved in 1.5% agarose gels, stained with ethidium bromide (0.5 μg/mL in 1X TBE buffer) and visualized under UV light.

Table 9. Oligonucleotides used for checking individual fragments insertion.

Primers	**Length (bp.)**	**Sequences**
M13pUCF	23	5'-CCCAGTCACGACGTTGTAAAACG-3'
M13pUCR	23	5'-AGCGGATAACAATTTCACACAGG-3'

The positive colonies were subcultured in 5 mL of LB broth with amplicillin, incubated overnight 37°C and continued shaking 100 rpm. Plasmid DNAs were extracted using a HiYield™ Plasmid Mini Kit (Cat. YPD100, RBC, Taiwan). Briefly (Figure 20), a fresh overnight grown bacterium was washed with DW and transferred to eppendorf tubes (Step1). Cells were resuspended, lyzed, and neutralized in buffer from commercial kit (Step 2). Later, DNA in supernatant

was transferred to PD column (Step3), washed (Step4) and eluted to a new eppendorf tube (Step5). The eluted DNA was kept at -20°C until use.

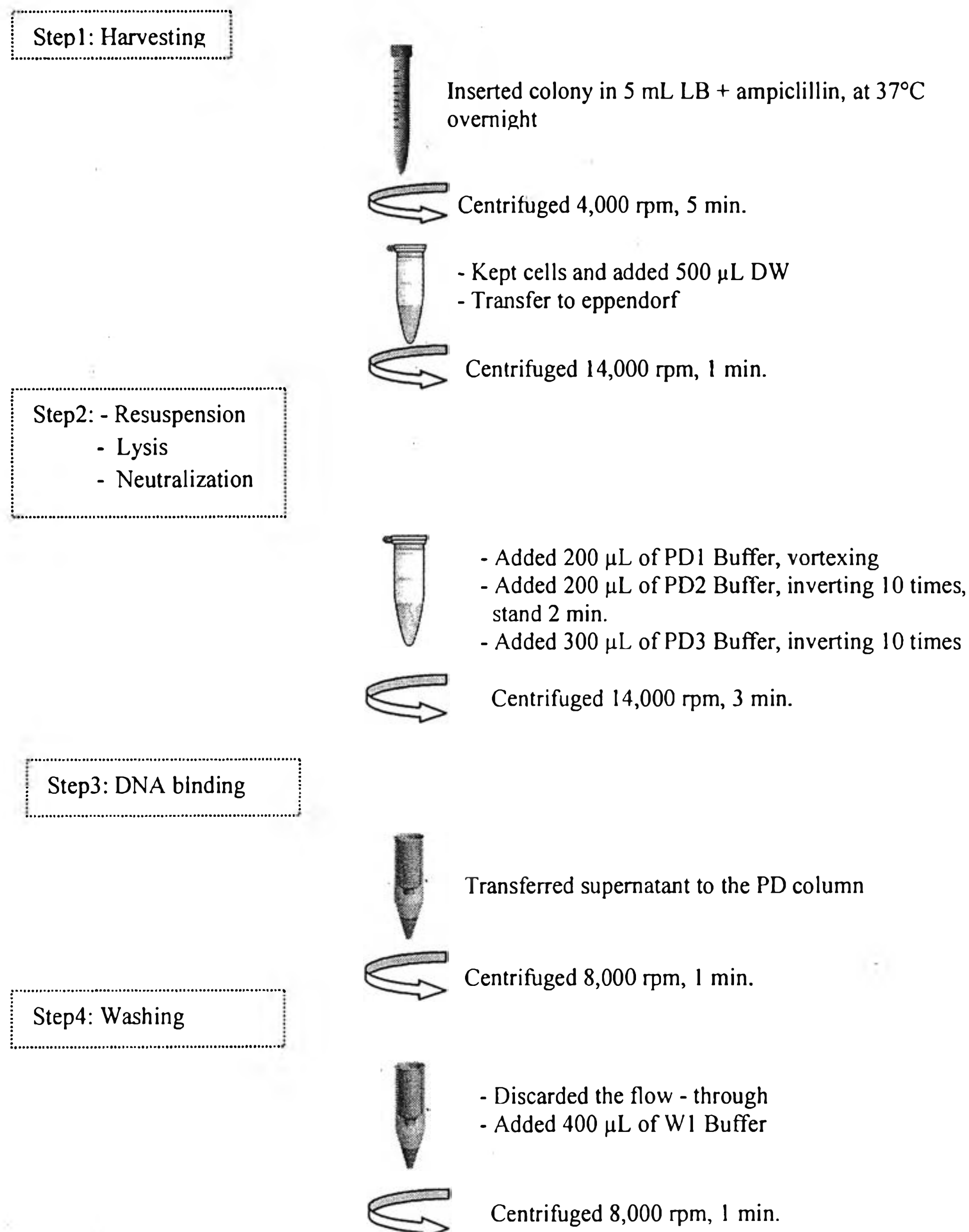

Figure 20. Drawing of HiYield™ Plasmid Mini Kit protocol. (HiYield™ Plasmid Mini kit, RBC,Taiwan).

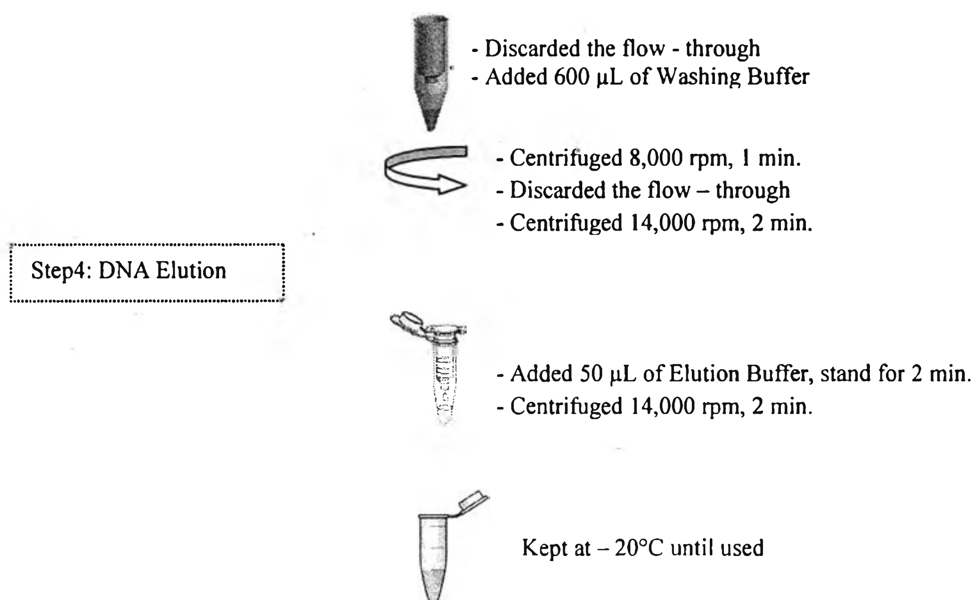

Figure 20. Drawing of HiYield™ Plasmid Mini Kit protocol. (HiYield™ Plasmid Mini kit, RBC, Taiwan) (cont.)

2.1.5.4 Sequencing and data analysis

Inserted genes were sequenced by 'Macrogen' (Korea) using ABI PRISM® 377 DNA Sequencer by using a T7 and SP6 primer; and the ABI BigDye kit. As described by the manufacturer, each reaction was performed in a 20 μL total volume containing approximately 300 ng of template DNA, 1 μL of 3.2 μM M13pUC primers, 4 μL of ready reaction premix and 2 μL of BigDye sequencing buffer. After thermocycling for 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extending at 60°C for 4 min, the extension products were purified by precipitation with ethanol acetate solution followed by washing with 70% ethanol. The samples were subsequently loaded on an ABI PRISM 377 DNA sequencher.

Each sequence was edited to correct sequencing and remove the vector sequence. The edited sequences were analyzed and aligned by using BLASTN and BLASTX (193) mode of the BLAST search program (http://ncbi.nlm.nih.gov/blast/) and ExPASy – PROSITE analyses of nucleotide and translated sequences for comparisons against the data in GenBank sequences. For functional annotation, the Swiss – Prot and MIP databases were an analysis tool (cutoff Expected (*E*) value < 2e-04).

2.2 GenBank databases

There were *P. aphanidermatum*, *P. deliense, Ph. sojae, Ph. infestans,* and *Ph. ramorum.* As *P. insidiosum* is able to cause the disease in mammals, eventhough the natural habitat is in swamp and damp soil. Therefore, the genes related to growth promotion and/or virulent related genes were the candidate groups. Three genes, cytochrome c oxidase subunit II (*COX* II), β- tubulin (*TUB*), and chitin synthase subunit II (*CHI* II) gene were studied in details. Only *COX* II's primers were referred from Villa *et al.* (194) whereas the others were designed in this study. Their sequences were listed in the Table 10.

3. Gene expression Analysis

3.1. Semi – Quantitative gene expression using PCR

3.1.1 First strand cDNA synthesis procedure

To digest an amount of DNA from total RNA sample, the DNase digestion reaction using RQ1 RNase – Free DNase (Cat. M610A, Promega, USA) was set up as follows: at a final volume of 10 μL; 5 μg of total RNA (from 2.1.2) was mixed with RQ1 RNase – Free DNase 1x reaction Buffer and 1 U/μg RNA of

RQ1 RNase – Free DNase. The reaction tubes were incubated at 37°C for 30 min, and 1µL of RQ1 DNase stop solution was added to terminate the reaction. The incubation at 65°C for 10 min was carried out to inactivate DNase, and DNA – free RNA was ready for the next experiment.

The cDNA was synthesized from a mRNA template using the RevertAid™ First Strand cDNA Synthesis kit (Cat.K1621, Fermentas, USA). The method was applied following a handbook kit. Twenty microlitre of reaction mixture containing 3µg DNA – free RNA and 0.5 µg oilgo $(dT)_{18}$ primer, 1x Reaction Buffer, 20U RiboLock™ Ribonuclease Inhibitor, 1mM dNTPs and 10U RevertAid™ A-MuLV Reverse Transcriptase. The reaction tubes were incubated at 42°C for 1 hr., and the reaction was stopped by incubating at 70°C for 5 min. The Figure 21 below described the main steps of the cDNA synthesis procedure.

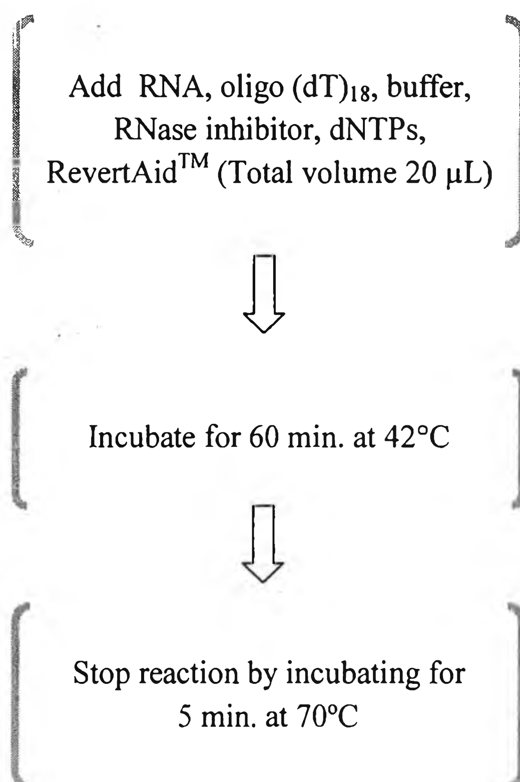

Figure 21. The cDNA synthesis procedures.

3.1.2 Polymerase Chain Reaction (PCR) Amplification

cDNA (from 3.1.1) of *P. insidiosum* strain PC7 (27°C & 37°C) was used as a template for PCR amplification. The semi–quantitative PCR for each candidate genes were carried out using the primers in Table 10. Amplification reactions were carried out with the total volume of 30 µL, containing 1 pg to 100 ng of cDNA, 1x *Taq* DNA polymerase buffer (100 mM Tris-HCl at pH 8.3, 500 mM KCl, 20 mM $MgCl_2$, Enhancer solution), 0.2 mM dNTPs, 0.2 pmol of each primer, and 1 U of *iTaq*™ polymerase (iNtRON Biotechnology, Korea). These reactions were performed in ThermoHybrid PCR thermocycle (Ashford, Middlesex, UK) with the cycling profile of pre-PCR at 94°C for 2 min, followed by 30 cycles of 94°C for 40 s, 55°C for 30 s and elongation at 72°C for 1.30 min. and

final extension at 72°C for 10 min. The positive control which was *P. insidiosum* strain CBS 574.85 (MTPI19), was run in parallel. To determine the size of PCR products, the samples were run in 1.5% agarose gel (Cat.75817, USB, USA) and stained with 0.05% ethidium bromide.

3.2 Quantification of gene expression

3.2.1 Real time reverse – transcriptase PCR (real time RT – PCR)

To quantitatively determine the level of gene expression, the LightCycler FastStart DNA Master SYBR Green I kit (Cat. 03003230001, Roche, Germany) was used. To start the PCR reaction, PCR mixture of a 20 µL standard reaction containing 1xLightCycler FastStart DNA Master SYBR Green I, 0.2 µM of each primer (Table. 10), 0.2 µM of $MgCl_2$ and 2 µL of cDNA templates were prepared. The protocol contains the following programs listed in the Table 11. Sixteen samples (27°C & 37°C) were run in parallel together with endogenous control (rRNA). Melting – curve analysis was performed in the range of 55 – 95°C to confirm that a single PCR product was generated from the cDNA template. The PCR products were checked for molecular size by electrophoresis and sequences were compared of the data available in the GenBank database.

Table 10. Primers use for RT – PCR and DNA sequencing.

Primers	Length (bp)	Sequences	References
***COX* II**			
FM58	21	5'- CCACAAATTTCACTACATTGA- 3'	Villa *et al.* (194)
FM66	19	5'- TAGGATTTCAAGATCCTGC- 3'	Villa *et al.* (194)
ITS region			
ITS-1	19	5'- TCCGTAGGTGAACCTGCGG- 3'	White *et al.* (195)
ITS-4	20	5'- TCCTCCGCTTATTGATATGC- 3'	White *et al.* (195)
β – tubulin			
TubuF	18	5'- AACGCCGAAGAGGTCATG-3'	This study
TubuR	18	5'- CCAGGAGATGTTCAAGCG-3'	This study
***CHI* II**			
ChiF	22	5'- GAACACTCACAGGCATCGCAAG-3'	This study
ChiR	19	5'- GGGTTACGATCCATGCTTC-3'	This study

Table 11. Real Time RT - PCR condition.

Analysis Mode	Cycles	Segment	Target Temperature	Hold time	Acquisition Mode
		Pre - Incubation			
None	1		95° C	10 min.	none
		Amplification			
Quantification	45	Denaturation	95° C	10 s	none
		Annealing	55° C	10 s	none
		Extension	72° C	25 s	single
		Melting Curve			
Melting Curves	1	Denaturation	95° C	0 s	none
		Annealing	55° C	15 s	none
		Melting	95° C	0.5 s	continuous
		Cooling			
None	1		40° C	30 s	none

3.2.2 Data analysis

The most common method for Comparative threshold (C_T) method is the $\Delta\Delta$ C_T which have been described by Livak *et al* (196). Where: $\Delta\Delta C_T = \Delta C_T$ 37°C – ΔC_T 27°C and ΔC_T = (C_T target gene – C_T reference gene (in this study we used rRNA)). If the expression increases in some samples and decreases in others, the DDC_T values will be a mixture of negative and positive ones. The last step in

quantification is to transform these values to absolute values. The formula for this is: comparative expression level = $2^{-\Delta\Delta C_T}$

Data were expressed as mean ± standard deviation (SD). The statistical difference of each pair was evaluated by Student's *t* test using generic approach, a wise investment is Graphpad Prism® 5 software (Graphpad Software Inc., San Diego, CA) for reporting data and performing statistical analyses.

4 Genetic diversity

4.1 Strains and Genomic DNA

In addition, thirty – three genomic DNA samples extracted from several *P. insidiosum* Thai strains and each one from Costa Rica and USA were also used (Table 12) to study genetic diversity.

4.2 PCR amplification and sequencing

The 580-bp DNA sequence of the partial *COX* II gene was amplified using the FM58 and FM66 primers (Table 10) as Villa *et al* (194). Amplification reactions were carried out with the total volume of 30 μL, containing 10-100 ng of genomic DNA, 1x *Taq* DNA polymerase buffer (100 mM Tris-HCl at pH 8.3, 500 mM KCl, 20 mM $MgCl_2$, Enhancer solution), 0.2 mM dNTPs, 0.2 pmol of each primer, and 1 U of *iTaq*^{tm} polymerase (iNtRON Biotechnology, Korea). These reactions were performed in ThermoHybrid PCR thermocycle (Ashford, Middlesex, UK) with the cycling profile of pre-PCR at 94°C for 2 min, followed by 35 cycles of 94°C for 40 s, 55°C for 30 s and elongation at 72°C for 1.30 min and final extension at 72°C for 10 min. The positive control, *P. insidiosum* strain CBS 574.85 (MTPI19), was run in parallel. To determine the size of PCR products, the samples were run in 1.5% agarose gel

(Cat.75817, USB, USA) and stained with 0.05% ethidium bromide. Whereas the amplification of 800 bp fragment of the rDNA ITS region using universal primers ITS-1 and ITS-4 (Table 10) was performed as described by White *et al* (197).

4.3 Phylogenetic analysis

DNA sequencing was performed by 'Macrogen' (Korea) using ABI PRISM® 377 DNA Sequencher. The amplified products were sequenced in both directions (sense strand and anti-sense strand) by using forward and reverse primers listed in Table 10. All sequences of *COX* II and ITS regions in this study were deposited in GenBank and their accession numbers are given in Table 17. The sequences of both regions were analyzed by BLAST comparisons against the database in GenBank sequences and aligned using the program Bioedit version 7.0.9.0 (198) together with program Mega4 version 4.0 and then inspected by eye (199). *Pythium* species, *Ph. sojae*, and *Lagenidium giganteum* were used as outgroups. Genetic distances were by Neighbor-Joining (NJ) and conditional clustering, with Kimura 2-parameter model (23), using the Mega4 program. Gaps obtained from the sequence alignments were treated as missing data. The confidences of internal branches of the resulting trees were statistically tested by bootstrap supporting analyses of 10,000 replications.

Table 12. List of 33 isolates of *P. insidiosum* and other *Pythium* species used in phylogenetic study.

Species	Isolate	Source	Location
P. insidiosum	MTPI04	Equine	Texas, USA
P. insidiosum	MTPI19	Equine	Costa Rica
P. insidiosum	MTPI12	Human	Thailand
P. insidiosum	PC2	Human	Thailand
P. insidiosum	PC3	Human	Central part, Thailand
P. insidiosum	PC5	Human	Central part, Thailand
P. insidiosum	PC6	Human	Northeastern part, Thailand
P. insidiosum	PC7	Human	Eastern part, Thailand
P. insidiosum	PC10	Human	Northeastern part, Thailand
P. insidiosum	PCM1	Human	Northern part, Thailand
P. insidiosum	PCM2	Human	Northern part, Thailand
P. insidiosum	PEC1	Water reservoir	Thailand
P. insidiosum	PECM3	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM5	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM6	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM7	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM8	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM9	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM10	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM11	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM12	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM13	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM14	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM15	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM16	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM17	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM18	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM20	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM21	Water reservoir [a]	Northern part, Thailand
P. insidiosum	PECM22	Water reservoir [a]	Northern part, Thailand

Table 12. List of 33 isolates of *P. insidiosum* and other *Pythium* species used in phylogenetic study (cont.).

Species	Isolate	Source	Location
P. insidiosum	PMS1	Human	Thailand
P. insidiosum	PMR2	Human	Northern part, Thailand
P. insidiosum	PMR3	Human	Thailand
P. catenulatum	ECU1	Water reservoir	Central part, Thailand
P. aphanidermatum		Ministry of Agriculture & Cooperative	Thailand
P. deliense		Ministry of Agriculture & Cooperative	Thailand

[a] 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).