IV. MATERIALS AND METHODS

4.1. Experimental design

An overview of the experimental plan was used to study the characteristics and differential expression of the *hsp70* gene.

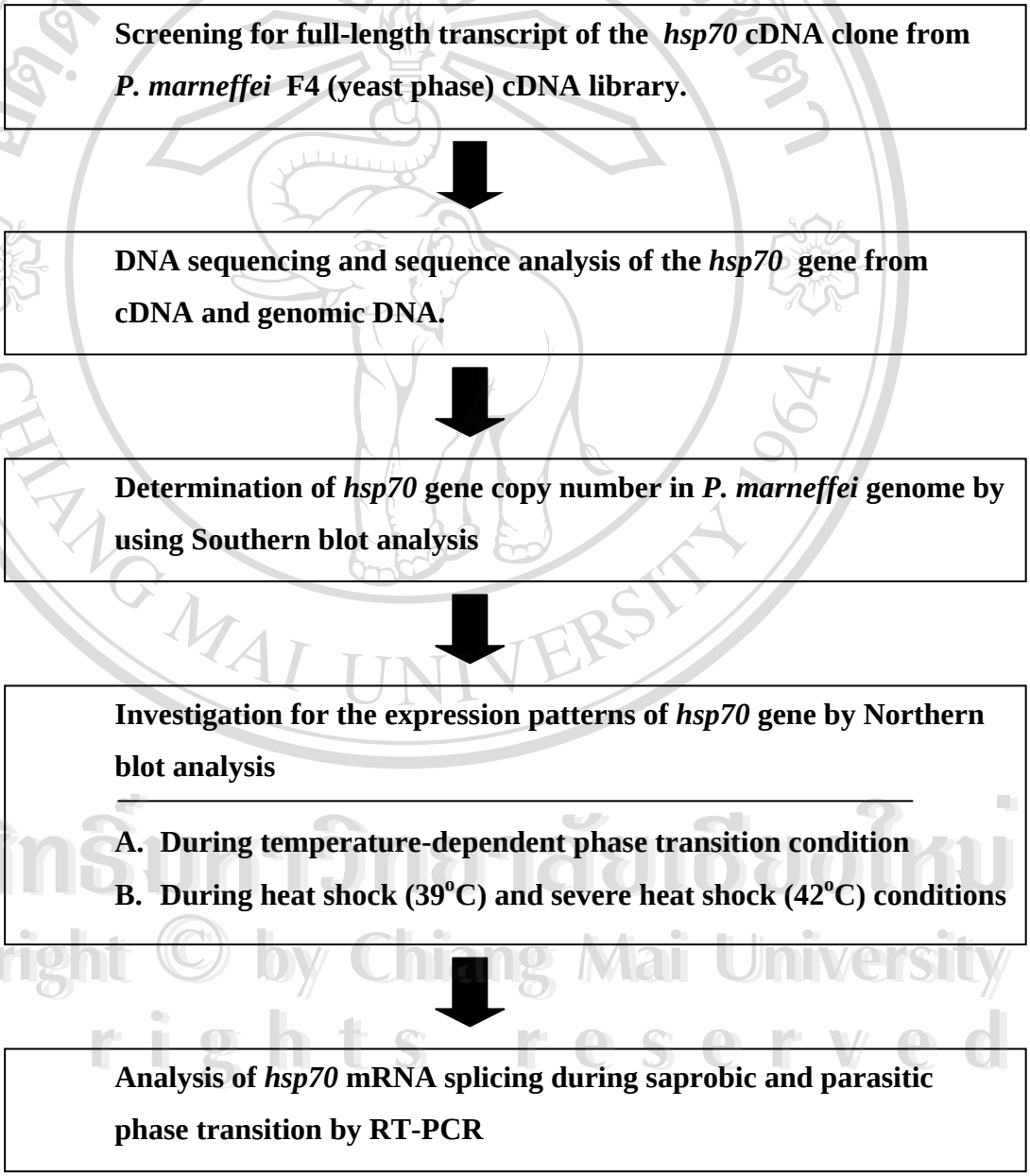

Figure 4. Schematic representation of the study of the *hsp70* gene.

4.2. Fungal strain and culture conditions

Penicillium marneffei F4 was obtained from the hemoculture of an AIDS patient from the Central Laboratory, Maharaj Nakorn Chiang Mai Hospital in January 1999. The primary isolate was kept in 20% glycerol at -80°C. Conidia were grown on malt extract agar (OXOID, England) for 7 days at 25-28°C. A conidial suspension for inoculating broth cultures was prepared by cotton swab-scraping in sterile normal saline and filtrating through sterile glass wool (Corning, Acton, MA, USA). To investigate saprophytic and parasitic phases transition, conidial suspensions of *P. marneffei* were inoculated into Brain Heart Infusion (BHI, OXOID, England) broth and incubated for 0, 6, 12, 24, 48, 72 and 96 h at 25°C or 37°C. To observe mycelium to yeast transition, complete mycelial cells in BHI (25°C, 72 h) were transferred to 37°C for 1, 3, 6, 12, 24, 48, 72 and 96 h. To generate heat shock and severe heat shock conditions, complete yeast cells in BHI (37°C, 96 h) were subjected to 39°C or 42°C heat for 0.5, 1 and 3 h. All cultures were maintained in shaking incubator (Jeio Tech, Kyunggi-Do, South Korea) or shaker bath (Precision Scientific 360) with continuous shaking at 135 rpm. At the indicated time of culture condition, the cells were harvested by centrifugation at 12,000 rpm for 5 min. Fungal cell differentiation was photographed under light microscope.

4.3. Genomic DNA isolation

Fungal genomic DNA was isolated by a modified method of Vanittanakom *et al.* (1996). The conidia from a 7-day-old culture were harvested, inoculated into Sabouraud dextrose broth (SDB) and incubated at 25°C in shaking incubator at 135 rpm for 12 h. The culture was centrifuged at 3,000 x *g* for 5 min, washed twice with phosphate-bufferred saline and washed once with 0.6 M $MgSO_4$. The cells were suspended in filter-sterilized osmotic medium (5 ml/g of cells), and the suspension was placed on ice. A filter-sterilized solution of lysing enzymes (Sigma, Steinheim, Germany; 20 mg/ml in osmotic medium; 2 ml/g of cells) was added, and the cells were incubated on ice for 5 min. Next, filter-sterilized solution of bovine serum albumin (12 mg/ml in osmotic medium; 0.5 ml/g of cells) was added. The suspension was then incubated at 37°C for 2 h. The spheroplasts were pelleted by centrifugation, washed twice with ST buffer, and resuspended in 10 ml of lysis buffer. The

suspension was mixed vigorously and was incubated at 65°C for 1 h. Three milliliters of 5M potassium acetate was added. The suspension was mixed by inversion and was incubated on ice for 1 h. The supernatant was pipetted into a new tube after centrifugation at 12,000 x *g* and 4°C for 10 min. DNA from supernatant was precipitated with an equal volume of 2-propanol and the solution was placed at -20°C for 1 h. DNA was pelleted by centrifugation at 12,000 x *g* and 4°C for 10 min and was resuspended in TE buffer containing RNase A (Sigma, St Louis, USA) 50 µg/ml. The DNA suspension was incubated at 37°C for 2 h, and an equal volume of phenol: chloroform (Pierce, Rockford, USA) was added. The suspension was mixed and centrifuged at 10,000 rpm for 10 min. The supernatants were transferred into a new tube. DNA was precipitated by adding 2 volume of ice-cold absolute ethanol, and then placed at -20°C for at least 30 min. The DNA pellet was washed twice with 70% ethanol, then air-dried. The DNA was resuspended in distilled water. The concentration and quality of DNA were determined by measuring the absorbance at 260 with a spectrophotometer (Eppendorf, Hamburg, Germany) and by gel electrophoresis.

4.4. Screening for full-length transcript *hsp70* from cDNA library

A cDNA library was constructed by Pongpom (2004). The system for construction of cDNA library was SuperScript™ Lambda by using ZipLox vector (Gibco BRL). The procedure of plaques hybridization was carried out following to the ECL Direct Nucleic Acid Labeling and Detection Kit (Amersham Pharmacia Biotech, Burkinghamshire, England)

4.4.1.) Titering of the cDNA library. Each 100 µl of tenfold serial dilutions (10^{-1} to 10^{-8}) of the cDNA library were used to infect the equal volume of *E. coli* Y1090 host (OD_{600}=4) for 15 min at 37°C. The infected bacteria were mixed with 3 ml of 0.6% LB top agar and plated on the LB agar in a 100-mm plate. The plates were incubated overnight at 37°C. The titer of cDNA library was calculated by the number of plaques at the appropriate dilution (30 to 300 plaques/plate) as follows;

$$\text{Titer of the cDNA library (pfu/ml)} = \frac{\text{Number of plaques x dilution factor x } 10^3(\mu l)}{100\ \mu l\ \text{(infection volume)}}$$

4.4.2.) Preparation of labelled probe. A probe was prepared by using *Not*I and *Sal*I enzymes, as adapters for construction of cDNA library, to excise the *hsp70* cDNA fragment from partial *hsp70* cDNA clone (1.9 kb, H3; Pongpom, 2004). The *hsp70* cDNA fragments were purified by using DNA gel extraction kit (Qiagen GmbH, Germany). Before use, a 1.9-kb fragment was labelled with the enzyme horseradish peroxidase, and performed according to the manufacturer's protocol (ECL Direct Nucleic Acid Labelling and Detection Kit, Amersham Pharmacia Biotech).

4.4.3.) Plaques hybridization. The plaque lifted membrane containing DNAs from the cDNA library was prepared as follows (adapted from cDNA library protocols, Ian and Carolin, 1997). Approximately 10,000 pfu of phage library were mixed with 0.6 ml of *E.coli* Y1090 (OD_{600}=4) and incubated for 15 min at 37 ^{o}C. Infected bacteria were added with 8 ml of molten top agarose (0.6% agarose). The mixture was poured onto a 150-mm LB agar plate and allowed to solidify at room temperature. The plate was incubated overnight at 37^{o}C to generate plaques. A 132-mm hybond-N+ membrane (Amersham Pharmacia Biotech, Burkinghamshire, England) was laid down on the surface of the agar for 2 min. The orientation of the nylon membrane was marked by piercing 3 locations asymmetrically with a 21-guage needle, and the membrane was carefully lifted from the plate. The membrane bound with library DNA was denatured by laying it on filter papers saturated with 0.5 M NaOH for 5 min and neutralized with 2X SSC. DNA was fixed to the membrane by UV crosslinker (GS Gene Linker^{Tm} UV Chamber, Bio-Rad, CA, USA) at C-L mode. Hybridization, washing and detection steps were done according to the manufacturer's protocol (Direct Nucleic Acid Labelling® and Detection kit, Amersham Pharmacia Biotech). Briefly, the membrane was prehybridized and hybridized at 42^{o}C for 1 h and overnight, respectively. The membrane was washed twice under high stringency condition by using a primary washing buffer with urea at 42^{o}C for 20 min, and washed twice with 2X SSC at room temperature for 5 min. The membrane was exposed for 1 h on Hyperfilm (ECL, Amersham Pharmacia Biotech). Positive signals on the Hyperfilm were aligned with the agar plate and plaques corresponding to the positive signals were picked and suspended in 250 µl SM buffer containing 0.3 % chloroform. Phage clones from primary screening were screened for the longest insert size by PCR using T7 and H70-2 primers.

4.4.4.) Screening for the longest clone by PCR using T7 and H70-2 primers. Phage clones were subjected to the PCR template by using sense primer T7, a binding site located in the λZiplox vector, and the antisense primer H70-2, which was designed based on the consensus sequence of HSP70 ORF from *Paracoccidioides brasiliensis*, *Histoplasma capsulatum* and *Trichophyton rubrum*. PCR was performed in a 25 µl reaction volume by using a master mix reagent (Qiagen GmbH, Germany). The PCR parameter was 94°C for 5 min, followed with 35 cycles of 94°C for 30 s, 55°C for 15 s, 72°C for 2 min and 30 s, and final extension at 72°C for 10 min. PCR products were determined by agarose gel electrophoresis, and stained with ethidium bromide. The size of amplicon was determined by comparing it to the size of 100 bp DNA ladder (New England Biolabs) and λ *Hin*dIII DNA marker (New England Biolabs). The longest clone was subjected to repeated screening to obtain the purified positive clones. The inserted size of the purified clone was determined by PCR using a pair of primers, T7 and SP6, which their binding sites were located in the λZiplox vector.

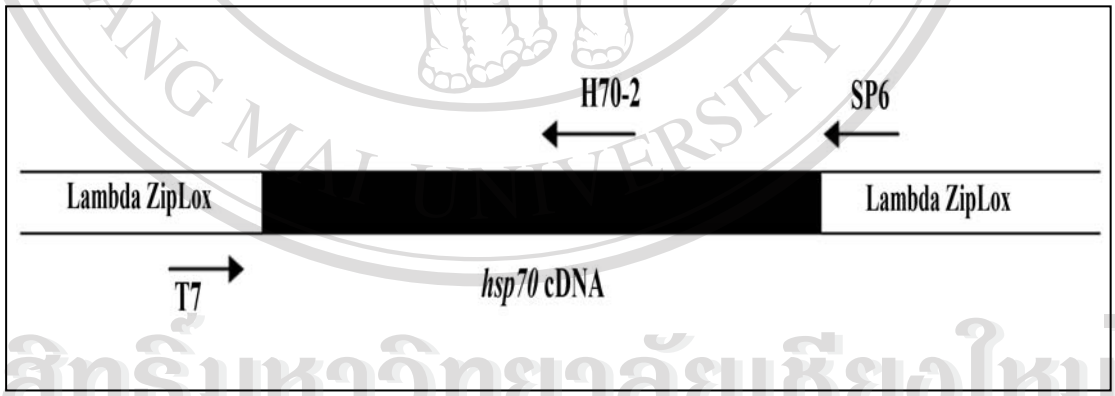

Figure 5. Site of primers for checking the *hsp70*-inserted clone sizes. A pair of T7 and H70-2 primers was used to amplify the 5'-end of cDNA encoding HSP70 and a pair of T7 and SP6 primers was used to amplify the whole inserted cDNA-encoding HSP70.

4.4.5) ***In vivo*** **excision of the phage clone**. A phage clone containing full-length transcript of *hsp70* cDNA was excised from the λZiplox vector. The *in vivo* excision process was performed by using *E. coli* DH10B according to the manufacturer's protocol (Invitrogen, Inc.). The resulting plasmid, pZL1, contained the inserted cDNA.

4.5. Isolation and purification of plasmid DNA

The method was adapted from the manual of Sambrook *et al.* (2001). A single colony of *E. coli* DH10B containing plasmid pZL1 was cultured overnight in LB medium containing ampicillin 100 µg/ml at 37°C, and then harvested by centrifugation at 3,000 rpm for 2 min. The cell pellet was resuspended by vigorous vortexing in 100 µl of ice-cold Solution I containing 50 µg/ml of RNaseA (Sigma, St Louis, USA). A freshly prepared lysis buffer (Solution II, 200 µl) was added, and then mixed by inverting the tube five times rapidly. The suspension was placed on ice, and an ice-cold Solution III (150 µl) was added. The suspension was gently inverted for 10 times, and stored on ice for 5 min. Plasmid DNA solution was separated by centrifugation at 12,000 rpm for 10 min. The supernatant was transferred to a new tube. An equal volume of phenol: chloroform was added, and then mixed by vortexing. After centrifuging at 12,000 rpm for 10 min at 4°C, the supernatant was transferred to a new tube. Plasmid DNA was precipitated by two volumes of absolute ethanol at -20°C for at least 20 min, and then centrifuged at 12,000 rpm for 30 min at 4°C. The plasmid DNA pellet was washed twice with 70 % ethanol, and air dried. Plasmid DNA was resuspended in distilled water. The concentration and quality of plasmid DNA were determined by measuring the absorbance at 260 with a spectrophotometer (Eppendorf, Hamburg, Germany) and by gel electrophoresis. An inserted size of *hsp70* cDNA was checked by using *Not*I and *Sal*I enzymes to excise a fragment from plasmid pZL1.

4.6. Cloning of the *hsp70* gene

The complete genomic sequence encoding HSP70 was obtained by PCR amplification of the genomic DNA of *P. marneffei*. Primers were designed based on the cDNA sequence. A 2,318 bp PCR product was obtained by using sense primer PMH70-F and the C-terminal antisense primer PMH70-R. The PCR was performed with 100 ng of genomic DNA of *P. marneffei*, and the amplification conditions were started at 95°C for 5 min; followed with 35 cycles at 95°C for 30 s, annealing at 54°C for 30s, and extension at 72°C for 5 min; and a final extension at 72°C for 10 min. The amplification was performed with automatic thermocycler (GeneAmp PCR System 2700, Applied Biosytem, Singapore) An amplified PCR product of 2,318 bp was gel-purified by using DNA gel extraction kit (Qiagen GmbH, Germany), subcloned into pCR®2.1-TOPO® vector (Invitrogen, California, USA) and sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

4.7. Sequencing of *P. marneffei* genomic *hsp70* gene and cDNA

DNA sequencing of the cDNA was performed by dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The procedure for extension products was performed according to manufacturer's protocol of BigDye Terminator Version 3.1 (Perkin-Elmer Applied Biosystems, Foster city, California). The conditions for amplifying extension products were as follows: 25 cycles of denaturation at 96°C for 10s, annealing at 50°C for 5s, and extension at 60°C for 4 min. The extension products were purified by adding 2 µl of 150 mM EDTA, then transferred to microcentrifuge tube containing 50 µl of ice-cold absolute ethanol with 2 µl of 3M sodium acetate. The suspensions were mixed by vortexing and placed on ice for 10 min. The extension products were pelleted by centrifugation at 13,000 rpm for 30 min, and washed twice with 70 % ethanol, then air-dried for 20 min at 40°C. The pellet was resuspended in Template Suppression Reagent (TSR) in 25 µl, and then vortexed and spinned. The suspension of purified-extension products were heated at 95°C for 2 min to denature. The sequencing products were analyzed on the automated sequencer (Genetic analyzer Model 310, Beckman).

4.8. Sequence analysis and phylogenetic tree construction .

The NCBI BLAST program (http://www.ncbi.nlm.nih.gov) was used to search for nucleotide and protein sequences similarity. The program "proteomics and sequence analysis tools" (http://www.expasy.org/) was used to predict an open reading frame and deduced amino acid sequences from nucleotide sequences. For analyses of intron numbers, lengths, and positions in other fungal *hsp70* genes, sequences available in GenBank databases (http://www.ncbi.nlm.nih.gov) were used. Deduced amino acid sequences of PmHsp70 and 48 other fungal Hsp70 sequences obtained from GenBank were used in multiple alignment and phylogenetic tree construction. Multiple sequence alignment was generated with ClustalW program (http://www. ebi.ac.uk/clustalw/index.html). The construction of the phylogenetic tree was carried out with MEGA3 program (Kumar *et al.*, 2004). Robustness of branches was estimated using 1000 boot-strapped replicates.

4.9. Southern blot analysis

Restriction enzyme mapping of *hsp70* gene was generated by using the program "NEBcutter" (http://tools.neb.com/NEBcutter2/index.php). Twelve micrograms of genomic DNA was digested with the restriction enzymes *Hin*dIII (Promega, Madison, WI, USA), *Bam*HI, *Sal*I, *Eco*RI, *Bgl*II, *Nde*I, *Xho*I, *Kpn*I and *Bss*I (New England Biolabs). The DNA fragments were electrophoresed on a 1% agarose gel and transferred onto nylon membranes (Hybond-N+, Amersham) by capillary blotting according to the manufacturer's instructions. DNAs were fixed to the membrane by UV crosslinker (GS Gene Linker^{Tm} UV Chamber, Bio-Rad, CA, USA) at C-L mode. The probe, a 2,063 bp nucleotide sequence including the complete PmHSP70 ORF, was prepared by PCR from Hsp70-encoding clone (ph20). Probe labelling, hybridization, and detection of the chemiluminescent signal were performed according to the manufacturer's protocol (ECL Direct Nucleic Acid Labelling and Detection Kit, Amersham Pharmacia Biotech).

4.10. Total RNA extraction

Total RNA was isolated from *P. marneffei* cells at the indicated time of culture by mechanical disruption method using RNeasy mini kit (Qiagen GmbH, Germany). Fungal cells were harvested and pelleted by centrifugation at 12,000 rpm for 2 min. The wet weight of each sample was about 0.2 to 1 g. Approximately 600 µl acid-washed glass beads were added to1.5 ml screw-cap microtubes. After adding 600 µl of Buffer RLT, the cell pellet was resuspended by vortexing. The sample was added to the glass beads prepared in the screw-cap microtubes. Then, the samples were agitated by bead beater (Biospec, Bartlesville. OK, USA) for 5 times at top speed for 30 seconds with cooling until cells were completely disrupted. The lysate was centrifuged at maximum speed for 2 min, and the supernatant was transferred to a new microcentrifuge tube. Seventy percent ethanol (350 µl) was added to the lysate and mixed by pipetting to adjust the binding conditions. The samples were applied to RNeasy mini column placed in a 2 ml collection tube, and centrifuged at 10,000 rpm for 15 s for adsorption of RNA to membrane. Buffer RW1 (350 µl) was added to RNeasy mini column, and the column was centrifuged at 10,000 rpm for 15 s. The mixture of DNase I (10 µl) and Buffer RDD (70 µl) was added to the column to decontaminate the genomic DNA. After 15 minutes, 350 µl of buffer RW1 was added to RNeasy mini column, and the column was centrifuged at 10,000 rpm for 15 s. The column was washed twice by using 500 µl of RPE buffer, and centrifuged at 10,000 rpm for 15 s and 2 min, respectively. The column was dried by centrifugation at full speed for 1 min. RNase-free water (30 µl for each sample) was added to the column and total RNA was eluted by centrifugation at full speed for 1 min. The RNA concentration and purity were determined by measuring the absorption at 260 and 280 nm (Eppendorf, Hamburg, Germany). The quality of RNA was determined by electrophoresis on denaturing 1 % agarose gel using the NorthernMax-Gly system (Ambion, Austin, USA). The suspension was then stored at -80^{o}C until used.

4.11. Northern blot analysis

The conditions of fungal growth and total RNA extraction were described above. Ten micrograms of total RNA from extracted cells at different condition and time points were separated by electrophoresis on denaturing 1% agarose gel using the NorthernMax-Gly system (Ambion) and transferred onto nylon membranes (Hybond-N+, Amersham) by capillary blotting. RNA was fixed to the membrane by UV crosslinker (GS Gene Linker^{Tm} UV Chamber, Bio-Rad, CA, USA) at C-L mode. The immobilized RNA was probed with a PCR-generated DNA fragment of the heat shock protein 70 clone (ph20). Probe labelling, hybridization, and detection of the chemiluminescent signal were performed according to the manufacturer's protocol (ECL Direct Nucleic Acid Labelling and Detection Kit, Amersham Pharmacia Biotech).

4.12. Reverse transcription-polymerase chain reaction (RT-PCR)

The splicing of the *hsp70* mRNA was investigated by RT-PCR. The preparation of DNase-treated total RNA samples was described in the RNA extraction method. Reverse transcription and polymerase chain reaction were performed in one tube by using QIAGEN® OneStep RT-PCR Kit (Qiagen GmbH, Germany). The *hsp70* gene-specific primers PMH70-F2 and H70-REV2, designed based on the sequence flanking 2 introns, were used as sense and antisense primers, respectively. The reactions were performed in a 25-µl reaction containing 100 ng of each RNA sample, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.6 µM of each primer, 1 µl of QIAGEN OneStep RT-PCR Enzyme Mix. Omniscript and Sensiscript reverse transcriptases and HotStar Taq DNA polymerase were included in the QIAGEN OneStep RT-PCR Enzyme Mix. The conditions were as follows: reverse transcription at 50^{o}C for 30 min; heat at 95^{o}C for 15 min; amplification were 30 cycles of 94^{o}C for 30 s, 56^{o}C for 30 s, 72^{o}C for 1 min; and a final extension at 72^{o}C for 10 min. PCR was performed to check for the absence of genomic DNA contamination on each RNA sample at various times during saprobic and parasitic phases transition. The reactions were performed in a 25-µl reaction containing 100 ng of each RNA sample, 3 mM $MgCl_2$, 0.4 mM of each

dNTP, 0.6 μM of each primer, 1.25 Unit of Taq DNA polymerase (Qiagen GmbH, Germany). The PCR was performed using only the amplification step as described in OneStep RT-PCR; and a final extension at 72°C for 10 min. The RT-PCR and PCR products were electrophoresed on 2 % agarose gel and visualized with ethidium bromide staining.

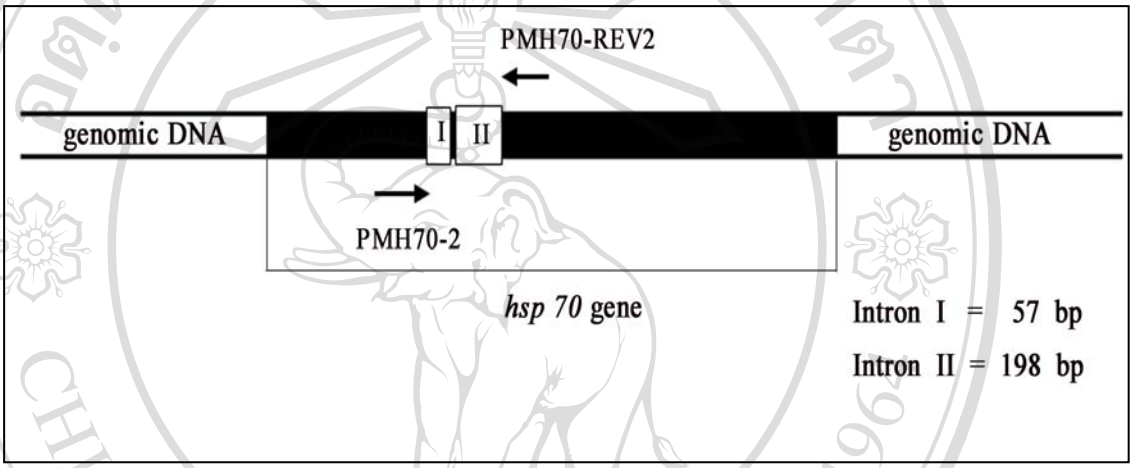

Figure 6. Site of primers used in RT-PCR. A pair of PMH70-2 and PMH70-REV2 primers, designed based on the sequence flanking 2 introns, was used in RT-PCR.

4.13. Accession number

The sequence of *hsp70* gene from cDNA containing open reading frame plus 92 nucleotides of an upstream and 201 nucleotides of a downstream sequences was submitted to the GenBank database under the accession number AY960135. The *hsp70* gene from genomic DNA, the nucleotide sequence containing open reading frame disrupted with 2 introns and 5', 3' flanking, was submitted to the GenBank database under the accession number AY960136.

Table 3. Sequence of primers used in this study

Primers	sequences	Total bases
T7	5’-TAATACGACTCACTATAGGG-3’	20
SP6	5’-ATTTAGGTGACACTATAG-3’	18
H70-PMW1	5’-CCTCCGTATCATCAACGAAC-3’	20
H70-PMW2	5’-TCTCTTCCGTTCCACCAT-3’	18
PMH70-F	5’-CCTCTTCCCATACACACC-3’	18
PMH70-F2	5’-CAGTCTTCGATGCTAAGCGT-3’	20
PMH70-F3	5’-GGACGGTATCTTCGAGGTCAA-3’	21
PMH70-R	5’-GAATCTCGCATAGCAAGG-3’	18
H70-REV1	5’-CTTCTGCTGAGCCTCGTA-3’	18
PMH70-REV2	5’-CTTAGCACGCTCACAGGCAGT-3’	21