CHAPTER IV

RESULTS

4.1 Quantification of commercial chlorpyrifos

By using technical grade chlorpyrifos as standard, the result of GC analysis of commercial chlorpyrifos used in this study was shown in Table 4.1. It was revealed that, at 10 mg/l of chlorpyrifos indicated by the product ingredient, only 6.81±0.09 (6.66-6.88) mg/l of chlorpyrifos was obtained. This indicated that commercial insecticide used in this study contained 27% chlorpyrifos instead of 40% as indicated by the product's label. Therefore, the concentration of chlorpyrifos presented in this study is used as the actual concentration detected by GC analysis in comparison with technical grade chlorpyrifos.

Table 4.1 GC analysis of commercial chlorpyrifos

Replication	Chlorpyrifos concentration (mg/l)		Yield (%)
	expected	Detected	
1	10	6.86	68.6
2	10	6.85	68.5
3	10	6.66	66.6
4	10	6.88	68.8
5	10	6.82	68.2
Average	10	6.81±0.09	68.1±0.9

Remark: Limit of detection (LOD) = 0.2 µg/l
Limit of quantification (LOQ) = 0.7 µg/l

4.2 Quantification of chlorpyrifos residue in water sample

After 1, 24, and 48 h of chlorpyrifos spiked to treatment water, water samples were collected and subjected to GC analysis. Results showed a rapid decrease of chlorpyrifos from the initial concentrations (0, 6.81, 13.62, 27.24, 54.48, and 68.1 µg/l) to almost less than the detection limit (0.2 µg/l) within 48 h (Table 4.2).

Table 4.2 Residue concentration of chlorpyrifos in treatment water

Innitial Concentration (µg/l)	Residue Concentration (µg/l) (%)		
	1 h	24 h	48 h
0	< LOD	< LOD	< LOD
6.81	2.02±0.83 (29.64)	< LOD	< LOD
13.62	3.04±0.45 (22.30)	0.31±0.23 (2.30)	< LOD
27.24	7.78±3.91 (28.55)	0.48±0.23 (1.76)	< LOD
54.48	12.37±1.86 (22.70)	1.08±0.16 (1.98)	0.53±0.09 (0.97)
68.1	18.37±8.49 (26.98)	1.37±0.35 (2.01)	0.68±0.27 (1.00)

Remark: Limit of detection (LOD) = 0.2 µg/l
Limit of quantification (LOQ) = 0.7 µg/l

4.3 Acute toxicity test of chlorpyrifos

The result of range finding test revealed that complete mortality was obtained in shrimp exposed to 68.1 and 681 µg/l of chlorpyrifos within 24 h while no mortality was found in shrimp exposed to 6.81 µg/l of chlorpyrifos throughout the experiment. As the result, definitive test for LC_{50} was conducted with 5 serial concentrations ranging from 6.81 to 68.1 µg/l of chlorpyrifos. For the result of definitive test, mortality of shrimp exposed to chlorpyrifos at the concentration of 6.81, 13.62, 27.24, 54.48, and 68.1 µg/l after 96 h exposure was 11.7, 25, 51.7, 95, and 98.3, respectively, while mortality of control shrimp was not found throughout the experiment. The calculated 24, 48, 72, and 96 h LC_{50} value of chlorpyrifos for *P. monodon* were 52.43, 28.21, 23.64, and 20.74 µg/l, respectively (Table 4.5).

Table 4.3 Accumulative mortality of shrimps from the range finding test

Chlorpyrifos Concentration (µg/l)	Accumulative mortality of shrimp (%) (N=20)			
	24 h	48 h	72 h	96 h
0	5	5	15	15
6.81	0	0	0	0
68.1	100	100	100	100
681	100	100	100	100

Table 4.4 Accumulative mortality of shrimps from the definitive test

Chlorpyrifos Concentration (µg/l)	Accumulative mortality of shrimp (%) (N=20)			
	24 h	48 h	72 h	96 h
0	0	0	0	0
6.81	5	8.3	10	11.7
13.62	8.3	15	25	25
27.24	11.7	21.7	33.3	51.7
54.84	45	93.3	95	95
68.1	75	93.3	96.7	98.3

Table 4.5 The LC_{50} and 95% confidence intervals at various exposure periods of chlorpyrifos to juvenile *P. monodon*

Time (h)	Concentration (µg/l)	95% Confidence Limits	Slope±SE	Intercept±SE
24	52.43	39.53-81.02	2.43 ± 0.52	0.82 ± 0.84
48	28.21	0.00-868.94	3.25 ± 1.02	0.28 ± 1.53
72	23.64	18.40-29.82	3.09 ± 0.49	0.76 ± 0.71
96	20.74	16.12-26.06	3.19 ± 0.50	0.79 ± 0.70

Remark. Theoretical Spontaneous Response Rate = 0.0000

4.4 Assay for Acetylcholinesterase (AChE) activity

For the result of AChE inhibition in the shrimp exposed to lethal concentration of chlorpyrifos, it was observed that AChE activity from the gill of shrimp decreased in corresponding to the increased concentrations of chlorpyrifos. The AChE activities observed in shrimp exposed to 68.1 and 681 µg/l of chlorpyrifos were significantly lower than that of control shrimp after 30 min of exposure ($P<0.05$) (Table 4.6).

For sub lethal exposure test, the AChE activity of shrimp was significantly lower than that of control shrimp after exposing to 0.681 µg/l of chlorpyrifos for 72 h ($P<0.05$). The result indicated that the inhibition of the enzyme activity increased in corresponding to the increasing levels of chlorpyrifos concentration. However, the inhibition was no longer detected after 96 h of exposure.

Table 4.6 Inhibitory effects of chlorpyrifos on AChE (mean±S.D.) in gills of juvenile *P. monodon* at the lethal concentration of chlorpyrifos (30 min post treatment).

Chlopyrifos concentration (µg/l)	**AChE Activity (nmol/min/mg protein) (N=5)**
0	4.33 ± 1.51^{a}
0.681	3.56 ± 0.71^{ab}
6.81	3.97 ± 1.42^{ab}
68.1	2.46 ± 0.82^{bc}
681	1.28 ± 1.13^{c}

Remark: The activity of AChE at 0 h = 3.45 ± 0.93.

The same superscripts indicated that the AChE activity was not significantly different ($P \geq 0.05$)

Table 4.7 Inhibitory effects of chlorpyrifos on AChE (mean±S.D.) in gills of juvenile *P. monodon* at the sub-lethal concentration of chlorpyrifos (24-96 h post treatment).

Chlopyrifos concentration (µg/l)	**AChE Activity (nmol/min/mg protein) (N=5)**			
	24 h	**48 h**	**72 h**	**96 h**
0	6.66 ± 1.32	3.25 ± 0.79	4.15 ± 1.17^{a}	3.61 ± 2.07
0.00681	7.16 ± 2.40	2.67 ± 0.61	3.55 ± 2.02^{ab}	2.09 ± 0.93
0.0681	6.21 ± 2.67	4.87 ± 4.44	2.83 ± 1.18^{ab}	2.74 ± 0.90
0.681	5.98 ± 1.77	2.25 ± 2.37	2.12 ± 0.86^{b}	1.94 ± 0.21

Remark: The activity of AChE at 0 h = 6.79 ± 3.51.

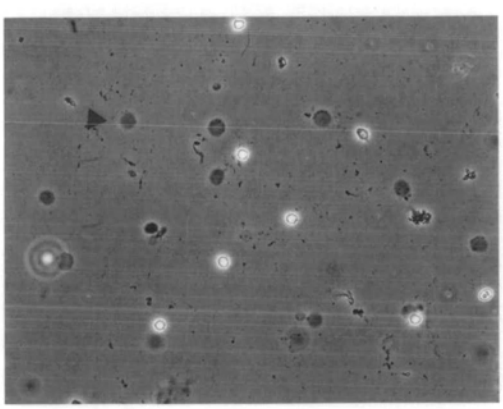
The same superscripts indicated that the AChE activity was not significantly different ($P \geq 0.05$).

4.5 Single cell gel electrophoresis analysis (Comet assay)

4.5.1 Viability of haemocyte

Viabilities of haemocytes exposed to 0, 0.007, 0.034, and 0.170 µg/l of chlorpyrifos were determined at 1, 6, 12, and 24 h of exposure. The result showed that the viability of the cells exposed to all concentrations of chlorpyrifos significantly decreased within the first hour of exposure ($P < 0.05$) when compared to control cells. Similar results were detected after 6 and 12 h of exposure. At 24 h, the viabilities of the haemocytes from all treatments appeared to be at the same level. Only haemocytes exposed to 0.007 µg/l of chlorpyrifos were significantly lower than that from other treatments ($P < 0.05$) (Table 4.8). No significant difference of cell viability was detected between haemocytes exposed to different concentration of chlorpyrifos. This indicates that in vitro cytotoxicity of chlorpyrifos to the haemocytes of *P. monodon* at a very low level (0.007 µg/l) can be detected within 1 h.

The viability of the cells from control decreased during the experiment, indicating the effect of the time factor for maintaining haemocytes in M199 media. To limit the interference of cytotoxic effect to the comet result, the experiment for detecting the DNA damage using single cell gel electrophoresis assay was conducted on 1 and 6 h of exposure where the number of dead cells were still low (25% or lower).


a.

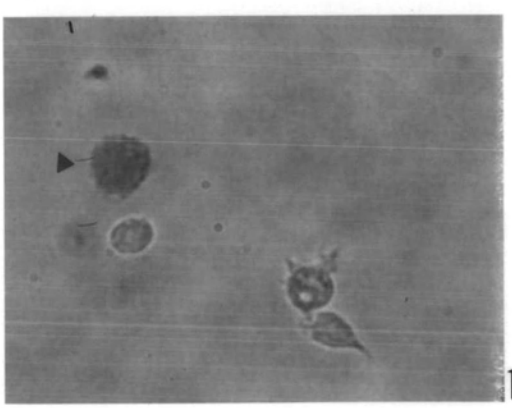

b.

Figure 4.1 200X (a.) and 1000X (b.) haemocytes of shrimp stained with trypan blue dye for cell viability test. Red arrows indicate dead cells.

Table 4.8 Viability of *P. monodon* haemocytes exposed to chlorpyrifos. Viability at time 0 was 97.2±3.3 %.

Time (h)	Chlorpyrifos Concentration (µg/l)			
	0 (control)	**0.007**	**0.034**	**0.170**
1	92.9 ± 3.0^{a}	83.7 ± 3.5^{b}	86.2 ± 2.2^{b}	86.8 ± 3.2^{b}
6	86.1 ± 4.1^{a}	77.3 ± 3.3^{b}	83.0 ± 1.2^{ab}	74.7 ± 6.7^{ab}
12	76.2 ± 3.2^{a}	66.1 ± 4.3^{b}	76.3 ± 5.3^{ab}	68.4 ± 3.6^{b}
24	57.4 ± 6.6^{a}	45.7 ± 5.4^{b}	60.9 ± 6.8^{a}	52.5 ± 3.8^{a}

Remark: The same superscripts indicated that the haemocyte viability was not significantly different ($P\geq0.05$) among group of treatment within the same period of exposure.

4.5.2 Non-genotoxic effect

DNA damage causing by non-genotoxic effect of chlorpyrifos can be detected by the presence of ghost cells in SCGE analysis. Ghost cells and their number detected in single cell gel electrophoresis analysis were shown in Figure 4.2 and Table 4.9. No significant difference between the numbers of ghost cells was detected from all treatments ($P > 0.05$). To prevent fault positive of DNA damage from non-genotoxic effect, ghost cells presented in the treatment were excluded from the analysis of comet assay.

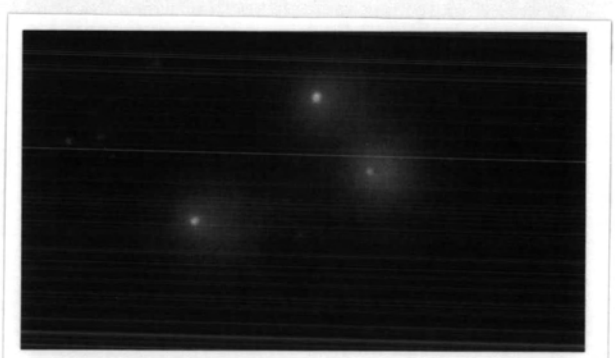


Figure 4.2 Ghost cells of haemocytes occurred during chlorpyrifos exposure and detected by single cell gel electrophoresis

Table 4.9 Percentage of ghost cells present in comet assay

Exposure time (h)	Chlorpyrifos concentration (µg/l)			
	0	0.007	0.034	0.170
1	46.84±12.72	52.35±5.34	73.66±11.77	48.35±8.70
6	48.00±31.24	50.67±15.53	17.42±20.60	17.22±21.26

4.5.3 DNA damage

The amount of DNA damage in the cell can be estimated from tail length as the extent of the migration of the genetic material in the direction of the anode and the tail moment which is calculated by multiplying the tail length with % of DNA in tail. Mean tail length of comets obtained by chlorpyrifos exposures are given in Table 4.10. The trend of increase in comet tail length with increase in concentration and duration is depicted in Figure 4.3. At 1 h exposure, the comet tail lengths of the haemocytes treated with 0.007, 0.034, and 0.170 µg/l of chlorpyrifos were 13.88, 23.17, and 22.11µm in length, which were 5.94, 17.23, and 14.17 µm longer than that of control.

All concentrations evoked significant DNA damage ($P<0.05$) when compared with controls. The results also showed significant differences between treatment groups. The tail lengths at 0.034 and 0.170 µg/l of chlorpyrifos treatments showed no significant difference between them. At 6 h exposure, the tail lengths of haemocytes exposed to 0.007 and 0.034 µg/l of chlorpyrifos were 15.20 and 14.69 in length which were slightly longer than that of control. However, there was no significant difference. A statistically significant increase in the extent of the tail moment took place only with the chlorpyrifos concentration of 0.170 µg/l. The tail moment was 10.7 longer than that of control. A gradual decrease in the tail length was observed when compared to the 1 h post-treatment with the lower doses of chlorpyrifos. This showed the commencement of the repair of the damaged DNA.

Table 4.10 DNA tail length (μm) (mean ± SD) from haemocytes after 1 and 6 h of chlorpyrifos exposure.

Exposure time (h)	Chlorpyrifos concentration (μg/l)			
	0	**0.007**	**0.034**	**0.170**
1	7.94 ± 1.68^{a}	13.88 ± 3.14^{b}	23.17 ± 2.03^{c}	22.11 ± 2.17^{b}
6	10.59 ± 3.68^{a}	15.20 ± 4.90^{a}	14.69 ± 1.35^{a}	21.29 ± 0.27^{b}

Remark: The same superscripts indicated that the DNA tail length was not significantly different ($P\geq0.05$) among group of treatment within the same period of exposure.

The values of comet tail moment of DNA are presented in Table 4.8. Significant differences on the DNA tail moment of haemocytes exposed to different concentrations of chlorpyrifos were obtained ($P<0.05$). Within 1 h of exposure, there was a small difference in the extent of comet tail moment between haemocytes treated with 0.007 μg/l chlorpyrifos and control group, although it was not statistically significant ($P>0.05$). Significant increase of the DNA tail moments were detected from the haemocytes exposed to 0.034 and 0.170 μg/l of chlorpyrifos when compared to that of control ($P<0.05$). At 6 h of exposure, significant increase of DNA tail moment was detected from haemocytes exposed to 0.170 μg/l of chlorpyrifos when compared to that of control ($P<0.05$). The increases of the tail moments from the chlorpyrifos-treated haemocytes indicated that the amount of DNA damage has increased proportionally according to the increasing concentrations of chlorpyrifos. A gradual decrease in the tail moment indicating the DNA repair mechanism was also obtained.

Table 4.11 DNA tail moment (mean ± SD) representing DNA damage after 1 and 6 h of chlorpyrifos exposure.

Exposure time (h)	Chlorpyrifos concentration (µg/l)			
	0	**0.007**	**0.034**	**0.170**
1	2.76±1.09 [a]	3.23±1.23 [a]	9.38±1.61 [b]	7.36±2.90 [b]
6	4.22+0.81 [a]	5.58+1.53 [a]	5.38+0.78 [a]	8.86+1.17 [b]

Remark: The same superscripts indicated that the DNA tail moment was not significantly different (P≥0.05) among group of treatment within the same period of exposure.

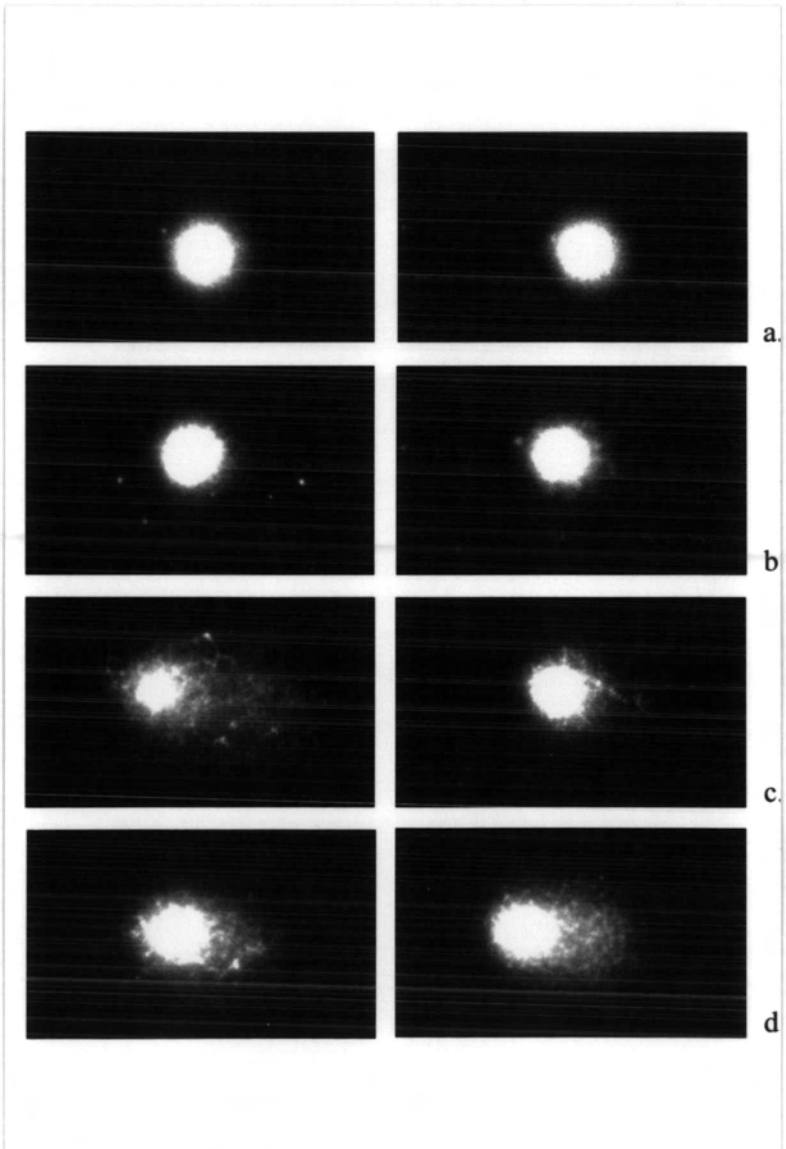


Figure 4.3 Haemocytes exposed to 0, 0.007, 0.034, and 0.170 µg/l (a-d) chlorpyrifos within 1 and 6 h.

4.6 Cloning and characterization of xenobiotic inducible genes in *P. monodon*

4.6.1 Isolation and Determination of partial sequence of xenobiotic inducible genes

Amplification of 7 target genes, including carboxylesterase, cytochrome P450, beta glucuronidase, glutathione-s-transferase, heat shock protein 70, heat shock protein 90, and vitellogenin using first strand cDNA from haemocyte, gill, and hepatopancreas of shrimp, the results showed variation of gene expression in different tissues (Figure 4.4-4.10). Expression of the genes was summarized in Table 4.12.

PCR products of cytochrome P450 (from F1R2 degenerated primer combination), beta glucuronidase, and glutathione-s-transferase (from generated primer combination) were subjected to cloning and sequencing analysis. Results from Blast X search (NCBI) showed similarity of the sequence to the genes reported in other animal species (Table 4.13).

Table 4.12 Expression of target genes in different tissues of *P. monodon*

Gene	Primer	Expected Size	Tissue		
			Haemocyte	Gill	Hepatopancreas
1. Carboxylesterase	F2R2	204	+	+	nd
2. Cytochrome P450	F1R1, F1R2, F2R1, F2R2	336, 402, 168, 234	+ nd nd nd	nd nd nd nd	+ + + +
3. Glucuronidase	F1R1	196	+	+	+
4. Glutathione-s-transferase	F1R1	225	nd	nd	+
5. Heat Shock Protein70	F1R1	719	+	+	+
6. Heat Shock Protein90	F1R1	612	+	+	+
7. Vitellogenin	F1R1	416	nd	+	+

Remark: + indicates expression in the target tissue was detected.

nd indicates expression was not detected.

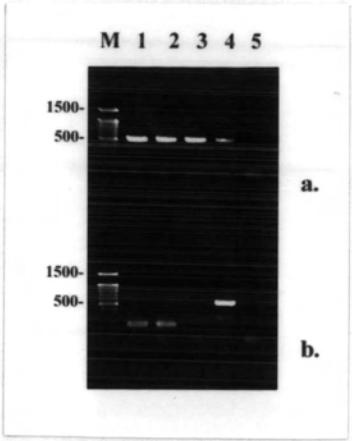


Figure 4.4 PCR products of carboxylesterase F2R2 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Elongation factor 1 alpha used as positive control is shown in Lane 1-4a. Lane M is 100 bp DNA ladder. Negative control is shown in Lane 5.

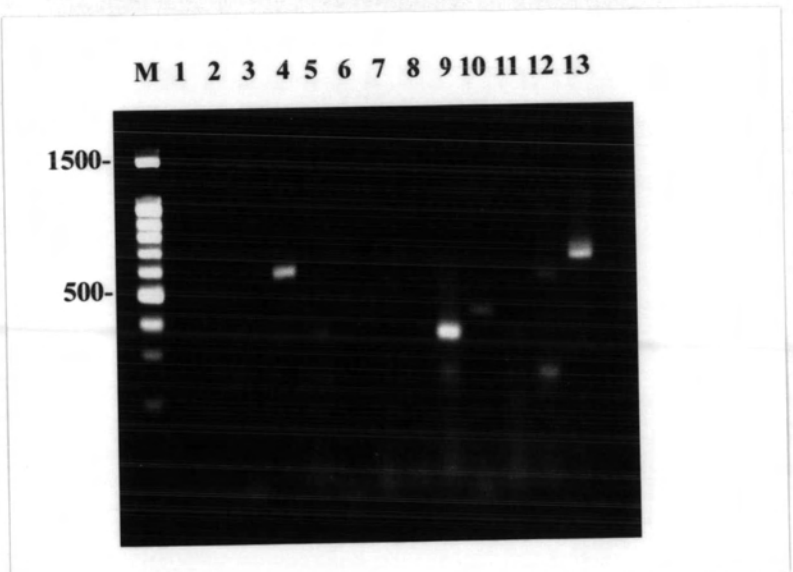


Figure 4.5 PCR products of cytochrome P450 primer combinations (F1R1, F1R2, F2R1, and F2R2) using first strand cDNA from gill (Lane 1-4), haemocyte (Lane 5-8), and hepatopancreas (Lane 9-12) as template. Lane M is 100 bp DNA ladder. Positive control (HSP70) is shown in Lane 13.

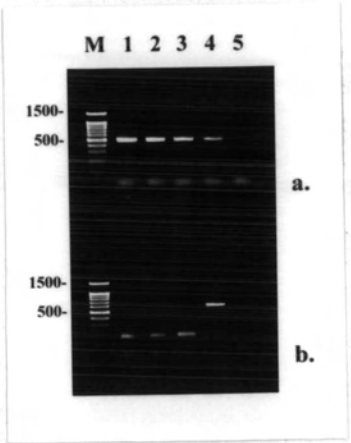


Figure 4.6 PCR products of beta glucuronidase F1R1 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Lane M is 100 bp DNA ladder. Elongation factor 1 alpha used as positive control is shown in Lane 1-4a. M is 100 bp DNA ladder. Negative control is shown in Lane 5.

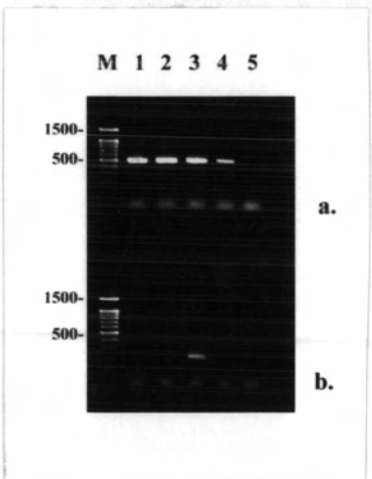


Figure 4.7 PCR products of glutathione-s-transferase F1R1 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Elongation factor 1 alpha used as positive control is shown in Lane 1-4a. Lane M is 100 bp DNA ladder. Negative control is shown in Lane 5.

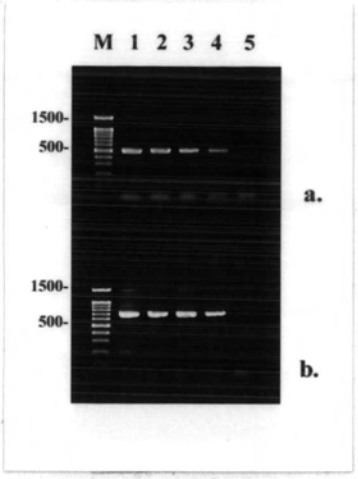


Figure 4.8 PCR products of heat shock protein 70 F1R1 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Lane M is 100 bp DNA ladder. Negative control is shown in Lane 5.

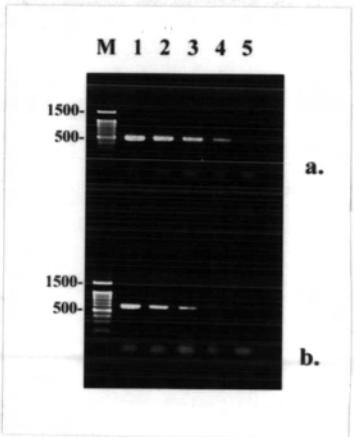


Figure 4.9 PCR products of heat shock protein 90 F1R1 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Lane M is 100 bp DNA ladder. Negative control is shown in Lane 5.

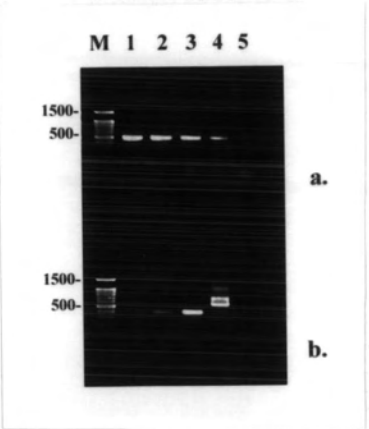


Figure 4.10 PCR products of vitellogenin F1R1 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Lane M is 100 bp DNA ladder. Negative control is shown in Lane 5.

Table 4.13 Summary of partial gene sequences from RT-PCR using hepatopancreas first strand cDNA as template

Gene	Primer	Size (bp)	Putative Gene	Species	Expect value	Figure
Cytochrome P450	F1R2	401	Cytochrome P450 *CYP4C17*	*Haliotis rufescens*	$3x10^{-51}$	4.7
Beta glucuronidase	F1R1	196	Beta-glucuronidase precursor (Beta-G1)	*Macaca mulatta*	$3x10^{-15}$	4.8
Glutathione-s-transferase	F1R1	225	Glutathione S transferase-1	*Culicoides variipennis*	$2x10^{-25}$	4.9

Remark: Nucleotide sequences are shown in Table B1, appendix B

4.6.2 Isolation and characterization of xenobiotic inducible genes using RACE-PCR

RACE-PCR was conducted to obtain 5' and 3' cDNA fragment of 3 target genes, including carboxylesterase, cytochrome P450, and glutathione-s-transferase. Sense and anti-sense gene specific primers were designed for 3' and 5' RACE-PCR and used in combination with universal primer that recognizes the SMART sequence. Fragments of expected size were subjected to cloning and sequencing analysis (Figure 4.11, 4.14, 17, Table 4.14).

Table 4.14 Summary of 5' and 3'nucleotide sequences from RACE-PCR

Gene	RACE PCR Product	Size (bp)	Figure
Carboxylesterase	5'	652	4.13
	3'	1,212	4.13
Cytochrome P450	5'	1,063	4.16
	3'	726	4.16
Glutathione-s-transferase	3'	692	4.19

Remark: Nucleotide sequences are shown in Table B2, appendix B

4.6.3 Determination of complete sequence of xenobiotic inducible genes

The sequences of carboxylesterase, cytochrome P450, and glutathione-s-transferase were obtained from partial sequence combination of RT-PCR, RACE PCR and EST from haemocyte and hepatopancreas libraries.

For carboxylesterase, combination of nucleotide sequence of carboxylesterase provided 2,206 bp in length. The ORF of carboxylesterase was 1,746 bp encoding a polypeptide of 582 amino acids. The 5' and 3' UTR were 41 bp and 389 bp (excluding the poly A tail). The complete sequence of carboxylesterase cDNA is shown in Figure 4.13. The consensus patterns of carboxylesterase were found (Table 4.15).

The full length of carboxylesterase cDNA was search against data in GenBank using Blast X and the closest homologue was carboxylesterase of *Athalia rosae* ($1e^{-72}$, accession number BAD91555). Carboxylesterase amino acid sequences of *P. monodon, Athalia rosae* (BAD91555), *Bombyx mori* (ABK27874), *Aedes aegypti* (EAT45545), *Bos aurus* (ABK27874) were multiple aligned and shown in Figure 4.15.

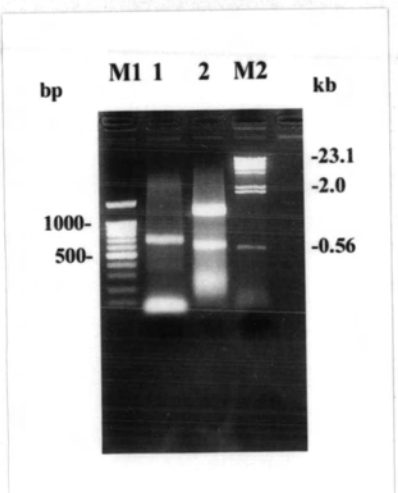


Figure 4.11 5' and 3' RACE-PCR of carboxylesterase (Lane 1-2). Lane M1 is 100 bp DNA ladder. Lane M2 is λ Hind III DNA ladder.

Table 4.15 Consensus pattern of carboxylesterase

Consensus pattern:	*P. monodon* Carboxylesterase
Carboxylesterases type-B signature 2 [EDA] - [DG] - C - L - [YTF] - [LIVT] - [DNS] - [LIV] - [LIVFYW] - x - [PQR] C is involved in a disulfide bond	E - D - C - L - Y - L - S - V - Y - T - P
Carboxylesterases type-B serine active site F - [GR] - G - x(4) - [LIVM] - x - [LIV] - x - G - x - S - [STAG] - G S is the active site residue	L - G - G - D - P - G - K - V - T - L - F - G - E - S - A - G

```
                                                      M  V  S  R  S  Y  A
    1 ACGCGGGGGGGGAGTCGTGGCTGTGCCTCCGGAGCGGAAAGATGGTTAGTCGAAGTTACG
        M  K  L  W  V  P  L  L  L  T  A  W  M  A  T  L  S  K  A  Q
   61 CAATGAAGCTGTGGGTCCCTCTTCTTCTGACGGCCTGGATGGCAACACTGTCGAAGGCGC
        Q  Q  E  T  T  T  S  T  E  P  S  I  E  V  R  L  R  Q  G  V
  121 AGCAGCAGGAAACCACGACATCCACAGAGCCTTCGATTGAGGTGCGTCTCCGGCAGGGCG
        I  T  G  A  Q  S  E  A  G  N  G  R  V  F  Y  S  F  K  T  I
  181 TGATCACAGGGGCCCAGTCAGAGGCCGGAAACGGTAGGGTCTTCTACAGCTTCAAGACCA
        P  F  A  E  P  P  V  E  D  L  R  F  R  D  P  V  P  A  R  P
  241 TTCCCTTCGCCGAGCCTCCTGTCGAGGACCTAAGGTTTAGGGACCCTGTTCCTGCAAGGC
        W  A  G  V  R  N  G  S  I  A  T  P  K  C  P  Q  L  G  N  A
  301 CATGGGCAGGAGTAAGAAATGGATCCATCGCCACACCGAAATGCCCACAGTTGGGAAATG
        T  V  E  G  Q  E  D  C  L  Y  L  S  V  Y  T  P  R  P  Y  A
  361 CTACTGTTGAGGGGCAGGAAGACTGTCTCTATCTCTCCGTCTACACACCTCGGCCTTACG
        S  D  L  P  V  M  V  W  I  H  G  G  G  F  T  N  G  Q  G  E
  421 CGTCGGACTTGCCTGTCATGGTGTGGATTCACGGCGGAGGATTCACGAACGGTCAAGGCG
        V  F  G  P  L  P  L  L  T  K  D  V  V  L  V  V  I  Q  Y  R
  481 AGGTCTTCGGGCCCCTTCCTCTCCTCACGAAGGATGTGGTCCTCGTGGTCATACAGTATC
        L  A  T  L  G  F  L  S  T  E  D  N  E  L  P  G  N  L  G  L
  541 GCCTGGCCACGCTGGGATTCTTATCGACTGAGGACAATGAGCTGCCTGGCAATCTAGGAC
        K  D  Q  R  M  A  L  L  W  V  Q  D  N  I  R  D  L  G  G  D
  601 TCAAGGACCAAAGGATGGCTCTCCTGTGGGTGCAAGACAACATCCGTGACCTCGGCGGCG
        P  G  K  V  T  L  F  G  E  S  A  G  A  G  A  V  H  F  H  V
  661 ACCCAGGCAAGGTCACCCTCTTCGGGGAGAGCGCCGGGGCAGGGGCAGTGCATTTCCACG
        L  S  P  M  S  S  G  L  F  S  R  A  I  L  Q  S  G  T  S  L
  721 TCCTGTCTCCCATGTCTTCAGGACTGTTCAGCCGTGCTATCCTGCAGTCGGGGACATCGC
        C  P  W  A  T  A  E  N  H  R  Q  V  A  A  K  I  G  Q  M  F
  781 TGTGTCCGTGGGCTACTGCGGAAAACCACAGACAGGTAGCCGCTAAGATTGGTCAGATGT
        N  C  S  G  I  S  D  Q  Q  L  T  S  S  S  A  F  V  A  C  L
  841 TCAACTGTTCAGGGATAAGTGATCAGCAACTTACCAGCAGCTCAGCCTTCGTCGCTTGTT
        R  N  V  P  Y  E  D  L  I  S  A  Q  K  K  F  V  I  F  N  E
  901 TGAGGAATGTCCCTTACGAAGACTTAATTTCAGCCCAGAAGAAATTCGTTATCTTCAACG
        S  P  Q  Y  M  L  P  R  V  D  G  H  F  L  P  D  Y  P  A  V
  961 AATCCCCTCAATACATGTTACCGCGCGTTGATGGCCATTTTCTACCCGACTACCCCGCCG
        L  L  R  R  G  R  Y  N  K  V  D  I  I  S  G  I  T  Q  D  E
 1021 TTCTGCTGAGAAGAGGACGGTATAACAAGGTGGACATTATATCTGGGATTACGCAAGATG
        A  A  V  I  G  L  I  F  T  L  D  K  A  A  A  N  S  L  V  Q
 1081 AGGCAGCCGTGATTGGCCTGATTTTCACCTTGGACAAAGCCGCTGCAAACAGCCTGGTCC
```

```
          N  F  S  V  N  G  P  V  S  L  I  F  E  A  W  E  D  D  P  E
1141  AGAACTTCTCTGTCAACGGACCAGTTTCACTGATCTTCGAAGCTTGGGAAGATGACCCGG
          Y  L  A  R  R  A  F  H  H  Y  L  G  A  I  E  V  T  E  E  K
1201  AGTACCTGGCACGCCGAGCCTTCCACCACTACCTGGGCGCCATTGAAGTGACAGAAGAGA
          R  D  S  L  I  R  L  F  S  D  R  M  F  D  M  C  H  L  D  A
1261  AACGGGATTCACTTATCAGGCTTTTCAGTGATAGAATGTTCGACATGTGTCATCTTGACG
          V  G  Q  H  L  R  T  S  H  Q  N  V  F  T  Y  K  L  Q  H  D
1321  CGGTCGGGCAACACCTCAGAACATCTCATCAGAACGTGTTTACATACAAACTGCAGCATG
          G  E  H  Q  F  V  F  G  L  F  P  T  T  P  D  W  Y  K  S  Y
1381  ACGGAGAACACCAGTTTGTTTTTGGTCTTTTCCCTACTACTCCGGATTGGTACAAAAGCT
          V  G  H  A  D  D  I  L  Y  L  F  G  Q  A  E  G  N  R  T  L
1441  ATGTCGGTCACGCAGATGATATTTTGTACCTGTTCGGTCAAGCTGAGGGCAACAGAACGT
          K  R  D  E  D  L  F  V  S  R  I  M  V  E  L  W  T  N  F  A
1501  TGAAGCGAGACGAGGATCTGTTCGTTAGCCGTATCATGGTAGAGCTGTGGACCAACTTCG
          S  V  G  H  P  T  P  D  M  S  L  G  F  K  W  N  P  T  S  F
1561  CTTCCGTCGGACACCCGACGCCTGACATGTCCCTCGGCTTCAAATGGAACCCGACGTCCT
          P  T  D  S  Y  L  S  I  T  S  S  P  T  M  K  T  F  E  D  C
1621  TCCCAACAGACTCCTACTTGTCCATCACCTCCTCACCCACCATGAAAACCTTCGAAGACT
          E  T  R  E  F  W  K  N  M  P  T  K  N  N  K  M  L  Y  P  E
1681  GCGAGACCCGTGAATTCTGGAAGAACATGCCTACCAAGAATAACAAAATGCTGTACCCTG
          R  F  Y  K  C  H  L  P  G  C  L  D  M  Y  L  *
1741  AGCGCTTCTACAAGTGTCACTTACCTGGCTGCTTAGACATGTACCTGTAATAAACTGATA
1801  GGATGGCGCTTCACTGATATCTAGTTGTCAGTGTCATTAGTGCCAAGAGACCCTGTTCTT
1861  ATTAGTTTAACAAATATTTAGCAGATAATATGTTCTTGGTGTGCAGTTTATTGAAATCTA
1921  ATAAATACAGGGTGAGTGTGATCTAATAACAGTAACGTAGCCATTGTTTACATTTTTTTT
1981  CTTTTTTTTGCACCATATTATCAAAATATTTTGAAAGTATGATTACCATCAGCTTCTGTG
2041  CCAAATCATATTTTCCAGTTTGTCAGAGATTATCAGTATTTCTCATAACCTTTGTGTCAC
2101  AATTTATGTAACATCGCAGTTATCCGTGATGCTGTTTTTGTGAACGTATATGTACACAAT
2161  TAATAAAGGAACTATTTCGAAAAAAAAAAAAAAAAAAAAAAAAAA  2206
```

Figure 4.12 The full lencth cDNA sequence of carboxylesterase of *P. monodon*. Start and stop codons are illustrated in boldface. The poly A additional signal is underlined. The consensus pattern of carboxylesterase is illustrated in italic-bolded.

```
A. rosae        ------MKKLTTIVPVLSFVIVFLYNRHFTYDYVEVQIDKGILKGFKTTTGRSNAD----
B. mori         ----------------------------MSESPLVTVEQGQLQGRIVNS-PSGKA----
A. aegypti      ------------MLKLIPILALAIAAARSDPARPIITTRGGQIQG-VTSSCGLFCS----
P. monodon      MVSRSYAMKLWVPLLLTAWMATLSKAQQQETTTSTEPSIEVRLRQGVITGAQSEAGNGRV
B. taurus       --MKPDGPRLRLRAVAFGFLLLLVPGQGQDSTRPVRTTHTGKVQGSLVYVNNADVG----
                                                          ::

A. rosae        YYAFKGIPYAKPPVGERRFKAPQEEAAWAGVRDALSHGNVCPHLDLAFG----------F
B. mori         FYSFQGIPYAKPPLGSLRFKAPKPAETWEGIRDATAEGNIAPQIDTSLTK---------T
A. aegypti      YFSFMGIPYGEPPVDELRFRNTVPHRGWEGIKDGGEHRASCPSG-ALVGD---------G
P. monodon      FYSFKTIPFAEPPVEDLRFRDPVPARPWAGVRNGSIATPKCPQLGN------------AT
B. taurus       VHTFLGIPFAKPPVGPLRFAPPEPPESWSGVKDGTSQPAKCPQDADGMKSMELWNVTLPS
                 .:*  **:.:**:   **  .    * *:::.      .*

A. rosae        LRGQEDCLYLNVYTPSVSSEGPLLPVMVWIHGGGFVLGSGNEEVYGSNYLLEAEVVLVTL
B. mori         YTGDENCLYLNVYTPHIAGN---LPVMIWIHGGAFKWGSGNETLYGPDYLVEKDVVVVTL
A. aegypti      YDGDEDCLYLNVYTQNIIGS---RPVMVWIHGGSFTGGSGDSWIYGPDHLIQENVVIVTI
P. monodon      VEGQEDCLYLSVYTPRP--YASDLPVMVWIHGGGFTNGQG-EVFGPLPLLTK-DVVLVVI
B. taurus       TSMSEDCLYLNIHTPAYSHEGSNLPVMVWIHGGGLVLGMA-SMYDGSALAAFGDVVVVVI
                   .*:****.::*          ***:*****.:  * . .          :**:*.:
```

```
A. rosae        NYRLGALGFLSIEDDEAPGNAGLKDQVAALRWVRRNIKHFGGDPERVTLFGESAGGASVH
B. mori         NYRCGPLGFLCLNTPEVPGNAGLKDVVQALRWLKQNIKSFGGDPDNFTVFGQSAGGAIVT
A. aegypti      NYRLGILGFFSTGDEHAQGNWGMKDCVEALRWVRDNIAAFGGDPNNVTVFGESAGGAAAH
P. monodon      QYRLATLGFLSTEDNELPGNLGLKDQRMALLWVQDNIRDLGGDPGKVTLFGESAGAGAVH
B. taurus       QYRLGLLGFFSTGDKHATGNWGYLDQVAALRWVQQNIAYFGGDPGRVTIFGESAGGISVS
                :** . ***:.    .  ** *  *   ** *:: **  :**** ..*:**:***.  .

A. rosae        LHLLSPLSAGLFSQAIGQSGSGANPWVISHNVSNNTIRLAECLGAKNIDGDKRLALQFLK
B. mori         ILTASPLSKNMINKAIVQSGTGISKWAVQNEPLTCAKALASHLGCEADNVD--EVLEFLN
A. aegypti      YLVLSPMATGLFHKAIIQSGTSLSPWAFQYNPREMSRHVADTFGYPTNNNA--ELVRLLR
P. monodon      FHVLSPMSSGLFSRAILQSGTSLCPWATAENHRQVAAKIGQMFNCSGISDQ--------Q
B. taurus       LHVISPMSQGLFHGAIMESGVALLPGLTINSSDKVAKVVANLSACG--------------
                   **:: .::  ** :** .       .    :  :..

A. rosae        TAPYGDIIKIQSTLRTSEEVRTRVAFLYTPSVETGV--NVDEAFLPDHPMEIIRSGKFNK
B. mori         TVTAKELVEATEIVNSFDSLVVDQNNFFSVVVEKEF--PGVEAVLTEPLLDFLTSGRTAE
A. aegypti      YTPKGEFVRLQQGWTDIPIPRGFKPFEFVPTAEPAN--SPEPTFLTQRPIDLLNAGNFNK
P. monodon      LTSSSAFVACLRNVPYEDLISAQKKFVIFNESPQYMLPRVDGHFLPDYPAVLLRRGRYNK
B. taurus       QVDSEALVDCLRHKNEEEVLAINKLVKIIPG-------VVDGIFLPKHPLELLASDDFQP
                 .    ::                                    .*..    ::  .

A. rosae        VPYITGYTSHEGYLFMKELTKPETMNAAYE-DTARYVPRDIKNEEFRN---ALGKSIRTF
B. mori         IPIMIGSTTLELLTNLRPS------------DLQMFIPSDLNIEKDSDESLAIAENLKGL
A. aegypti      MPMVFGYTDAESLFMIHEHRIDSTVWNEFSRNPQFFVPHYWRITPGTAASNGVSQGFRDF
P. monodon      VDIISGITQDEAAVIGLIFTLDKAAANSLVQNFSVNGPVSLIFEAWEDDPEYLARRAFHH
B. taurus       VPSIIGVNNDEYGWIIPLSVNNSDTRREISRESVRN--ALQELSTMTTMPPEFGELLMEE
                :  : * .  *                      :                    ...

A. rosae        YFEDKPIGTDNISNLVDLYTDTTFVAGINIATKLQLSVGQSPIYFYPFSYDGGLN-LLKY
B. mori         YFTSDTE-ENKAEGLNRLHSDLLLNININRYIKYLVQNSNQPIYFYKFDYVGQFNFAHKS
A. aegypti      YWQDRPLGPDIMLEWTRFHTDQQFIYPIDKTIRLTAQHNTSPTFYYQFSFDGDLNLVKRL
P. monodon      YLGAIEVTEEKRDSLIRLFSDRMFDMCHLDAVGQHLRTSHQNVFTYKLQHDGEHQFVFGL
B. taurus       YIEDSEDHQTLQNQLHEIMGDYLFVIPALQVAMFHR--SHAPVYFYEFQHQ--SSFFKDV
                *                 :  *  :              .    : * :..    .

A. rosae        FFKIS---LPGAAHGDELGYLFNHQLLFWRKAEPASEDEDVMLMMVRLWTNFAKYGNPTP
B. mori         FFDTE---LKYALHMDDLGYLFKNDFQKD-VDDPSPQDVKMRERMVRLWTNFAKFGNPTP
A. aegypti      ILLSD---WPGAVHADELPYMWSMTNLPITPILPGNPALTVRNRMVRLWTNFALHSNPTP
P. monodon      FPTTPDWYKSYVGHADDILYLFGQAEGNR--TLKRDEDLFVSRIMVELWTNFASVGHPTP
B. taurus       RPSS-----VRADHGDEVLFLFRNEQ-----IQFTEEEELLSRKMIKYWANFARNQNPN-
                          . * *:: :::                   :   *:. *:***   :*.

A. rosae        KG--------------------------------------DWTGSRLEAYNGGQIQLF
B. mori         EENHYLPTKWLPVTNDTLYYLNLGQELNLLQNPDEEIMKFWEDLYSKHFKIWEHTKINTI
A. aegypti      NSDSN-----------------------------------LQNVIWAPIQNQNMAFLDIN
P. monodon      DM--------------------------------------SLGFKWNPTSFPTDSYLSIT
B. taurus       ----------------------------------------GEGLPHWPMFDQEDQYMQLN
                                                         .          :.

A. rosae        AHLRKG------------------------------------------------------
B. mori         EPLRKLVYPDNEATEEQASIDDGQPSLEEKVVFEEIVPISSVLNPPVNGDAKSNADFKPK
A. aegypti      ANLVAGHYPNTARLNTWYDLESRYANGPFEYPMT--------------------------
P. monodon      SSPTMKTFEDCETREFWKNMPTKNNKMLYPERFYKCHLPGCLDMYL--------------
B. taurus       TQPAVGRALKAHRLQFWMKTLPQKTQELMETKEKHTEL----------------------


A. rosae        ------------------------------------------------
B. mori         ISNEIKMAQTSAPKDVVRASDPPEDDLPKNIGVNKFVNFYESLGGKK
A. aegypti      ------------------------------------------------
P. monodon      ------------------------------------------------
B. taurus       ------------------------------------------------
```

Figure 4.13 Multiple alignment of amino acid sequence of carboxylesterase of *P. monodon, Athalia rosae, Bombyx mori, Aedes aegypti, Bos taurus*

For cytochrome P450, combination of nucleotide sequence of cytochrome P450 provided 1,701 bp in length. The ORF of cytochrome P450 was 1,530 bp encoding a polypeptide of 510 amino acids. The 5' and 3' UTR were 22 bp and 116 bp (excluding the poly A tail). The complete sequence of cytochrome P450 cDNA is shown in Figure 4.15. The consensus patterns of cytochrome P450 were found (Table 4.16).

The full length of cytochrome P450 cDNA was search against data in Gen Bank using Blast X and the closest homologue was cytochrome P450 *CYP4C39* of *Carcinus maenas* ($8e^{-175}$, accession number JC8026). Cytochrome P450 amino acid sequences of *P. monodon*, *Carcinus maenas* (JC8026), *Orconectes limosus* (AAF09264), *Cherax quadricarinatus* (AAL56662), and *Aedes aegypti* (EAT35570) were multiple aligned and shown in Figure 4.16.

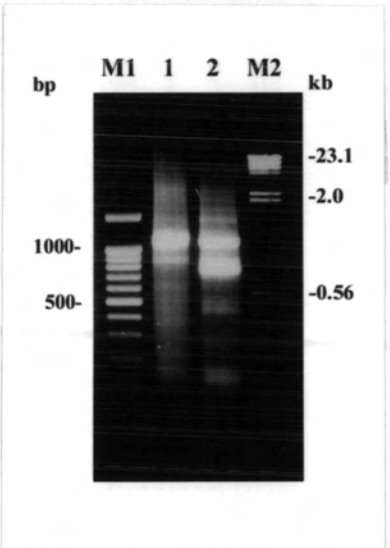


Figure 4.14 5' and 3' RACE-PCR of cytochrome P450 (Lane 1-2). Lane M1 is 100 bp DNA ladder. Lane M2 is λ Hind III DNA ladder.

Table 4.16 Consensus pattern of cytochrome P450

Consensus pattern:	***P. monodon* Cytochrome P450**
Cytochrome P450 cysteine heme-iron ligand signature [FW] - [SGNH] - x - [GD] - {F} - [RKHPT] - {P} - C - [LIVMFAP] - [GAD] C is the heme iron ligand	F - S - A - G - P - R - N - C - I - G

```
                                  M  W  P  K  N  D  T  L  V  W  A  S  S
   1  ACGCGGTGGAGTTGCTGCTGCGATGTGGCCCAAGAACGACACCCTCGTCTGGGCCTCGAG
         A  F  T  Y  L  A  F  T  V  T  L  A  L  G  L  A  W  F  L  Q
  61  TGCCTTCACGTACCTAGCCTTCACCGTGACGCTGGCCCTCGGCCTGGCCTGGTTCTTGCA
         R  Q  R  K  S  W  L  L  A  K  I  P  G  P  K  A  H  V  L  F
 121  GAGGCAACGGAAGTCATGGCTTCTTGCCAAGATCCCTGGTCCCAAAGCCCACGTCCTCTT
         G  S  H  R  A  G  G  A  A  E  D  R  I  Q  W  L  I  K  T  S
 181  CGGTTCTCACCGTGCCGGTGGCGCTGCAGAGGACCGCATCCAATGGCTCATCAAGACAAG
         T  L  G  E  V  V  K  F  W  I  G  F  L  P  T  C  M  I  C  S
 241  CACCCTGGGGGAAGTCGTCAAATTCTGGATTGGCTTTCTCCCGACGTGCATGATCTGCAG
         A  R  G  A  E  V  I  L  T  S  Q  K  H  I  N  K  G  N  N  Y
 301  CGCCAGAGGGGCAGAAGTTATTCTTACAAGTCAGAAGCACATCAATAAAGGCAATAATTA
         N  F  L  R  D  W  L  G  D  G  L  L  T  A  T  G  S  K  W  H
 361  CAACTTTCTCAGAGACTGGCTTGGCGACGGCCTCCTGACAGCCACAGGAAGCAAGTGGCA
         S  R  R  K  L  L  T  P  A  F  H  F  K  I  L  E  D  F  L  D
 421  CTCCCGAAGGAAGCTGCTGACGCCCGCCTTCCACTTCAAGATCCTGGAGGACTTCCTGGA
         V  F  N  S  Q  S  T  T  M  I  Q  Q  L  R  G  K  A  D  G  K
 481  CGTCTTCAACAGCCAGAGCACGACGATGATCCAGCAGCTGCGAGGGAAAGCCGACGGGAA
         P  F  D  V  F  P  F  I  T  R  C  A  L  D  I  I  C  E  T  A
 541  GCCGTTCGACGTTTTCCCTTTCATCACGCGCTGCGCCCTCGACATCATATGTGAAACTGC
         M  G  R  T  V  N  A  Q  C  N  A  N  S  E  Y  V  K  A  L  N
 601  GATGGGCCGAACGGTGAATGCCCAGTGCAATGCAAATTCCGAGTACGTGAAAGCCCTCAA
         R  F  A  E  L  L  I  K  R  S  T  T  P  W  L  R  P  D  W  L
 661  TAGGTTCGCTGAGCTACTGATTAAGCGGTCGACGACGCCGTGGCTCCGCCCCGACTGGCT
         Y  T  L  F  G  Q  R  S  D  Y  D  A  C  L  K  V  L  H  G  F
 721  CTACACTCTCTTCGGCCAGAGGAGTGACTACGACGCCTGCCTGAAGGTCCTCCATGGCTT
         S  Q  E  T  I  V  E  R  R  A  L  L  Q  N  S  K  R  N  G  K
 781  CTCCCAGGAGACCATCGTGGAGAGGAGGGCGCTACTGCAGAACTCCAAGAGGAATGGCAA
         E  E  D  T  E  E  V  F  G  K  K  K  R  L  A  F  L  D  L  L
 841  GGAAGAGGACACAGAGGAAGTCTTTGGCAAGAAGAAGCGGCTGGCGTTCCTGGACCTCCT
         L  E  Y  S  E  D  G  A  K  L  S  D  E  D  I  R  E  E  V  D
 901  GCTGGAGTACTCGGAAGACGGCGCCAAGCTCTCCGACGAGGACATCCGCGAGGAGGTGGA
         T  F  M  F  E  G  H  D  T  T  T  A  A  M  N  W  V  L  Y  L
 961  CACCTTCATGTTCGAGGGCCACGACACCACCACGGCGGCCATGAACTGGGTTCTCTACCT
         L  G  H  H  P  E  I  Q  A  R  V  H  Q  E  L  D  S  I  F  G
1021  CCTGGGTCATCACCCAGAGATACAGGCTCGGGTCCACCAGGAGCTGGACTCGATCTTCGG
         D  E  D  R  P  A  T  M  D  D  L  R  S  M  K  L  L  E  N  C
1081  TGACGAGGACCGCCCGGCGACGATGGACGACCTGCGCTCCATGAAGCTGCTGGAGAACTG
         I  K  E  G  L  R  L  F  P  S  V  H  R  F  A  R  T  L  R  E
1141  CATCAAGGAGGGCCTGAGGCTATTCCCGTCCGTCCACAGGTTTGCCAGGACGCTGCGAGA
         D  V  R  I  C  D  Y  V  I  P  A  G  T  N  I  M  L  F  V  Y
1201  AGACGTCCGCATATGCGACTACGTCATCCCGGCCGGAACCAACATCATGCTCTTCGTGTA
         R  I  H  R  D  P  K  Q  F  P  D  P  E  R  F  D  P  D  R  F
```

```
1261    CCGAATCCACCGCGACCCGAAGCAGTTCCCCGACCCGGAGAGGTTCGACCCGGACCGCTT
          L  P  E  N  S  K  H  R  H  P  Y  A  Y  I  P  F  S  A  G  P
1321    CCTGCCCGAGAACAGCAAACACCGCCACCCGTACGCTTACATTCCCTTCAGCGCCGGACC
          R  N  C  I  G  Q  K  F  A  Q  M  E  E  K  V  L  L  S  S  I
1381    CAGGAACTGCATCGGCCAGAAGTTCGCCCAGATGGAGGAGAAGGTCCTTCTCAGCAGCAT
          L  R  K  F  R  V  E  S  T  V  P  R  E  S  L  R  T  M  D  H
1441    CCTCCGCAAGTTCCGCGTCGAGAGCACTGTCCCTCGAGAGTCCCTGAGGACGATGGACCA
          F  V  L  R  P  K  G  G  N  N  L  R  L  F  P  R  S  *
1501    CTTTGTCTTGCGGCCAAAGGGAGGGAATAACCTGAGGCTCTTCCCGAGGTCGTGACGACA
1561    AACACGCCAAGACGAGCTAACAATCCTGGGTATCGATTGATGGGATCAGTGTACAGTGAT
1621    TGGGGGATCTTGCTTGGATGGAAGGAAATACACTCGTGTAATGTGAATAAAAAAAAAAAA
1681    AAAAAAAAAAAAAAAAAAAAA 1701
```

Figure 4.15 The full length cDNA sequence of cytochrome P450 of P. *monodon*. Start and stop codons are illustrated in boldface. The poly A additional signal is underlined. The consensus pattern of cytochrome P450 is illustrated in italic-bolded.

```
C. maenas          MALLLGREFVWWS-SVASY-SLGTACLALLLTWFIRRQQTVWLIEKLPGPRSLPILGNAL
O. limosus         MSWLLDGALDLETGSVITY-LIVTTLITLTLVWYFKRQQKVWLLEQIPGPRGLPILGNVL
C. quadricarina    MEWLRSQVVVMEEVGVVTY-LVITALLALTLAAFFRRQYEVWIINRIPGPISLPLVGNAL
P. monodon         -MWPKNDTLVWAS-SAFTY-LAFTVTLALGLAWFLQRQRKSWLLAKIPGPKAHVLFGSHR
A. aegypti         ----------MSELTTFIYGILVFLIFAPFLQWWVKRARLVQIIDKIPGPKAYPFIGTTY
                                 .  *      ::  *  :.:*     :: ::*** .   :.*.

C. maenas          DVN-VAPRELFLK-IMEFCEYGNTVKIWLGMYPYCLVSEAKSAEVLLSSNKHLDKSRDYN
O. limosus         YLN-VDPPELFER-FLAVAEYGEVSRLWLCNMCTCLLSSATTAEVILSSTKHLDKSEDYT
C. quadricarina    SVT-TDSEVLFKLGVWLVREYGQMVRVWIGMSPPVIISGARQAEVVLNNTKHLDKSHQYD
P. monodon         AGG-AAEDRIQWL--IKTSTLGEVVKFWIGFLPTCMICSARGAEVILTSQKHINKGNNYN
A. aegypti         TFFGKKHYELFYIIDERTRRYPDIHRIWTGMRPEIRISKPEYVETIIGASKHMEKSHGYD
                             :          :  :.*        :. .  .*.::   **::*.. *

C. maenas          FLHPWLG-TGLLTSTGKKWHSRRKILTPAFHFKILEDFVEVFNSQSNKMLDKLTPKAD-G
O. limosus         LLHPWLG-ESLLNSAGSRWHARRKLLTPAFHFKILEQFMEVFNSQTNKLVHKLLKKAD-G
C. quadricarina    FFHPWLGTTGLFISKTSDWHTRKKLLTPAFHLKVLEQFVDVFNIQSNKLVSKLKKEAD-G
P. monodon         FLRDWLG-DGLLTATGSKWHSRRKLLTPAFHFKILEDFLDVFNSQSTTMIQQLRGKAD-G
A. aegypti         FLFDWLG-EGLLTSKGERWFQHRKLITPTFHFNILDGFCDVFAEQGAVLAERLEPFANTG
                   ::  ***  .*: :  . *. ::*::**:**:::*: * :**  *   :  :*   *: *

C. maenas          KAFDIFPYITLCTLDIICETAMGININAQGNSNSEYVNAVYRIGALVQHRQTRPWIQPDF
O. limosus         SPFDISDDITHCVLDIICETAMGRSINAQDNSESEYVQAVRKISGLIQYRQFRPWMYYEF
C. quadricarina    CVFDIFPYITNCTLDIICETAMGCSVNAQDNPESDYIMAIHRIQHLIQQRMIVLWMQPDF
P. monodon         KPFDVFPFITRCALDIICETAMGRTVNAQCNANSEYVKALNRFAELLIKRSTTPWLRPDW
A. aegypti         KPVDVFPFITKAALDIICETAMGVKVNAQTGGENNYVNAIYRMSEIFVDRSIKPWLHPEF
                     .*:   ** ..********** .:*** . :.:*: *: ::  :.  *    *:  ::

C. maenas          LFRLFGYAKLHDEYLRVLHHFSNSAIENRRKEYQL-EKLNAKENIDDD-VIGKKRRLAFL
O. limosus         LFKLMGPIKEYNACFKTLHDMSNSTIKER-KESRK-DKANTEVLEEEE-VFGKKKRQAFL
Ch.                IFRLLGYAREQEELLKTLHSFTRNIVKARRKLYEQ-QKQQGGAGSDDEQHLGKKQRLAFL
P. monodon         LYTLFGQRSDYDACLKVLHGFSQETIVERRALLQN-SKRNGKE-EDTEEVFGKKKRLAFL
A. aegypti         IFKRTEYGRQHKKALDIVHGYTKKVIRDRKEALQVKENSTGAGDTGEDLYFGTKKRLAFL
                   ::          .  :  :*  :.. :  *    .  .:         :  :*.*:* ***

C. maenas          DLLLNYSETQMPLSNEDIREEVDTFMFEGHDTTAAALNWSVYLLGCHPEIQAKVHEELDA
O. limosus         DLMLEYAEDNPELTDEEIRKEVDTFMFAGHDTTASAINWVLYTLGLHPDIQTRVQEELDD
C. quadricarina    DLLLEYSEGGTVLTDEDIREEVDLFVFAGHDTTTVAINWCLYILGRHPEIQARVHEELDS
P. monodon         DLLLEYSEDGAKLSDEDIREEVDTFMFEGHDTTTAAMNWVLYLLGHHPEIQARVHQELDS
A. aegypti         DLLLEGNAKHKQLTDDDVREEVDTFMFEGHDTTTAGMSWALFLLGLHPDWQDRVHQEIDS
                   **:*:       *:::::*:*** *:* *****: .:.* :: ** **: *  :*::*:*

C. maenas          LFGDSDRPVTMADLREMKYTENCIKEALRLFPSVPFLARELREEAVINNYRIPVGTTVMV
O. limosus         IFGSSDRPATMDDLRQMKYAEMCIKETMRLFTPVPVISRDIKEEVVINNYRIPANTIVAV
C. quadricarina    IFEGTDRPATMDDIRQMKYTENCIKEALRLFPSVPYVGRQLSGDINIGKYRIPAGASVMV
```

```
P. monodon       IFGDEDRPATMDDLRSMKLLENCIKEGLRLFPSVHRFARTLREDVRICDYVIPAGTNIML
A. aegypti       IFAGSDRPATMKDLGEMKLLERCLKETLRLYPSVSFFGRKLSEDVTLGQYHIPAGTLMGI
                 :* . ***.** *: .**  * *:** :**:..*  ..* :  :  : .* **..: : :

C. maenas        ITYRLHRDPEQFPNPETFDPDRFLPENVAKRHPYSYVPFSAGPRNCIGQKFAIMEEKIVL
O. limosus       VIYKIHRDPEQFPDPEVFDPDRFLPENALKRHPYAYVPFSAGPRNCIGQKFAMLELKTVV
C. quadricarina FTYALHRDPEQFPDPEVFDPDRFLPENASKRHPFAYNAFSAGPRNCIGQKFGMIEEKVMV
P. monodon       FVYRIHRDPKQFPDPERFDPDRFLPENSKHRHPYAYIPFSAGPRNCIGQKFAQMEEKVLL
A. aegypti       HAYHVHRDERFYPDPEKFDPDRFLPENTEHRHPFAYIPFSAGPRNCIGQKFAILEEKSIV
                   * :*** . :*:** **********  :***::* .**********. :* * ::

C. maenas        SSIMRRFRVESTTRREELKLLGELILRPENGNTVKLIPRTPKV----------------
O. limosus       SSIFRKLRVESVIPRKDLKMTAEIILRPANGNILKLSPRTK----------------
C. quadricarina SSVLRKFRIESITPMKKLKLLSEIVLRPKDGNHVRLFPRTAESPTSARGSNSVQDNNTRC
P. monodon       SSILRKFRVESTVPRESLRTMDHFVLRPKGGNNLRLFPRS-----------------
A. aegypti       SSVLRKFRVRSANTRDEQKICQELITRPNEGIRLYLEKRQ-----------------
                 **::*::*:.*    .. :   .:: ** *  : *  *

C. maenas        -----
O. limosus       -----
C. quadricarina QGEGS
P. monodon       -----
A. aegypti       -----
```

Figure 4.16 Multiple alignment of amino acid sequence of cytochrome P450 of *P. monodon*, *Carcinus maenas*, *Orconectes limosus*, *Cherax quadricarinatus*, and *Aedes aegypti*

For glutathione-s-transferase, combination of nucleotide sequence of glutathione-s-transferase provided 1,030 bp in length. The ORF of glutathione-s-transferase was 654 bp encoding a polypeptide of 218 amino acids. The 5' and 3' UTR were 35 bp and 311 bp (excluding the poly A tail). The complete sequence of glutathione-s-transferase cDNA is shown in Figure 4.18.

The full length of glutathione-s-transferase cDNA was search against data in GenBank using Blast X and the closest homologue was glutathione-s-transferase 1-1 of *Tribolium castaneum* (1e-^{67}, accession number XP_974273). Glutathione-s-transferase amino acid sequences of *P. monodon*, *Tribolium castaneum* (XP_974273), *Lygus lineolaris* (ABC46449), *Culicoides variipennis* (AAB94639), *Anopheles dirus* (AAG38507), and *Bombyx mori* (NP_001036974) were multiple aligned and shown in Figure 4.19.

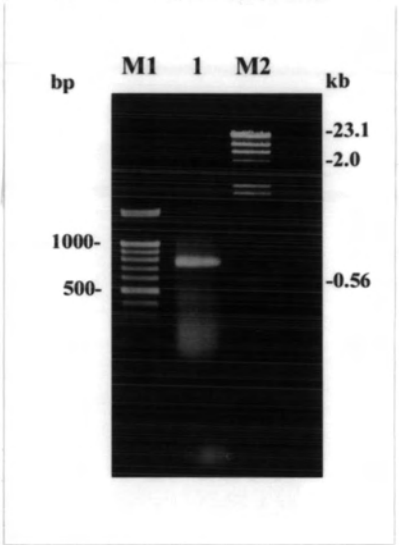


Figure 4.17 3' RACE-PCR of glutathaione-s-transferase (Lane 1). Lane M1 is 100 bp DNA ladder. Lane M2 is λ Hind III DNA ladder.

```
                                                    M  P  I  D  L  Y  Y  M  P
   1    GTGAGATAAGGTCGCAGACTAACCTACAAACCAAGATGCCCATAGACCTGTACTACATGC
          L  S  A  P  C  R  S  V  M  L  T  A  K  A  V  G  V  E  L  N
  61    CCCTGTCAGCGCCCTGCAGGTCGGTAATGCTAACGGCCAAGGCAGTGGGCGTCGAACTCA
          L  K  L  L  N  L  S  A  G  E  H  M  K  P  E  F  V  A  I  N
 121    ACCTGAAGCTGCTCAACCTGTCGGCGGGAGAGCATATGAAGCCCGAGTTCGTGGCCATCA
          P  Q  H  C  I  P  T  L  V  D  G  N  L  K  L  W  E  S  R  A
 181    ACCCACAGCACTGCATCCCCACCTTGGTCGACGGGAACCTGAAGCTGTGGGAGAGCCGCG
          I  C  T  Y  L  I  A  K  Y  A  E  D  D  S  L  Y  P  S  D  P
 241    CCATCTGCACCTACCTGATCGCCAAATACGCCGAGGACGACTCACTCTACCCGTCCGACC
          K  T  R  A  L  V  D  R  L  L  Y  F  D  M  G  T  L  Y  H  R
 301    CGAAGACTCGTGCCCTCGTCGATCGCCTCCTCTACTTCGACATGGGCACGCTCTACCACA
          F  G  E  Y  V  Y  P  V  M  F  Y  G  Q  E  K  L  E  P  A  K
 361    GGTTCGGGGAGTACGTGTACCCCGTGATGTTCTACGGGCAGGAGAAACTCGAGCCGGCGA
          L  E  K  L  H  E  A  L  G  W  L  D  G  F  L  A  G  H  D  W
 421    AGTTGGAGAAGCTGCACGAAGCCCTGGGCTGGCTCGACGGGTTCCTGGCCGGCCACGACT
          A  A  G  N  N  I  T  V  A  D  F  V  L  V  A  S  V  S  S  F
 481    GGGCCGCCGGCAACAACATCACCGTCGCCGACTTCGTGCTGGTCGCTTCCGTGTCCTCCT
          E  V  C  G  I  D  L  S  K  H  R  N  V  T  T  W  L  A  R  C
 541    TCGAGGTCTGCGGCATCGACCTGAGCAAGCACAGGAACGTGACGACGTGGCTGGCGCGCT
          K  A  G  L  R  G  Y  D  E  A  N  A  P  G  V  K  D  L  A  R
 601    GCAAGGCCGGCCTTCGGGGCTACGACGAGGCGAATGCTCCCGGCGTGAAAGACCTCGCCA
          M  T  E  A  K  L  A  G  K  *
 661    GGATGACGGAGGCGAAGCTGGCGGGCAAGTAGGGGCGGCGGCGAGCATGGGGGCTCTTCT
 721    GCTTGCAAAGGCTCTATTACATGCAAGACTTAATCACCCAAATGGTTAGACGAGAGAGAG
 781    GCAGAGCGAGCGGAGATTTTAGAATGAAGAATACCACTCGTATAACAGAAAGAAATGTAT
 841    TATAAGCAGTTACATACTGAAATGAAAAGGAAATGAAGATTAGGGAATAGGTGTCGGAAA
 901    TCACTTTATCAGTATATTTTCTTTGTTGAACTTTGTCCAAAAATTGTTCGTTACATAATC
 961    TGCTGGAATCTAGCGCTCTCTCAGCCAAAATAAAGGAGTGACTAANAAAAAAAAAAAAAA
1021    AAAAAAAAAA 1030
```

Figure 4.18 The full length cDNA sequence of glutathione-s-transferase of *P. monodon*. Start and stop codons are illustrated in boldface. The poly A additional signal is underlined. The consensus pattern of cytochrome P450 is illustrated in italic-bolded.

```
B. mori          MTIDLYYVPGSAPCRAVLLTAKALNLNLNLKLVDLHHGEQLKPEYLKLNPQHTVPTLVDD
T. castaneum     MPIDLYYLPGSAPCRAVLLAAKAVGVELNLKLTDLMKGEHLTPEFIKINPQHTIPTMVDN
P. monodon       MPIDLYYMPLSAPCRSVMLTAKAVGVELNLKLLNLSAGEHMKPEFVAINPQHCIPTLVDG
L. lineolaris    MTIDFYYTPGSSPCRNVLLAAKAVGVDLNLKLLDLMKGEHLAPDFVKINPQHCVPTLVDN
A. dirus         --MDFYYLPGSAPCRAVQMTAAAVGVELNLKLTNLMAGEHMKPEFLKLNPQHCIPTLDDN
C. variipennis   MGLDFYYLPGSSPCRAVQMTAKAVGVDLNLKLTNLMAGEHMKPEFLKLNPQHCIPTLVDN
                   :*:** * *:*** * ::* *:.::***** :*  **:: *::: :**** :**: *.

B. mori          GLSIWESRAIITYLVNKYAKGSSLYPEDPKARALVDQRLYFDIGTLYQRFSDYFYPQVFA
T. castaneum     GFALWESRAIMTYLADQYGKNDALYPKDPKKRALVDQRLYFDIGTLYARFADYYYPVIFG
P. monodon       NLKLWESRAICTYLIAKYAEDDSLYPSDPKTRALVDRLLYFDMGTLYHRFGEYVYPVMFY
L. lineolaris    GFVLLESRAIMTYLASKYGKDDSLYPKDPQKRAVVDQRLYFDMGTLYQRFGELYYPIIFG
A. dirus         GFSLWESRAIQIYLVEKYGKDDKLYPKDPQKRAVVNQRLFFDMGTLYQRFGDYWYPQIFA
C. variipennis   GFSLWESRAIQVYLVEKYGKDDSLYPKDVQQRALVNQRLYFDMGTLYQRFADYWYPQLFA
                 .: : *****  **  :*.:.. ***.* : **:*:: *:**:**** **.:  ** :*

B. mori          G-APADKAKNEKVQEALQLLDKFLEGQKYVAGPNLTVADLSLIASVSSLEASDIDFKKYA
T. castaneum     G-AEYEPAKLEKIKDAFKFLEIFLEGQDFVAGNQLTLADLSLLATVTTFEAVNFDLSPYK
P. monodon       GQEKLEPAKLEKLHEALGWLDGFLAGHDWAAGNNITVADFVLVASVSSFEVCGIDLSKHR
L. lineolaris    G-APYDEEKAKKLDDAFKFLDGYLGKSEWAAGGNLTVADLALVASVSTAESCDWDVSKYP
A. dirus         K-QPANAENEKKMKEAVGFLNTFLEGQEYAAGSDLTIADLSLAASIATYEVAGFDFAPYP
C. variipennis   K-QPANPENFKXMEEAMGFLNTFLEGHKYAVGDKFTVADLALAASVATYEVSGFDFKPYP
                      :  : : :.:*.  *: :*   .:..* .:*:**: * *:::: *  . *.  :

B. mori          NVKRWYETVKSTAPGYQEANEKGLEAFKGLVNSMLKK----
T. castaneum     NVVNWLARAKAAAPGYEEANGKGAVIFKQMVENLTKK----
P. monodon       NVTTWLARCKAGLRGYDEANAPGVKDLARMTEAKLAGK---
L. lineolaris    NVAKWYAKCKTTIPGYAEANQAGADKFKGMYQAAKSK----
A. dirus         NVAAWLARCKANAPGY-ALNQAGADEFKAKFMS--------
C. variipennis   NVQKWFALCKTTLPGY-DLNEAGVKNSRIFPLSLNACSGLN
                 ** *    *:   **   * *
```

Figure 4.19 Multiple alignment of amino acid sequence of glutathione-s-transferase of *P. monodon*, *Bombyx mori*, *Tribolium castaneum*, *Lygus lineolaris*, *Anopheles dirus*, and *Culicoides variipennis*

4.7 Identification of the genes differentially expressed during chlorpyrifos exposure

4.7.1 DDRT-PCR

Genes differentially expressed in hepatopancrease of shrimps exposed to sub-lethal concentration of chlorpyrifos (0, 0.681, and 6.81 µg/l) were examined using mRNA DDRT-PCR with 90 pairs of 30 arbitrary primers (10 OPA, 10 OPB, and 10 UBC primers) in combination with 3 types of anchored oligodT primer (i.e., 5'-TTTTTTTTTTT-N-3' where N was either A, G, or C). PCR products shown differential display among group of treatment were observed.

A total of 44 differential displayed transcripts were subjected to cloning and sequencing analysis. Result from BLASTx (NCBI) search identified 22 transcripts (16 up-regulated and 6 down-regulated) were known genes. Twenty two transcripts were unknown genes and hypothetical proteins found in other species (8 up-regulated and 14 down-regulated). The results are shown in Table 4.17-4.19.

Nucleotide sequences of UBC119A-650-F-5, UBC101C-1000-D-3, OPA07G-350-27-1, OPA18G-600-4-1, OPA01G-415-1, and OPA02G-450-2 product showing similarity to *CYP330A1*, Esterase, LDL receptor member LR3, Ubiquitin-like-7, Leucine zipper protein 5, and sequence of unknown gene respectively were subjected to semi quantitative RT-PCR to examine expression level of the genes in hepatopancreas of shrimps exposed to broad range of chlorpyrifos concentration (0-27.24 µg/l within 96 h).

4.7.2 Isolation and tissue distribution of chlorpyrifos inducible genes from mRNA DDRT-PCR

Nucleotide sequences of UBC119A-650-F-5 (down regulated), UBC101C-1000-D-3 (up regulated), OPA07G-350-27-1 (up regulated), OPA18G-600-4-1 (up regulated), OPA01G-415-1 (down regulated), and OPA02G-450-2 (down regulated) showing differential expression by chlorpyrifos exposure were designed for specific primer to examine the expression in 3 tissues, including haemocyte, gill, and hepatopancreas. Results showed expression of the genes in hepatopancreas of shrimp (Figure 4.20-4.25)

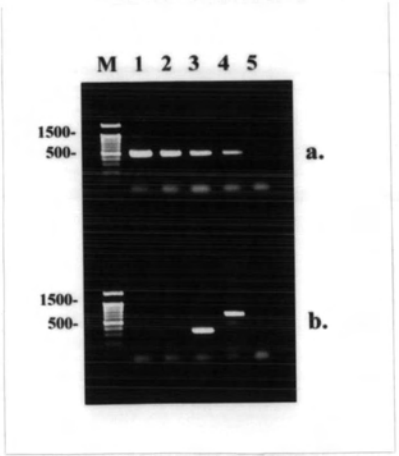


Figure 4.20 PCR products of UBC119A-650-F-5 F1R1 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Elongation factor 1 alpha used as positive control is shown in Lane 1-4a. Lane M is 100 bp DNA ladder. Negative control is shown in Lane 5.

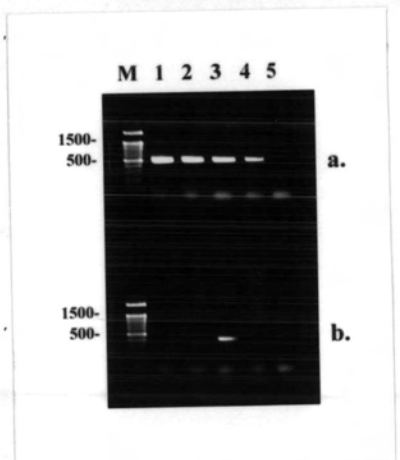


Figure 4.21 PCR products of UBC101C-1000-D-3 F1R1 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Elongation factor 1 alpha used as positive control is shown in Lane 1-4a. Lane M is 100 bp DNA ladder. Negative control is shown in Lane 5.

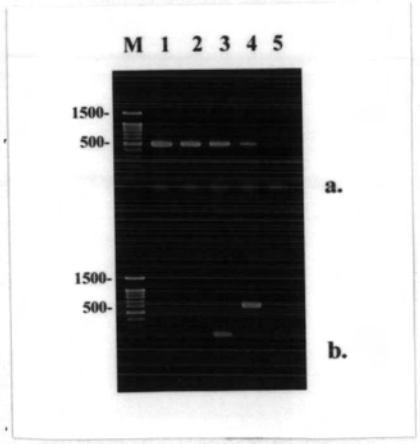


Figure 4.22 PCR products of OPA07G-350-27-1 F1R1 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Elongation factor 1 alpha used as positive control is shown in Lane 1-4a. Lane M is 100 bp DNA ladder. Negative control is shown in Lane 5.

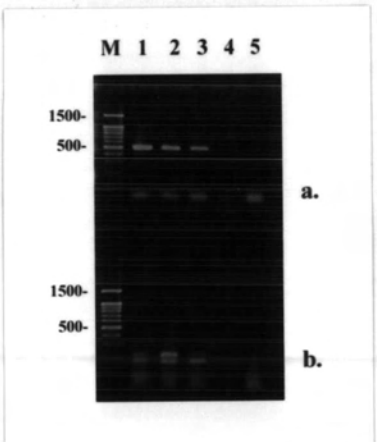


Figure 4.23 PCR products of OPA18G-600-4-1 F1R1 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Elongation factor 1 alpha used as positive control is shown in Lane 1-4a. Lane M is 100 bp DNA ladder. Negative control is shown in Lane 5.

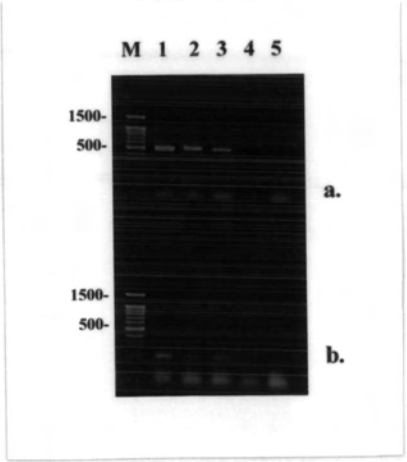


Figure 4.24 PCR products of OPA01G-415-1 F1R1 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Elongation factor 1 alpha used as positive control is shown in Lane 1-4a. Lane M is 100 bp DNA ladder. Negative control is shown in Lane 5.

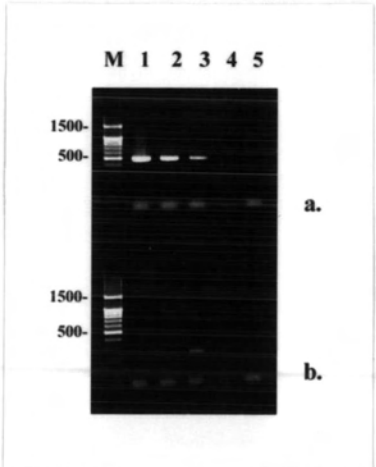


Figure 4.25 PCR products of OPA02G-450-2 F1R1 primer combination using first strand cDNA from haemocyte (Lane 1b), gill (Lane 2b), hepatopancreas (Lane 3b), and genomic DNA (Lane 4b) as template. Elongation factor 1 alpha used as positive control is shown in Lane 1-4a. Lane M is 100 bp DNA ladder. Negative control is shown in Lane 5.

Table 4.17 Summary of differential display PCR product from mRNA DDRT-PCR using A anchored oligodT first strand cDNA as template

Product	Primer	Detected size (bp)	Size (bp)	Expression Direction	Putative Gene	Species	Expect value	Figure
OPA07A-350-7-1	OPA07	350	332	up regulated	LDL Receptor member LR3	*Mus musculus*	$3x10^{-17}$	4.37
OPA18A-500-19-1	OPA18	500	543	up regulated	Sequence of unknown	*M. musculus*	2.6	4.41
OPA18A-550-18-1	OPA18	550	559	up regulated	Hypothetical protein	*Tribolium castaneum*	$2x10^{-03}$	4.41
OPA18A-550-18-2	OPA18	550	569	up regulated	Ubiquitin-like 7	*T. castaneum*	$5x10^{-21}$	4.41
OPA18A-600-17-1	OPA18	600	569	up regulated	Ubiquitin-like 7	*T. castaneum*	$5x10^{-21}$	4.41
OPA18A-600-17-3	OPA18	600	559	up regulated	Sequence of unknown	*T. castaneum*	0.006	4.41
OPA18A-650-16-3	OPA18	650	627	up regulated	Apical early endosomal glycoprotein	*Macaca mulatta*	$3x10^{-19}$	4.41
OPB07A-225-15-3	OPB07	225	235	down regulated	Hypothetical protein	*Homo sapien*	$1.2x10^{-01}$	4.40
OPB07A-250-14-1	OPB07	250	258	up regulated	Protein of unknown function DUF11	*Psychrobacter cryohalolentis*	$7x10^{-08}$	4.40
OPB07A-250-14-2	OPB07	250	256	up regulated	Sequence of unknown	*Trichomonas vaginalis* G3	1.4	4.40
OPB07A-650-13-1	OPB07	650	665	up regulated	Transporter	*Brucella melitensis* 16M	$2x10^{-65}$	4.40
OPB08A-275-8-3	OPB08	275	277	down regulated	Unknown seq. not match			4.38
OPB12A-800-20-5	OPB12	800	781	up regulated	Formin-like 2 isoform B	*Apis mellifera*	$5x10^{-91}$	4.42
UBC119A-250-11-2	UBC119	250	248	down regulated	Unnamed protein product	*Tetraodon nigroviridis*	$1x10^{-32}$	4.39

Product	Primer	Detected size (bp)	Size (bp)	Expression Direction	Putative Gene	Species	Expect value	Figure
UBC119A -250-11-4	UBC119	250	249	down regulated	Exonuclease V gamma subunit RecC	*Pseudomonas syringae*	$2x10^{-20}$	4.39
UBC119A -650-F-5	UBC119	650	633	down regulated	Cytochrome P450 enzyme, *CYP330A1* enzyme	*Carcinus maenas*	$3x10^{-53}$	4.39
UBC119A -700-9-1	UBC119	700	665	down regulated	Sequence of unknown	*Porphyromonas gingivalis* W83	0.02	4.39

Remark: Nucleotide sequence with expect value $\leq 10^{-4}$ was assigned as gene.
Nucleotide sequences are shown in Table B3, appendix B

Table 4.18 Summary of differential display PCR product from mRNA DDRT-PCR using C anchored oligodT first strand cDNA as template

Product	Primer	Detected size (bp)	Size (bp)	Expression Direction	Putative Gene	Species	Expect value	Figure
UBC101C -1000-D-3	UBC101	1000	783	up regulated	Esterase	*T. castaneum*	$3x^{-17}$	4.35
UBC119C -350-5-1	UBC119	350	332	down regulated	Sequence of unknown	*Litopenaeus vannamei*	0.47	4.35
UBC122C -375-6-1	UBC112	375	329	up regulated	RPGR embryo globulin	*Ovis aries*	$2x10^{-08}$	4.36

Remark: Nucleotide sequence with expect value $\leq 10^{-4}$ was assigned as gene.
Nucleotide sequences are shown in Table B4, appendix B

Table 4.19 Summary of differential display PCR product from mRNA DDRT-PCR using G anchored oligodT first strand cDNA as template

Product	Primer	Detected size (bp)	Size (bp)	Expression Direction	Putative Gene	Species	Expect value	Figure
OPA01G 415 1	OPA01	415	408	down regulated	Leucine zipper protein 5	*Xenopus laevis*	$3x10^{-15}$	4.27
OPA01G 415 3	OPA01	415	415	down regulated	Hypothetical protein GuraDRAFT_1187	*Geobacter uraniumreducens* Rf4	$8x10^{-09}$	4.27

Product	Primer	Detected size (bp)	Size (bp)	Expression Direction	Putative Gene	Species	Expect value	Figure
OPA01G 600 3	OPA01	600	619	down regulated	Sequence of unknown	*Dictyostelium discoideum* AX4	0.005	4.27
OPA02G 450 2	OPA02	450	442	down regulated	Sequence of unknown	*Anolis pulchellus*	0.009	4.27
OPA02G 450 3	OPA02	450	480	down regulated	Sequence of unknown	*Homo sapiens*	5.3	4.27
OPA07G-350-27-1	OPA07	350	332	up regulated	LDL receptor member LR3	*M. musculus*	$3x10^{-17}$	4.29
OPA09G-3-5	OPA09	250	241	down regulated	Phosphoglucomutase	*T. castaneum*	$1x10^{-30}$	4.29
OPA11G-350-21-2	OPA11	350	331	down regulated	Unnamed protein product	*T. nigroviridis*	$2x10^{-07}$	4.30
OPA18G-600-4-1	OPA18	600	569	up regulated	Ubiquitin-like 7 (bone marrow stromal cell-derived)	*T. castaneum*	$5x10^{-21}$	4.26
OPA18G-650-5-1	OPA18	650	588	up regulated	Nitrogen regulatory protein P-II	*Serratia proteamaculans* 568	$1x10^{-52}$	4.26
OPA18G-650-5-2	OPA18	650	569	up regulated	Ubiquitin-like 7 (bone marrow stromal cell-derived)	*T. castaneum*	$5x10^{-21}$	4.26
OPB04G-6-1	OPB04	450	467	up regulated	Retina aberrant in pattern CG3000-PA, isoform A isoform 1	*A. mellifera*	$4x10^{-72}$	4.26
OPB10G-700-23-2	OPB10	700	692	up regulated	Sequence of unknown	*Leishmania infantum*	0.064	4.31
OPB10G-700-23-3	OPB10	700	679	up regulated	RNA-directed DNA Polymerase from mobile element jockey (Reverse transcriptase)	*T. castaneum*	$2x10^{-14}$	4.31
OPB10G-850-22-1	OPB10	850	784	down regulated	Hypothetical protein Y39B6A.1	*Caenorhabditis elegans*	$8x10^{-05}$	4.31
OPB10G-850-22-2	OPB10	850	786	down regulated	Sequence of unknown	*M. musculus*	0.021	4.31
UBC101G -225-A-1	UBC101	225	190	up regulated	Unknown seq. not match			4.33

Product	Primer	Detected size (bp)	Size (bp)	Expression Direction	Putative Gene	Species	Expect value	Figure
UBC119G -225-29-3	UBC119	225	220	down regulated	Trypsin	*L. vannamei*	$2x10^{-23}$	4.34
UBC119G -275-C-1	UBC119	275	272	down regulated	Sequence of unknown	*Verminephrobacter eiseniae* EF01-2	1.8	4.34
UBC119G -275-C-3	UBC119	275	275	down regulated	Thrombospondin	*Penaeus monodon*	$1x10^{-53}$	4.34
UBC119g-350-5-3	UBC119	350	260	up regulated	Trypsin	*L. vannamei*	$2x10^{-27}$	4.34
UBC122G -7-2	UBC122	250	221	down regulated	Sequence of unknown	*Roseiflexus sp.* RS-1	0.63	4.28
UBC135G -200-24-2	UBC135	200	200	up regulated	Sequence of unknown	*Aspergillus oryzae*	6.9	4.32
UBC135G -200-25-2	UBC135	200	260	up regulated	Acetyl-Coenzyme A carboxylase alpha	*Gallus gallus*	$2x10^{-25}$	4.32

Remark: Nucleotide sequence with expect value $\leq 10^{-4}$ was assigned as gene.
Nucleotide sequences are shown in Table B5, appendix B

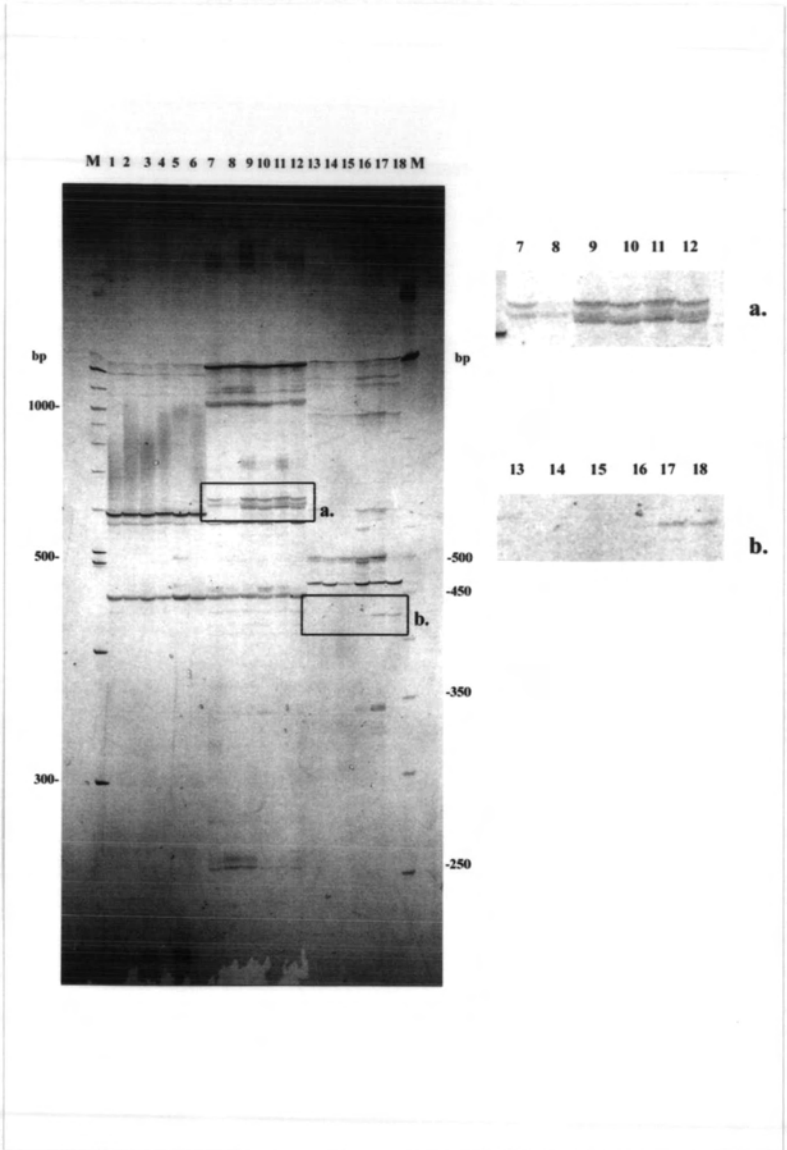


Figure 4.26 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer G anchored olig dT in combination with OPA17 (Lane 1-6), OPA18 (Lane 7-12), and OPB04 (13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

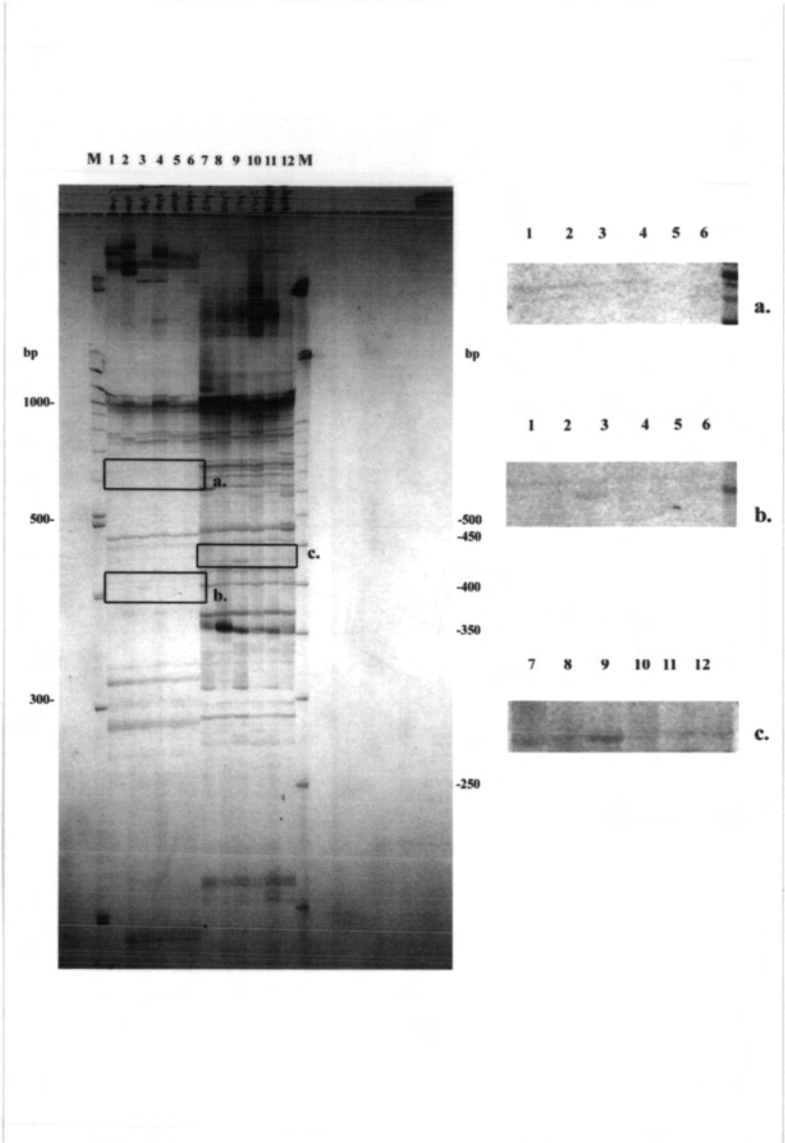


Figure 4.27 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer G anchored olig dT in combination with OPA01 (Lane 1-6) and OPA02 (Lane 7-12). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8 = Control
Lane 3, 4, 9, 10 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12 = 6.81 µg/l chlorpyrifos exposure

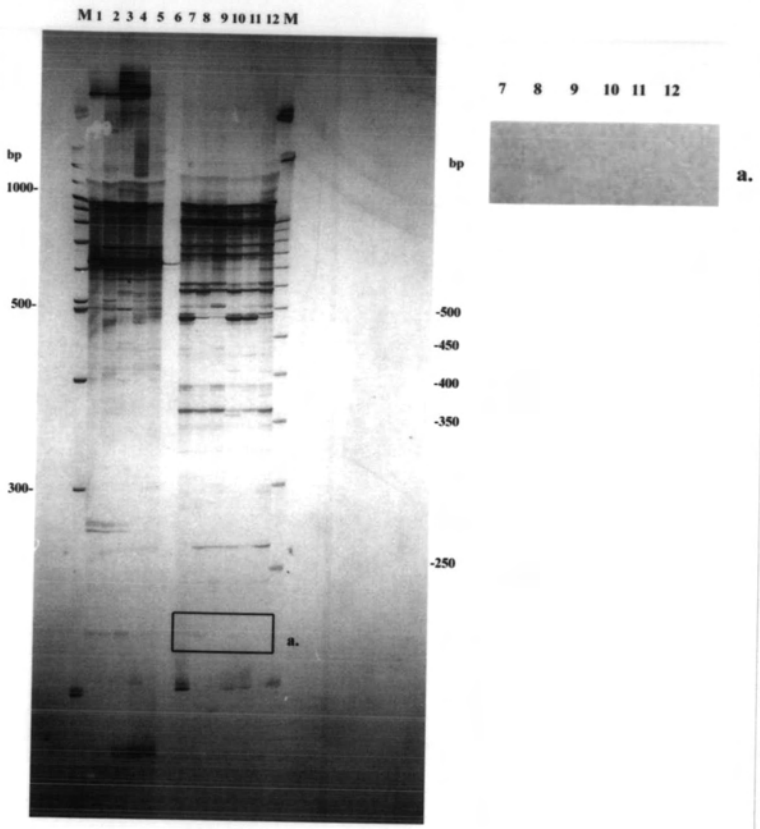


Figure 4.28 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer G anchored olig dT in combination with UBC122 (Lane 7-12). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8 = Control
Lane 3, 4, 9, 10 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12 = 6.81 µg/l chlorpyrifos exposure

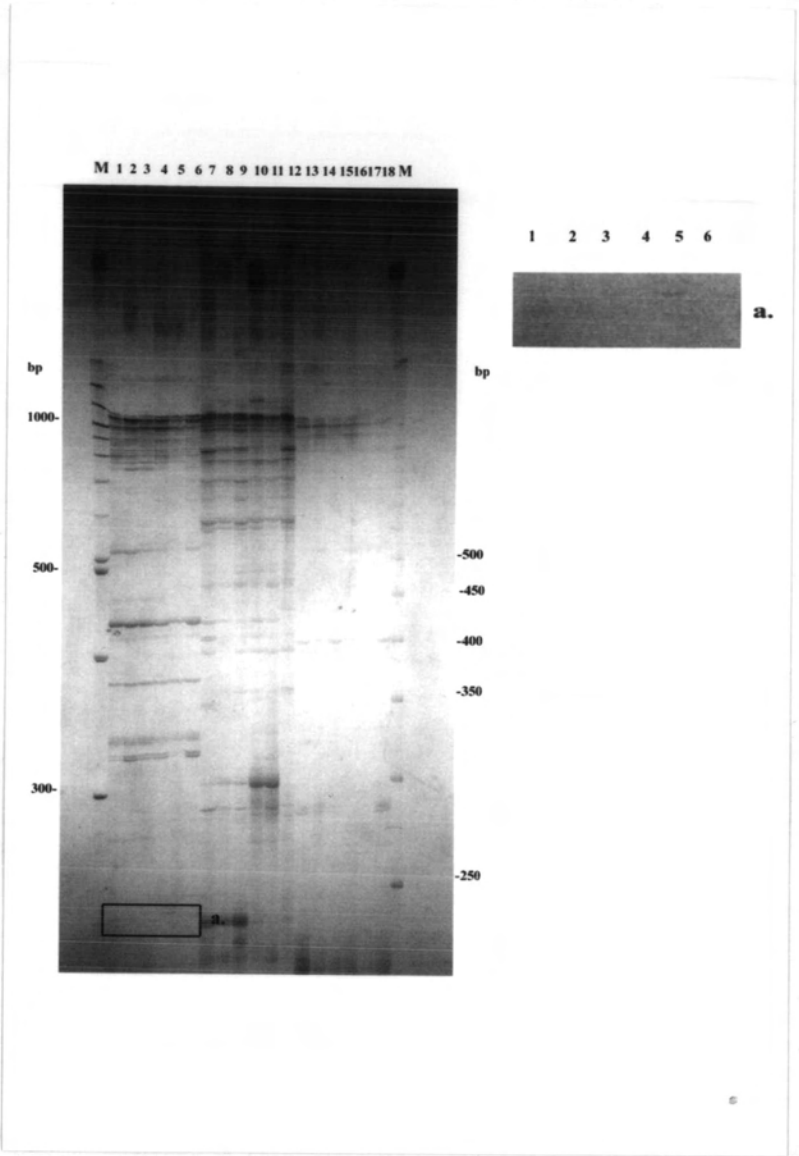


Figure 4.29 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer G anchored olig dT in combination with OPA09 (Lane 1-6), OPA07 (Lane 7-12), and OPA08 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

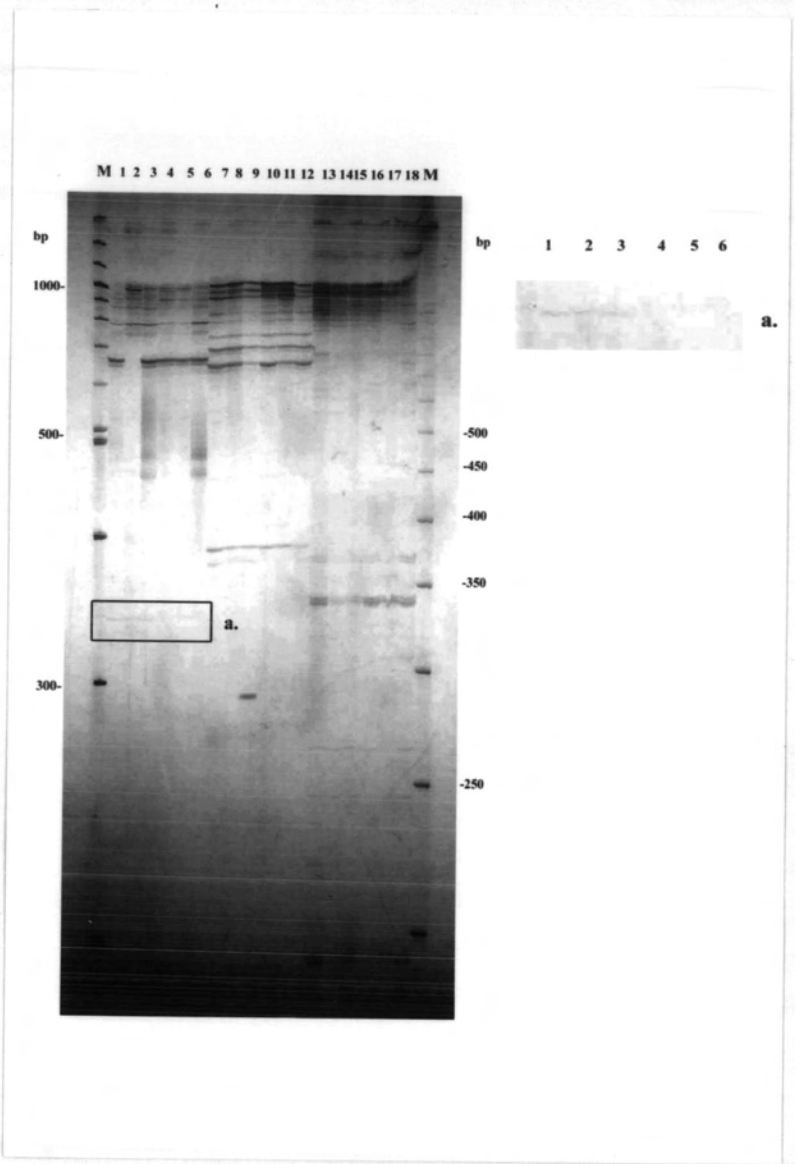


Figure 4.30 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer G anchored olig dT in combination with OPA11 (Lane 1-6), OPA14 (Lane 7-12), and OPA16 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

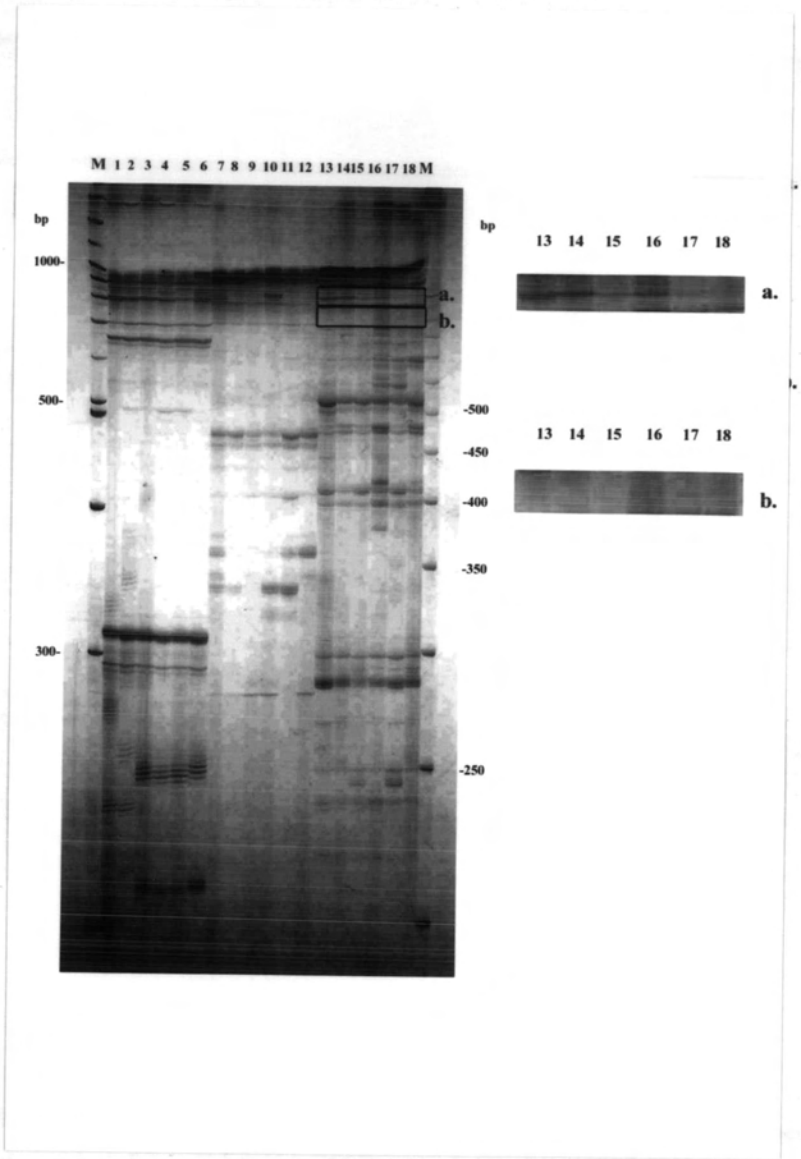


Figure 4.31 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer. G anchored olig dT in combination with OPA15 (Lane 1-6), OPB09 (Lane 7-12), and OPB10 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

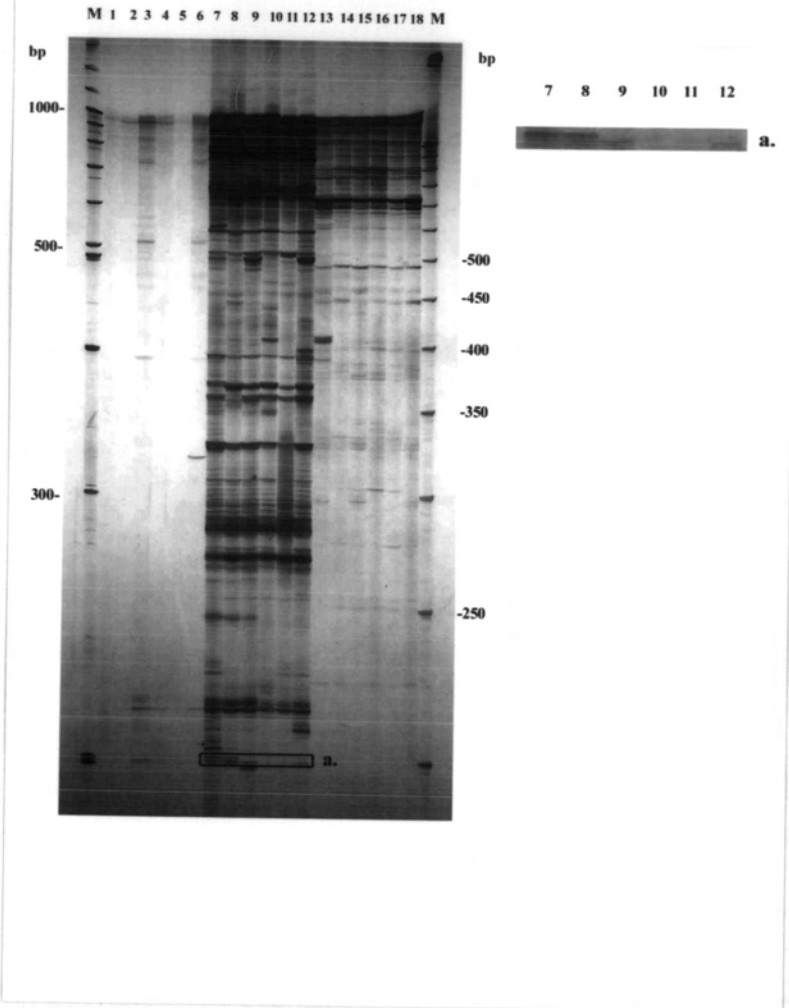


Figure 4.32 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer G anchored olig dT in combination with UBC128 (Lane 1-6), UBC135 (Lane 7-12), and UBC191 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, .16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

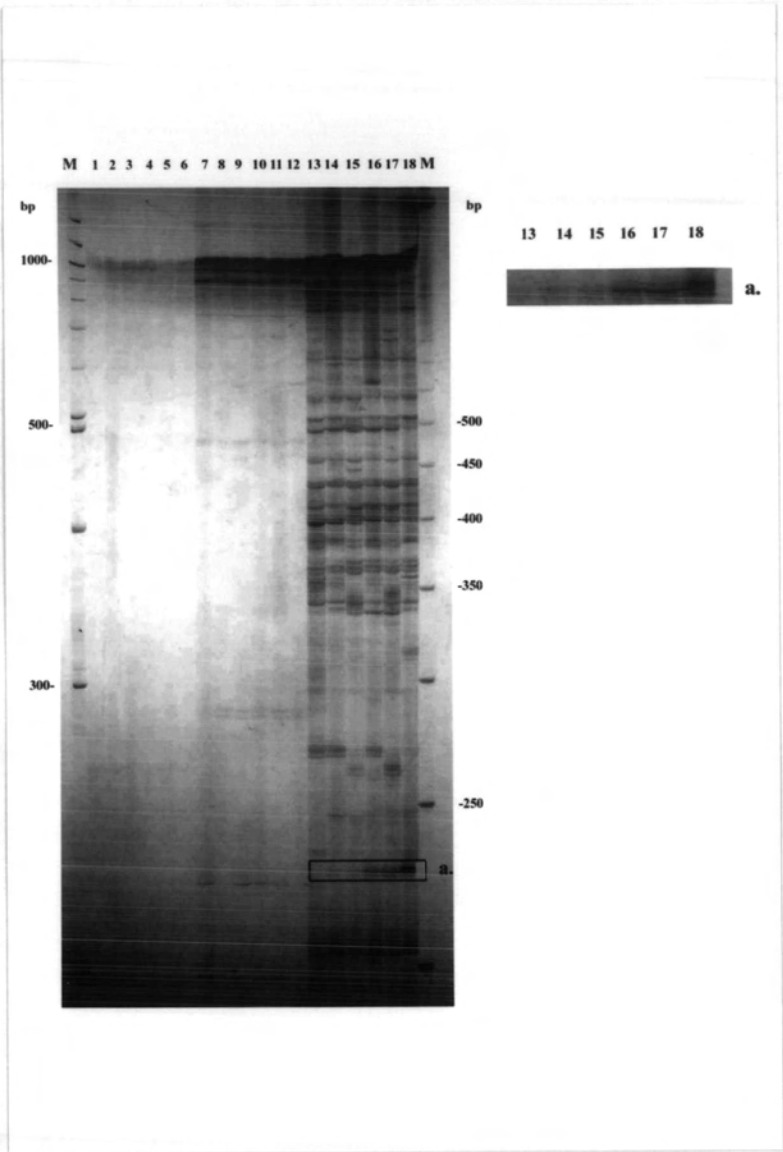


Figure 4.33 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer G anchored olig dT in combination with OPB12 (Lane 1-6), OPB16 (Lane 7-12), and UBC101 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

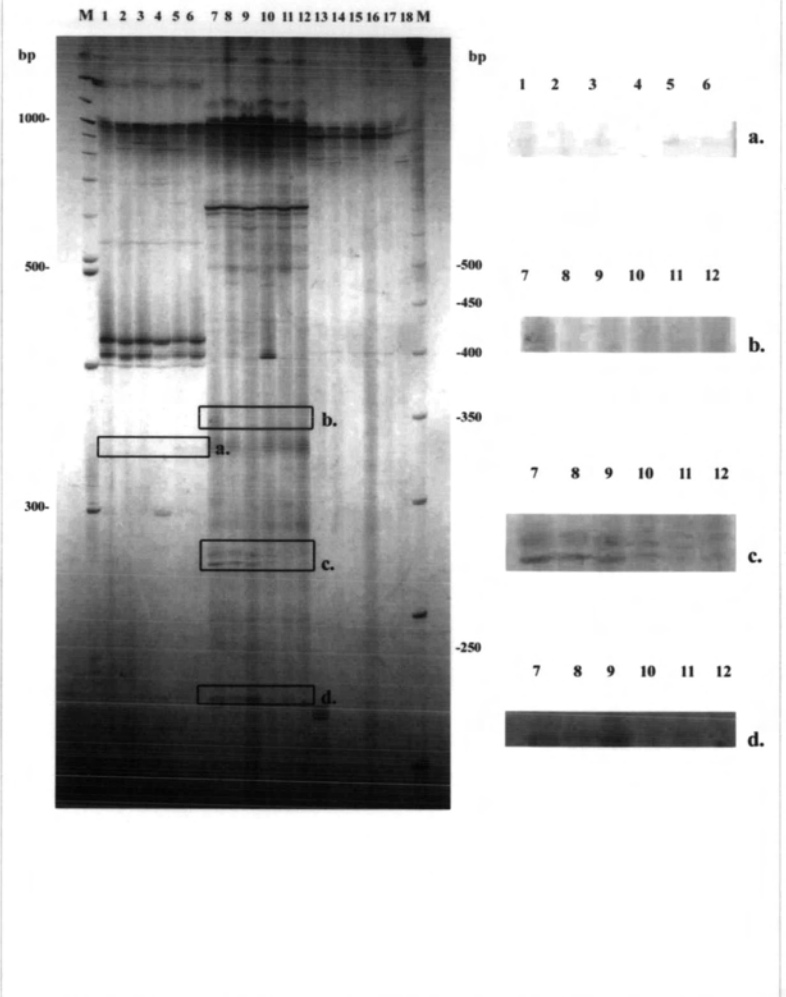


Figure 4.34 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer G anchored olig dT in combination with OPA07 (Lane 1-6), UBC119 (Lane 7-12), and UBC169 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

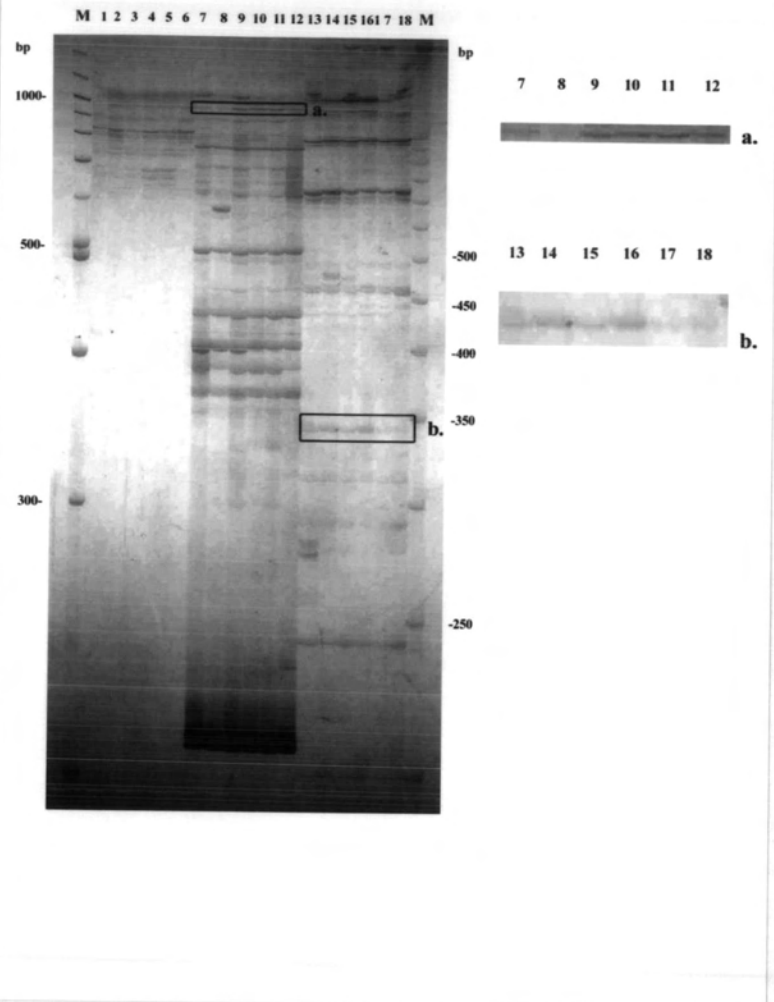


Figure 4.35 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer C anchored olig dT in combination with OPB16 (Lane 1-6), UBC101 (Lane 7-12), and UBC119 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

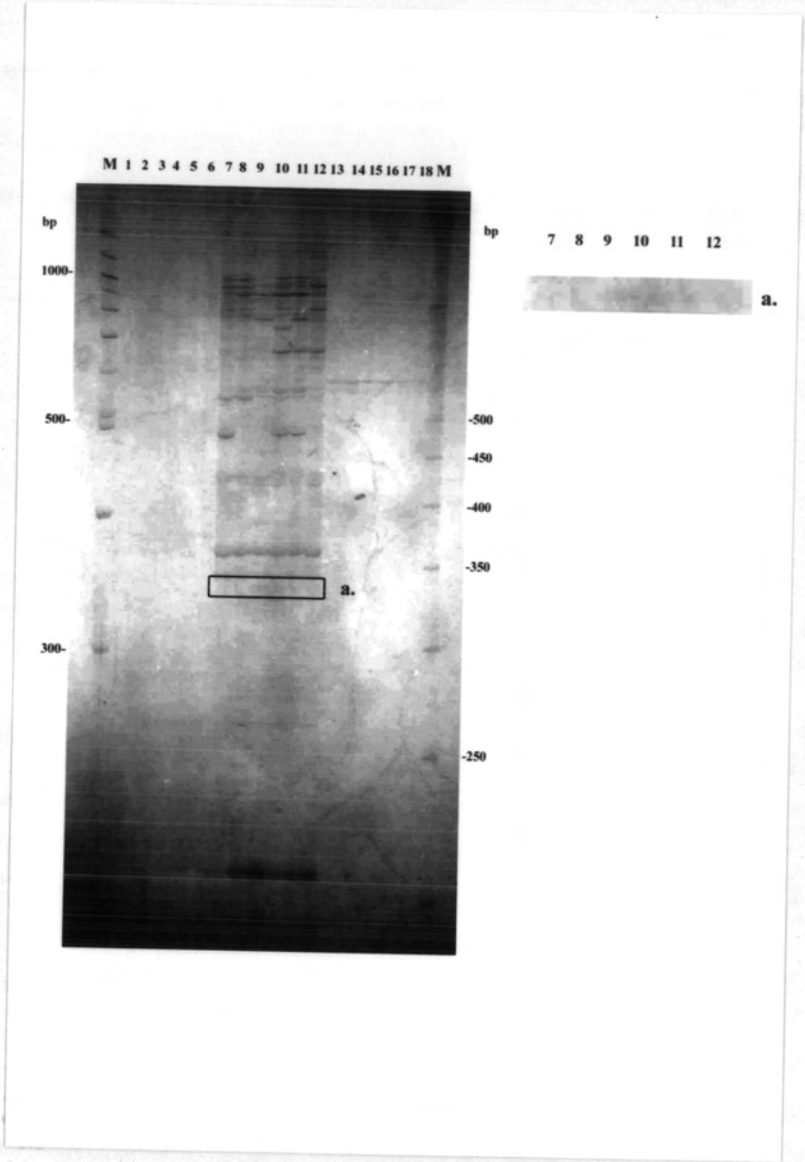


Figure 4.36 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer C anchored olig dT in combination with OPB12 (Lane 1-6), UBC122 (Lane 7-12), and UBC128 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

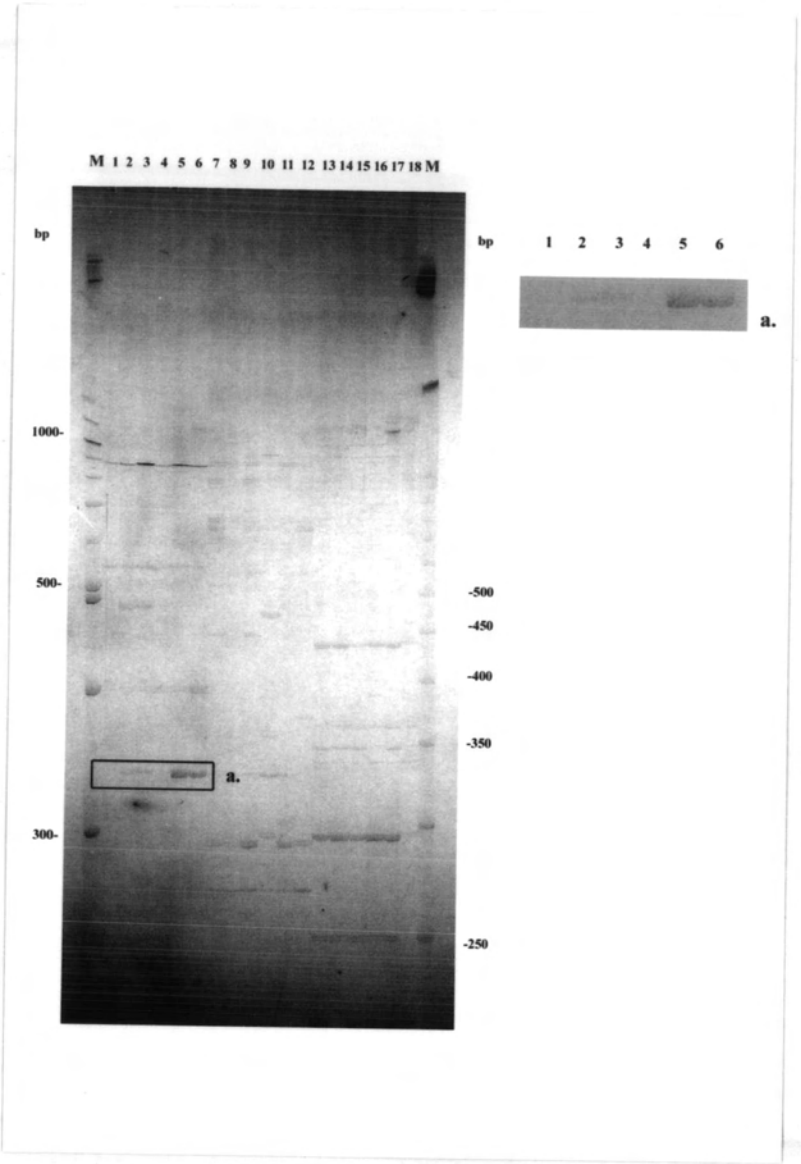


Figure 4.37 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer A anchored olig dT in combination with OPA07 (Lane 1-6), OPA15 (Lane 7-12), and OPA16 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

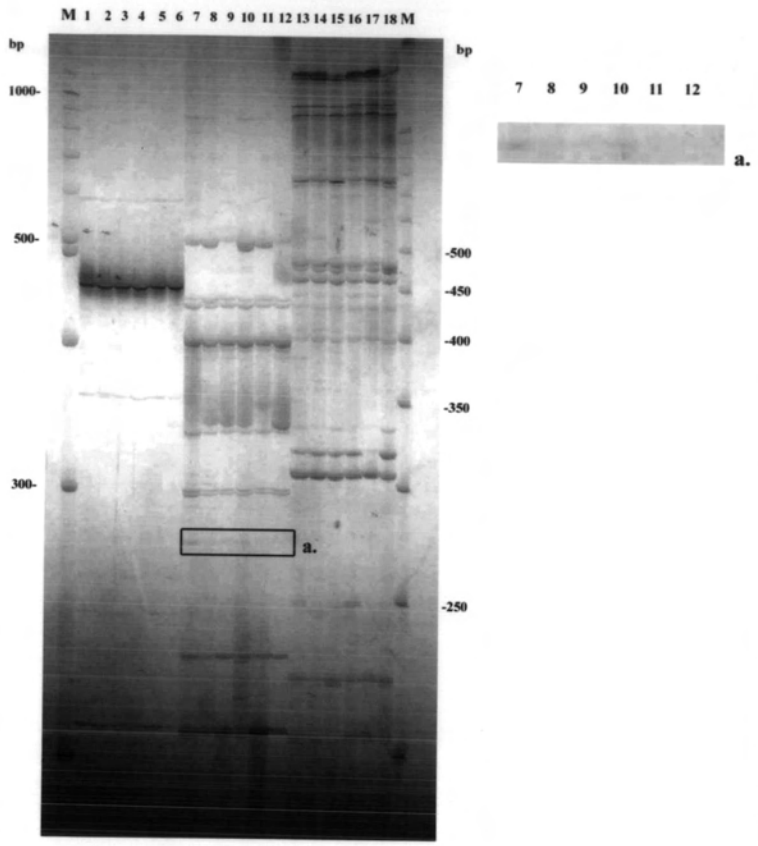


Figure 4.38 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer A anchored olig dT in combination with OPB04 (Lane 1-6), OPB08 (Lane 7-12), and OPB10 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

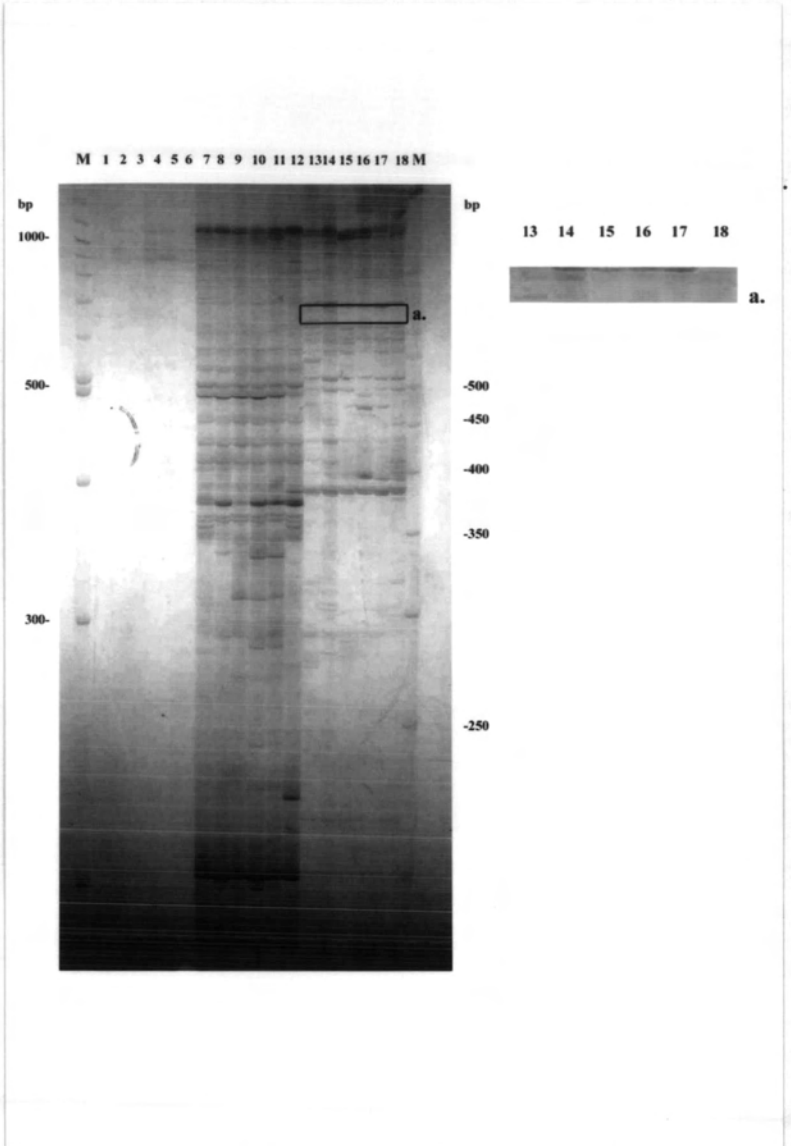


Figure 4.39 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer. A anchored olig dT in combination with OPB16 (Lane 1-6), UBC101 (Lane 7-12), and UBC119 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
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Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

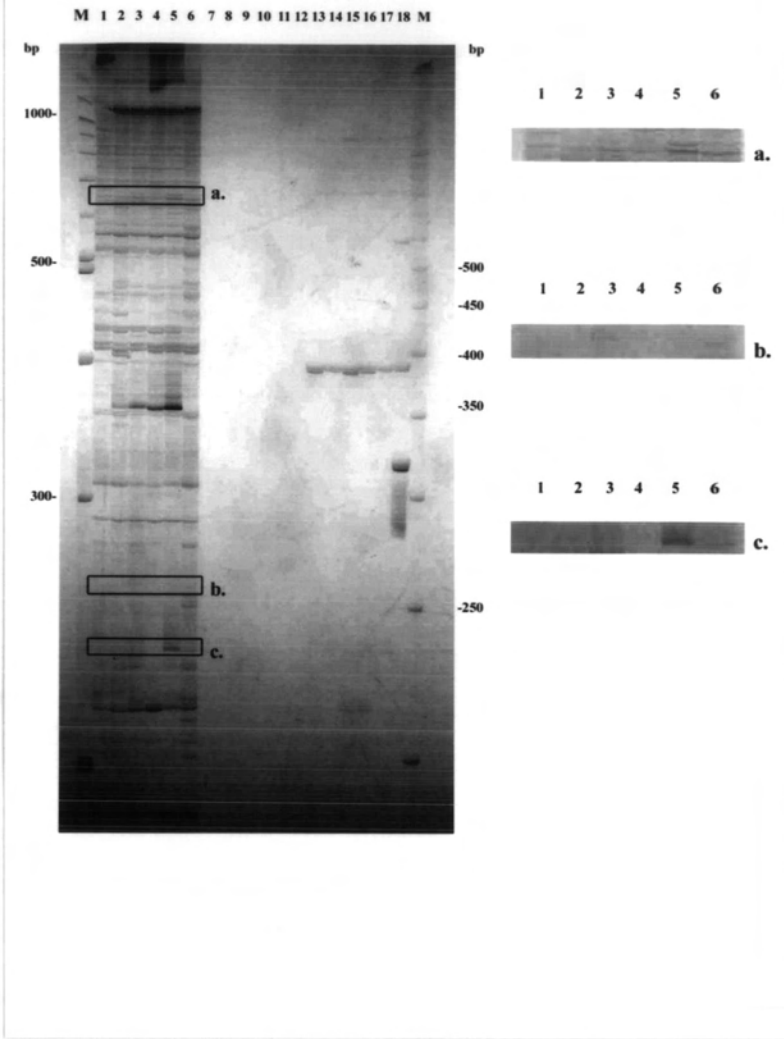


Figure 4.40 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer A anchored olig dT in combination with OPB07 (Lane 1-6), OPB09 (Lane 7-12), and UBC128 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

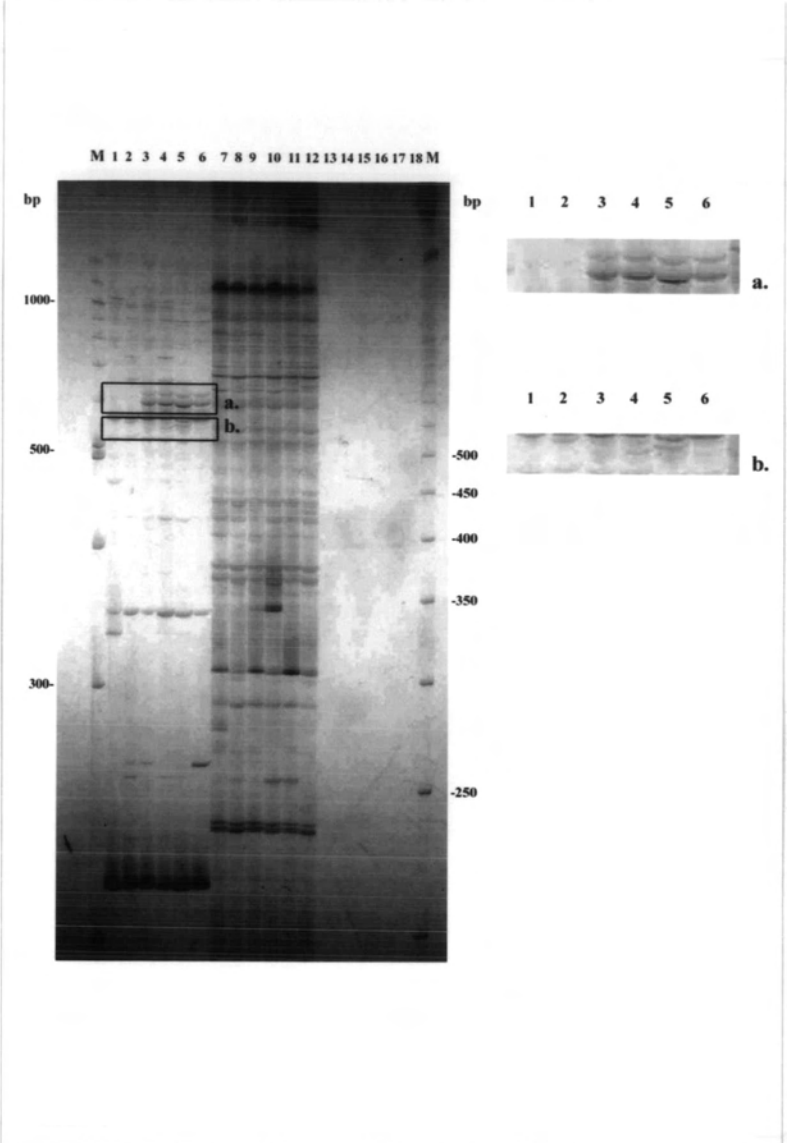


Figure 4.41 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer A anchored olig dT in combination with OPA18 (Lane 1-6), UBC174 (Lane 7-12), and UBC169 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

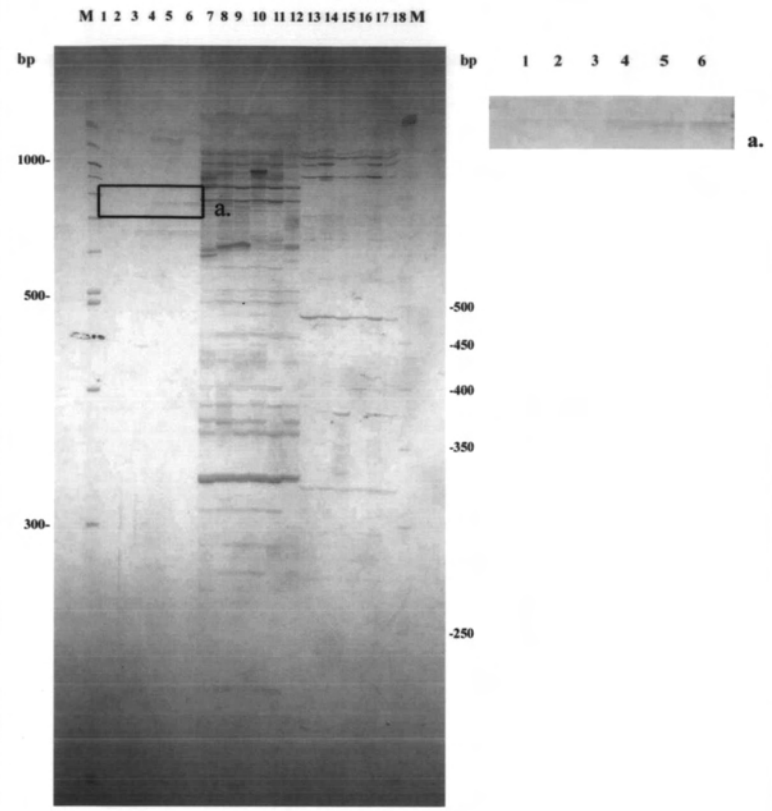


Figure 4.42 Differential display of gene expression in hepatopancreas of *P. monodon* after chlorpyrifos exposure analyzed on 4.5% denaturing polyacrylamide gel electrophoresis from primer A anchored olig dT in combination with OPB12 (Lane 1-6), UBC135 (Lane 7-12), and UBC191 (Lane 13-18). Box indicates differential expression display product that was cloned and sequenced.

Lane M = DNA ladder
Lane 1, 2, 7, 8, 13, 14 = Control
Lane 3, 4, 9, 10, 15, 16 = 0.681 µg/l chlorpyrifos exposure
Lane 5, 6, 11, 12, 17, 18 = 6.81 µg/l chlorpyrifos exposure

4.8 Expression analysis of the genes in chlorpyrifos-exposed shrimp

Expression levels of target genes, including cytochrome P450 (*CYP4C39*), beta glucuronidase, heat shock protein 70, heat shock protein 90, vitellogenin, OPA07G350-27-1 (LDL receptor member LR3), UBC101C-1,000-D-3 (esterase), UBC119A-650-F-5 (*CYP330A1*), glutathione-s-transferase, OPA18G-600-4-1 (Ubiquitin-like-7), OPA01G-415-1 (leucine zipper protein 5), and OPA02G-450-2 (sequence of unknown gene) in chlorpyrifos-exposed shrimp were analyzed using semi-quantitative RT-PCR analysis. Elongation factor 1 alpha was used as internal control gene.

4.8.1 Optimization of PCR condition

Prior to the quantitative analysis, the appropriate PCR conditions including temperature, template concentration, number of cycles, and $MgCl_2$ concentration for each of target genes and reference gene were verified based on the criteria that the PCR product must be on the log phase of amplification.

PCR was performed in a PCR thermal cycler (Hybraid Limited, England). The PCR reaction was based on the standard condition consisted of 1X PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, and 0.1% Triton X-100), 0.2 mM each of dNTPs, and 1 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes) in a final volume of 25 µl reaction. The standard PCR profiles consisted of predenaturing step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 50-65 °C for 45 sec (depending on the melting temperature of the primers), and 72 °C for 45 sec, and a final extension at 72 °C for 5 min. The condition was optimized as follow.

First, the annealing temperature for each target gene was adjusted within several degrees to obtain the best intensity and specificity of the target band. Then, PCR reactions with selected annealing temperature were conducted with various concentrations of $MgCl_2$ (0.5, 1, and 1.5 mM) and the concentration that provided the best and specific target band was chosen. The optimal primer concentration was examined with the concentration ranging from 0.05, 0.1, 0.15, and 0.2 µM using PCR with optimal $MgCl_2$ concentration and the concentration that gave highest yield and specificity was chosen. Finally, optimal $MgCl_2$ and primer concentration was used to identify the suitable PCR cycle number with various concentration of DNA template

(between 100 to 1,000 ng). The cycle number and amount of template that amplified the PCR product in the exponential range and did not reach a plateau level was chosen.

The optimal condition of PCR for each target gene is shown in Figure 4.43 - 4.55 and Table 4.20.

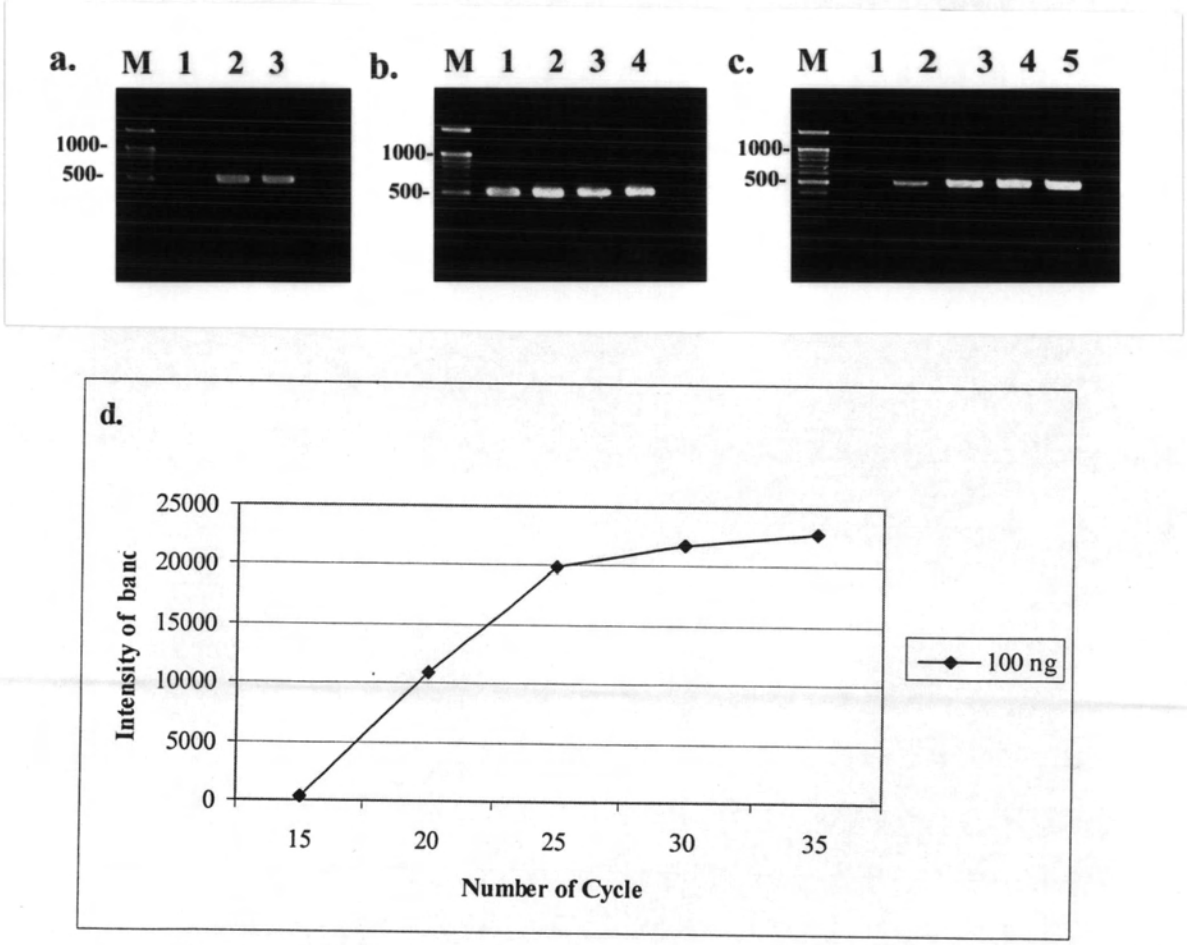


Figure 4.43 Optimization for suitable PCR condition of elongation factor 1 alpha. $MgCl_2$ concentration was examined from the varied concentration of 0.5, 1.0, and 1.5 mM (Lane a1-a3). Primer was examined from the varied concentration of 0.05, 0.10, 0.15 and 0.2 µM (Lane b1-a4). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles (Lane c1-c5) for 100 ng of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).

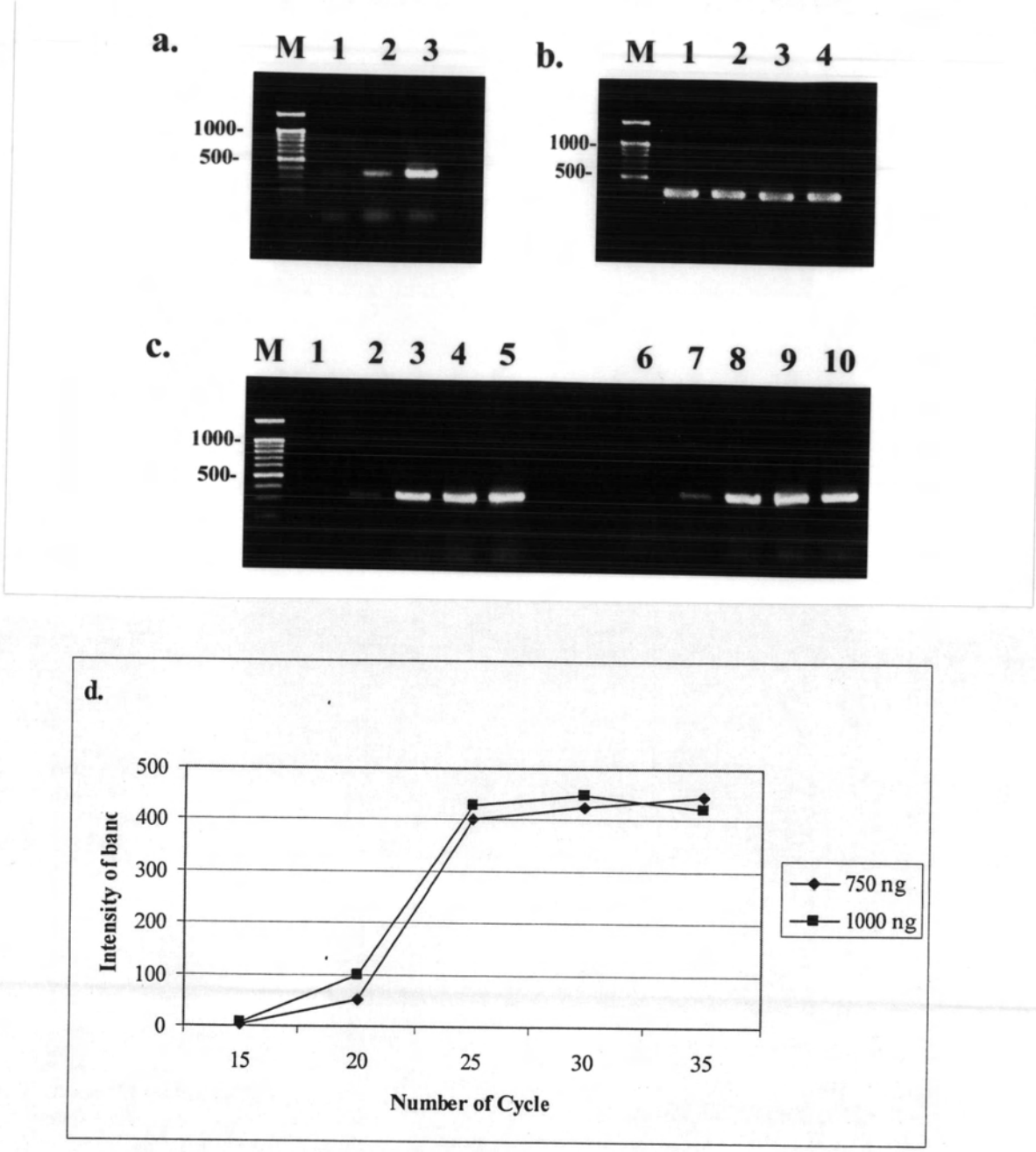


Figure 4.44 Optimization for suitable PCR condition of cytochrome P450 (*CYP4C39*). $MgCl_2$ concentration was examined from the varied concentration of 0.5, 1.0, and 1.5 mM (Lane a1-a3). Primer was examined from the varied concentration of 0.05, 0.10, 0.15 and 0.2 µM (Lane b1-a4). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles for 750 ng (Lane c1-c5) and 1,000 ng (Lane c6-c10) of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).

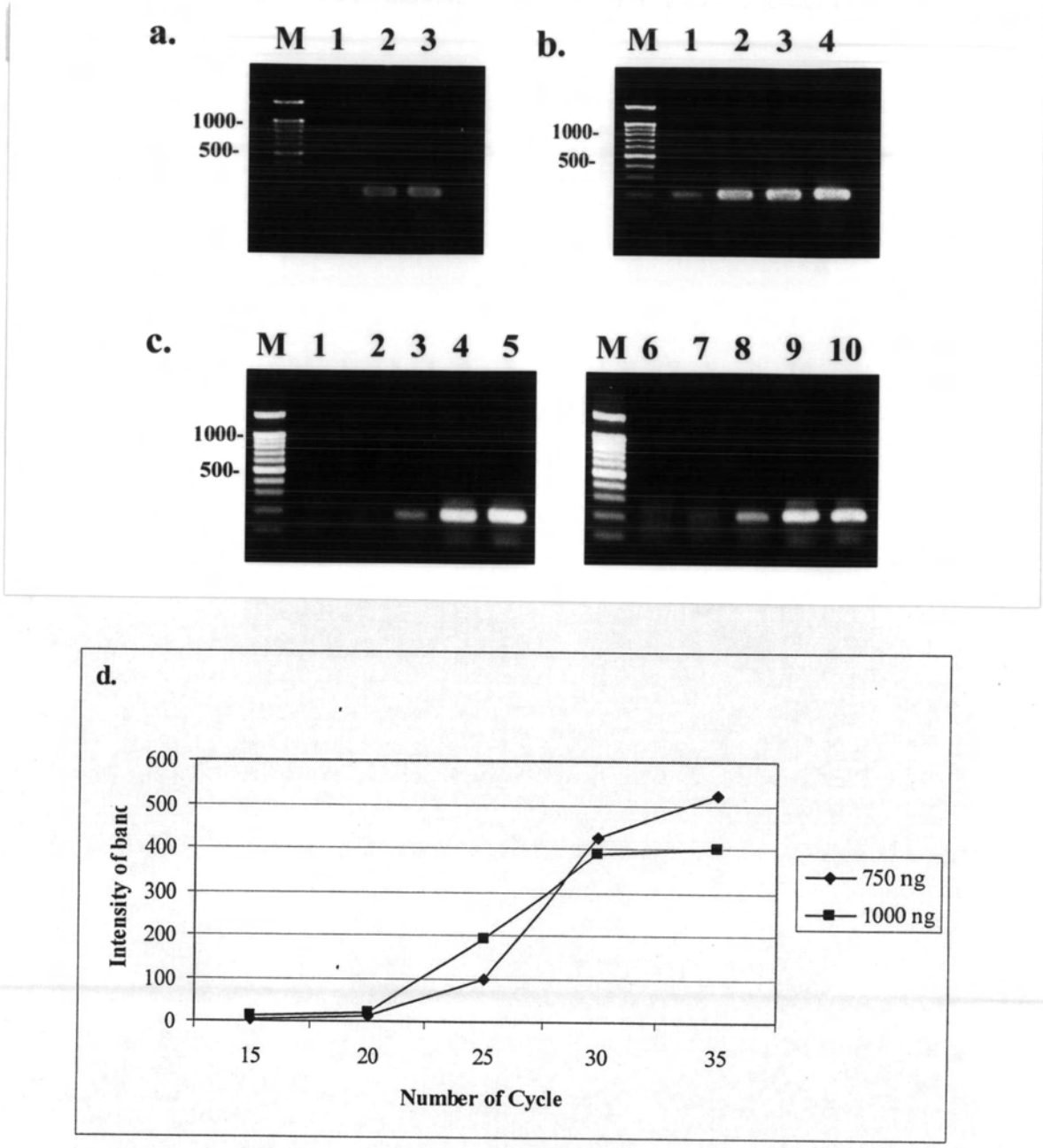
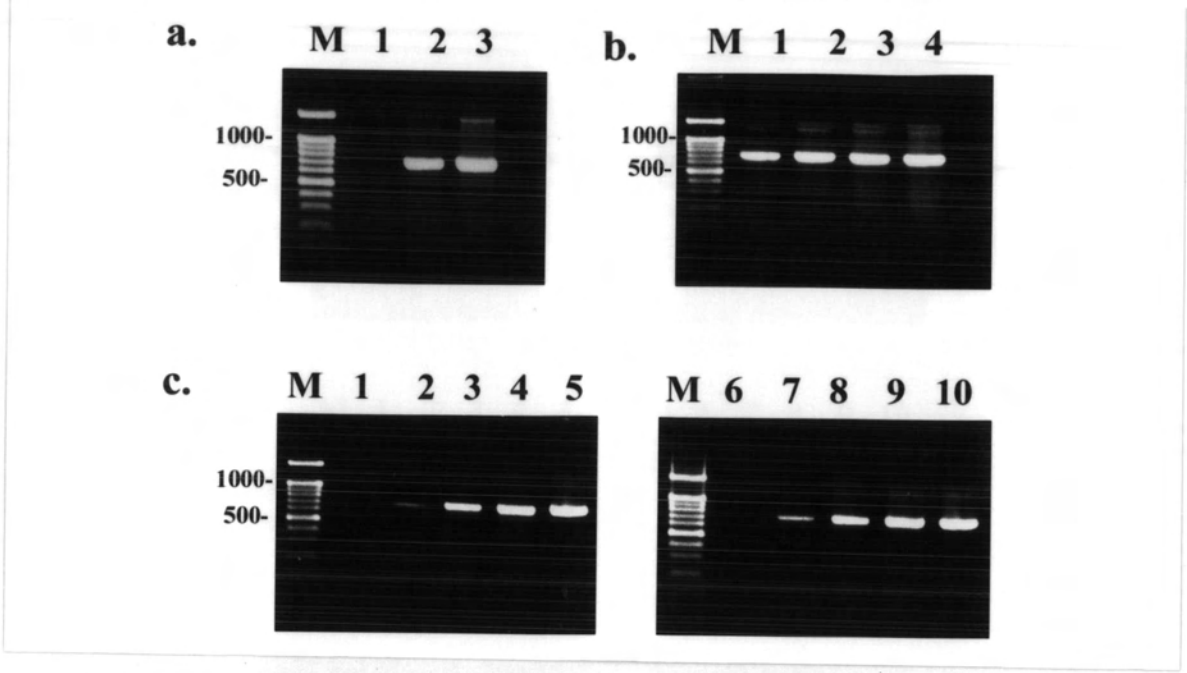


Figure 4.45 Optimization for suitable PCR condition of beta glucuronidase. $MgCl_2$ concentration was examined from the varied concentration of 0.5, 1.0, and 1.5 mM (Lane a1-a3). Primer was examined from the varied concentration of 0.05, 0.10, 0.15 and 0.2 μM (Lane b1-a4). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles for 750 ng (Lane c1-c5) and 1,000 ng (Lane c6-c10) of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).



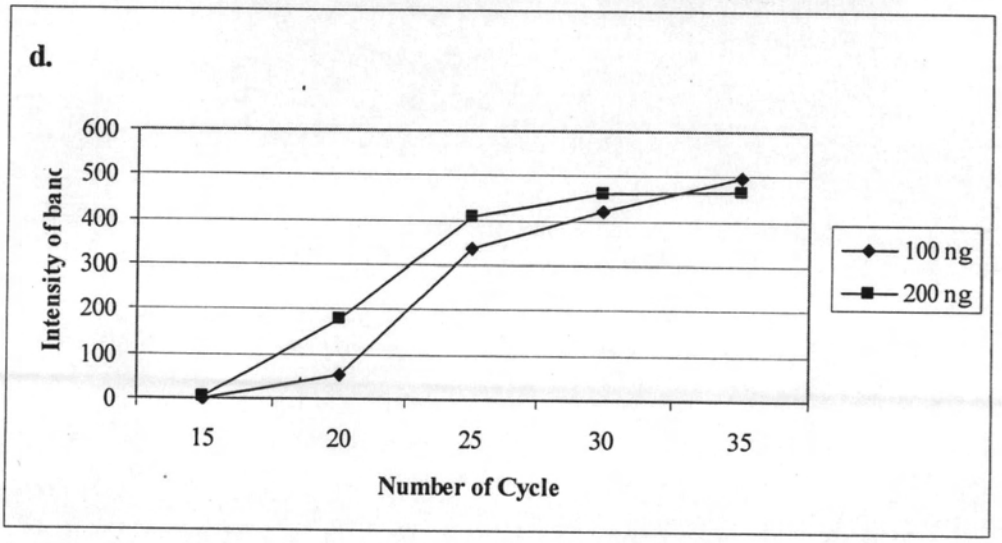


Figure 4.46 Optimization for suitable PCR condition of heat shock protein 70. $MgCl_2$ concentration was examined from the varied concentration of 0.5, 1.0, and 1.5 mM (Lane a1-a3). Primer was examined from the varied concentration of 0.05, 0.10, 0.15 and 0.2 μM (Lane b1-a4). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles for 100 ng (Lane c1-c5) and 200 ng (Lane c6-c10) of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).

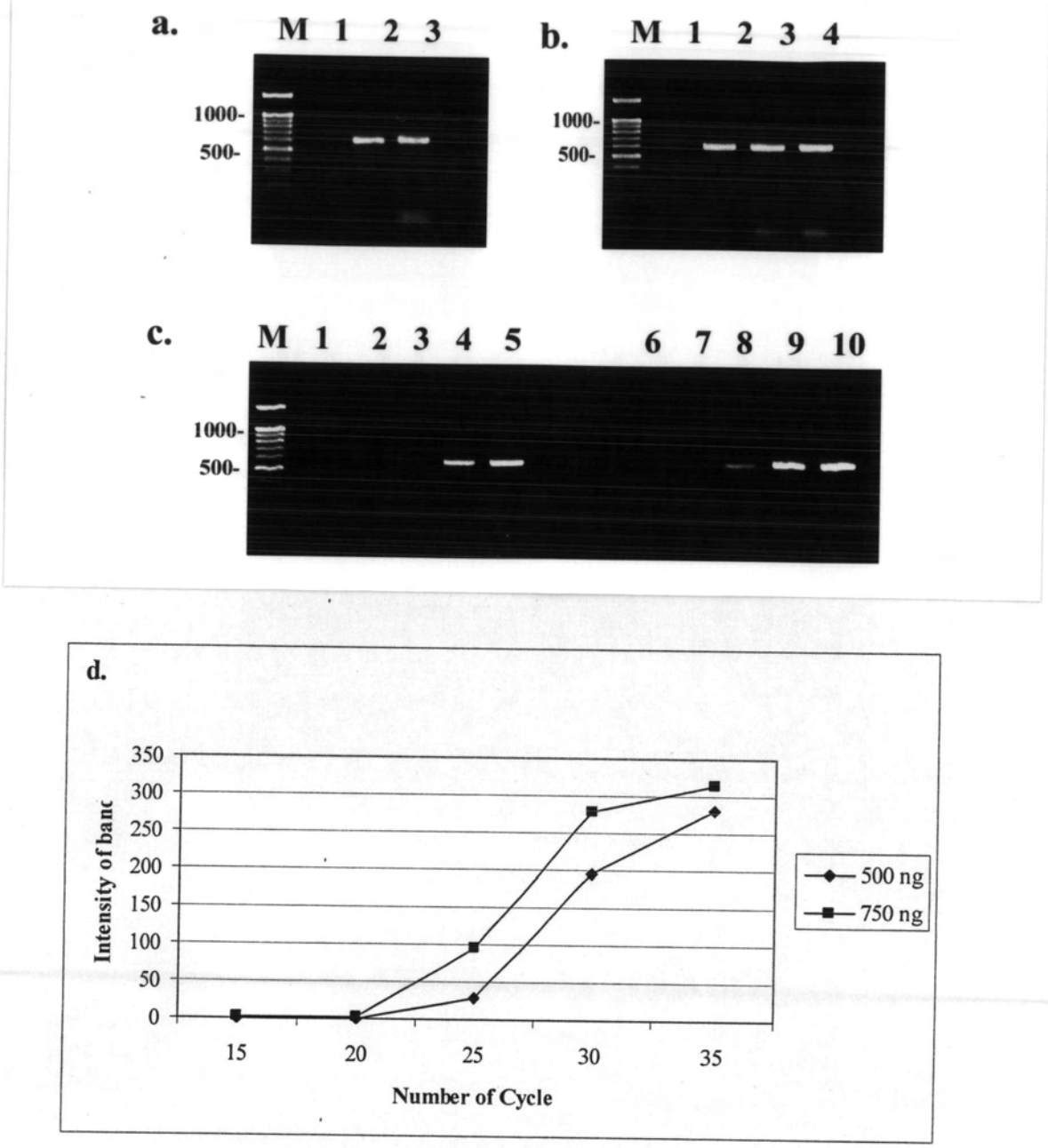


Figure 4.47 Optimization for suitable PCR condition of heat shock protein 90. $MgCl_2$ concentration was examined from the varied concentration of 0.5, 1.0, and 1.5 mM (Lane a1-a3). Primer was examined from the varied concentration of 0.05, 0.10, 0.15 and 0.2 μM (Lane b1-a4). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles for 500 ng (Lane c1-c5) and 750 ng (Lane c6-c10) of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).

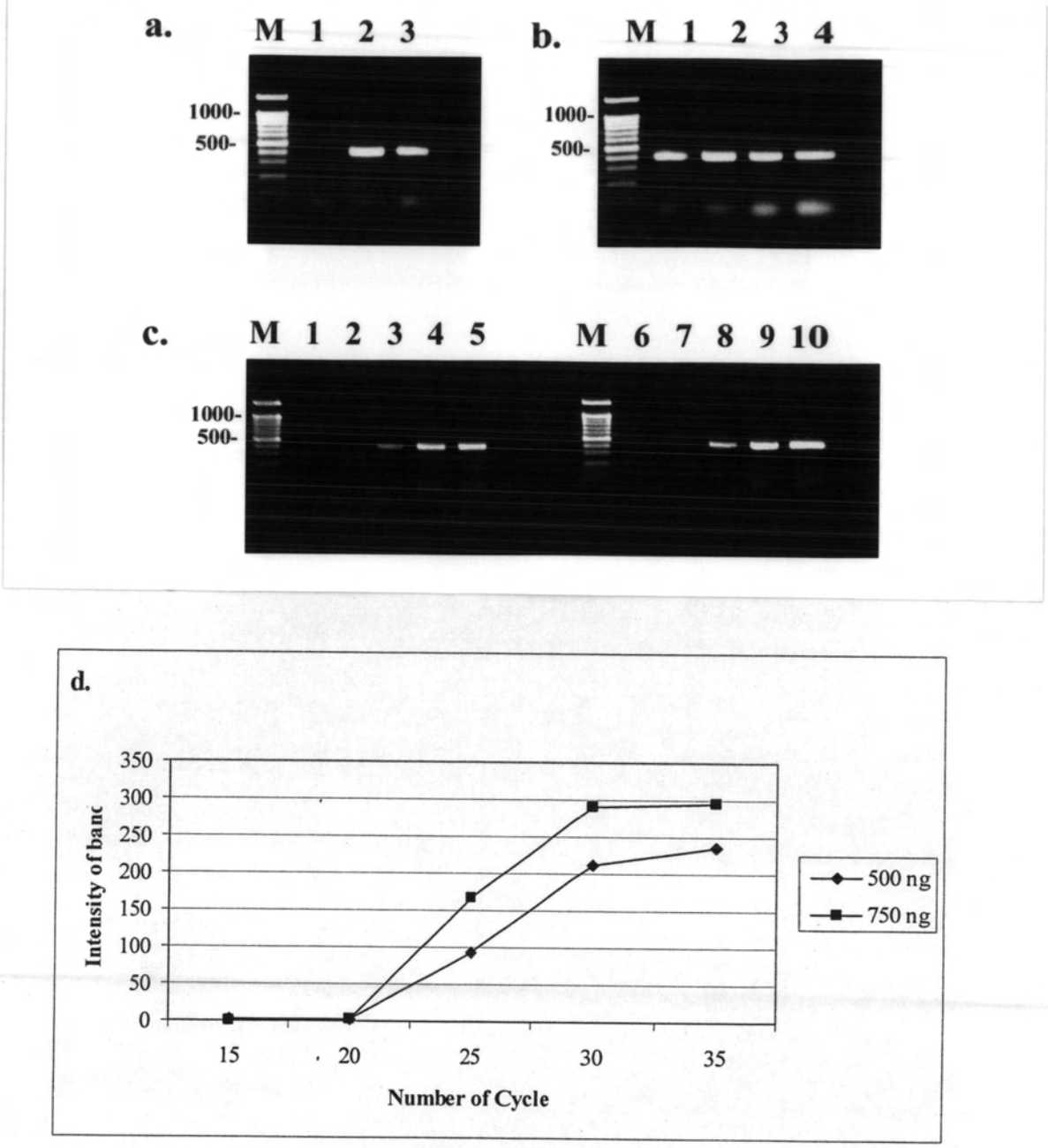


Figure 4.48 Optimization for suitable PCR condition of UBC101C-1,000-D-3 (Esterase). $MgCl_2$ concentration was examined from the varied concentration of 0.5, 1.0, and 1.5 mM (Lane a1-a3). Primer was examined from the varied concentration of 0.05, 0.10, 0.15 and 0.2 μM (Lane b1-a4). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles for 500 ng (Lane c1-c5) and 750 ng (Lane c6-c10) of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).

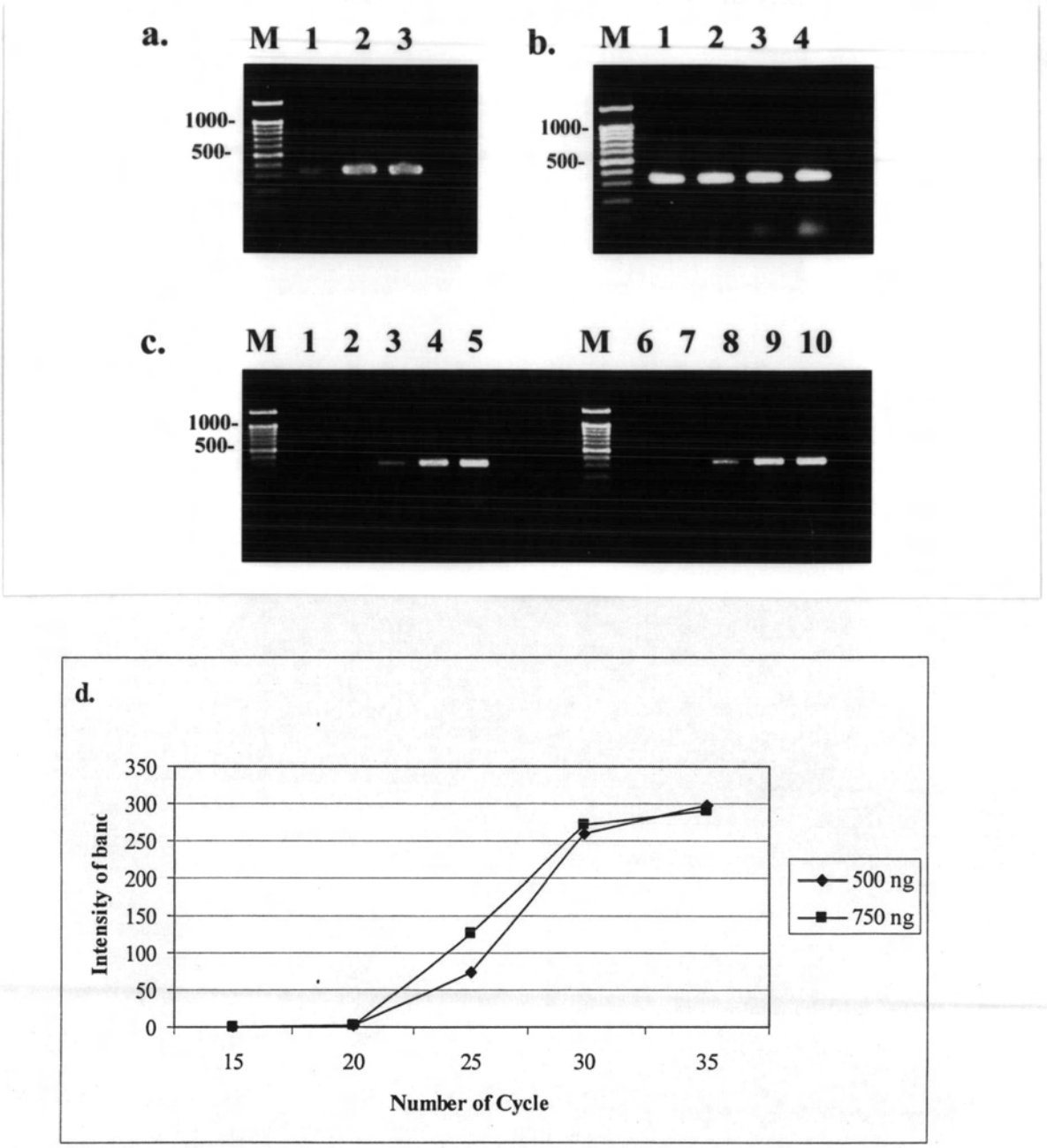
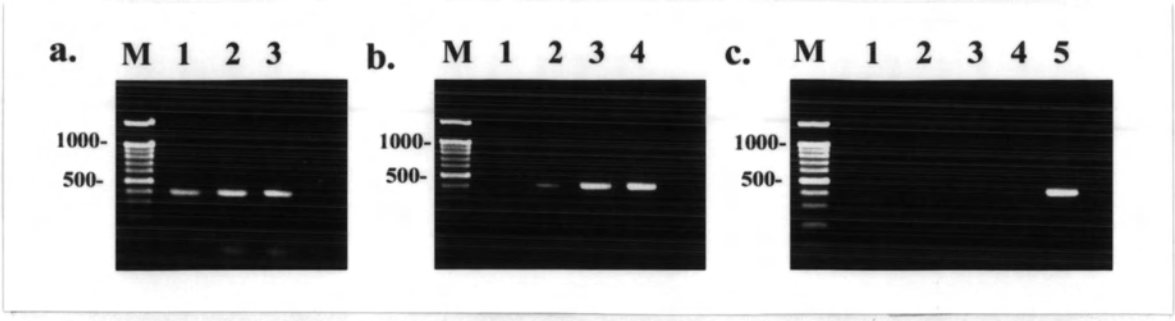


Figure 4.49 Optimization for suitable PCR condition of UBC119A-650-F-5 (*CYP330A1*). $MgCl_2$ concentration was examined from the varied concentration of 0.5, 1.0, and 1.5 mM (Lane a1-a3). Primer was examined from the varied concentration of 0.05, 0.10, 0.15 and 0.2 μM (Lane b1-a4). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles for 500 ng (Lane c1-c5) and 750 ng (Lane c6-c10) of hepatopancreas first strand cDNA template.



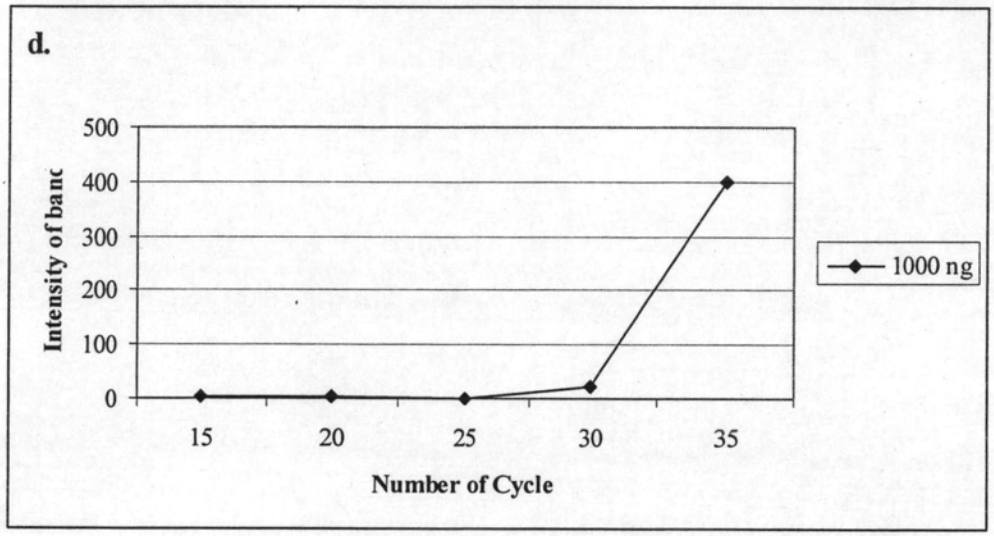
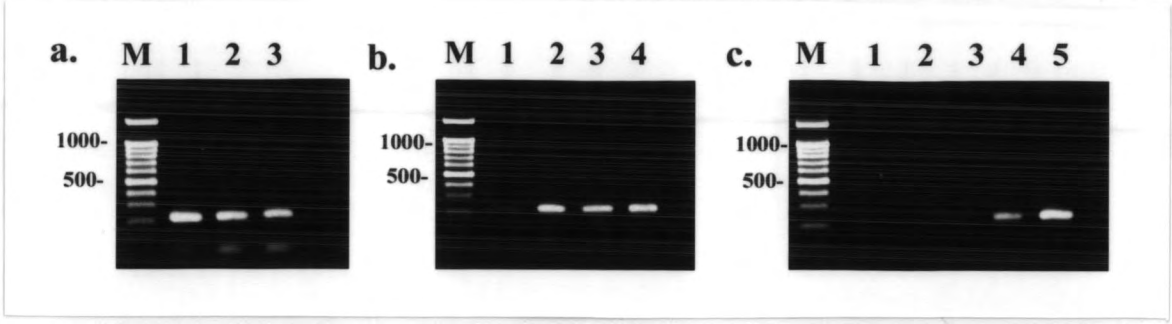


Figure 4.50 Optimization for suitable PCR condition of vitellogenin. $MgCl_2$ concentration was examined from the varied concentration of 0.5, 1.0, and 1.5 mM (Lane a1-a3). Primer was examined from the varied concentration of 0.05, 0.10, 0.15 and 0.2 µM (Lane b1-a4). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles (Lane c1-c5) for 1,000 ng of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).



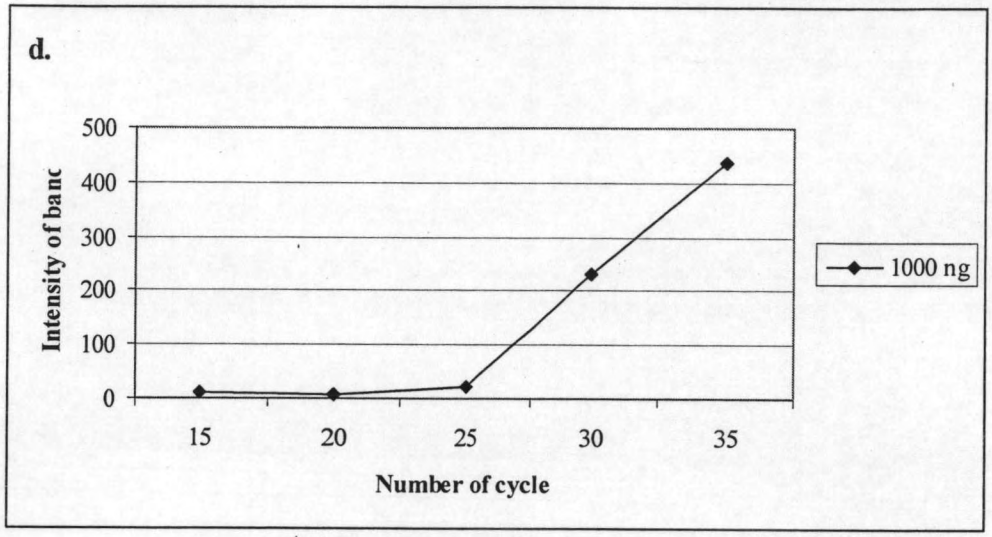
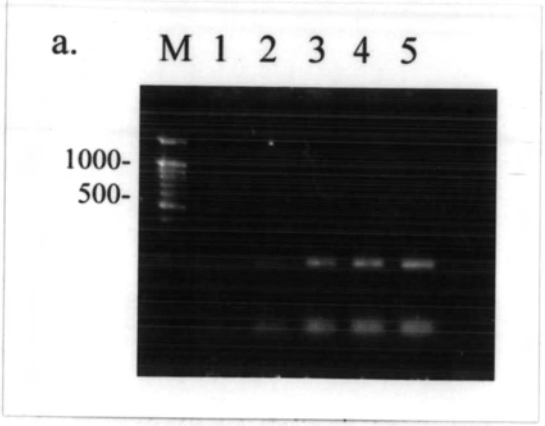


Figure 4.51 Optimization for suitable PCR condition of OPA07G350-27-1 (LDL receptor member LR3). $MgCl_2$ concentration was examined from the varied concentration of 0.5, 1.0, and 1.5 mM (Lane a1-a3). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles (Lane c1-c5) for 1,000 ng of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).



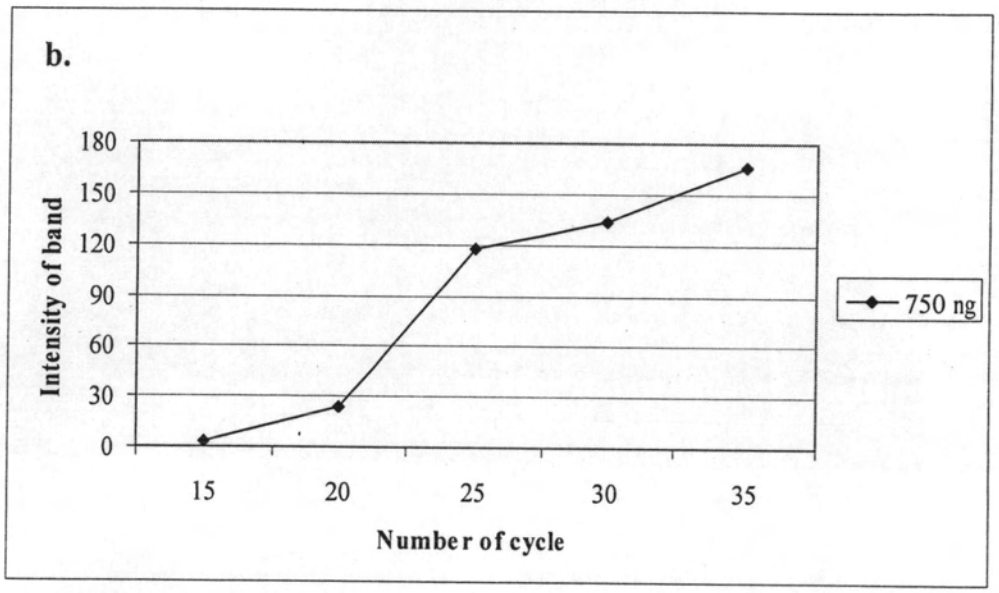


Figure 4.52 Optimization for suitable PCR condition of glutathione-s-transferase. Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles (Lane A1-A5) for 750 ng of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (b.).

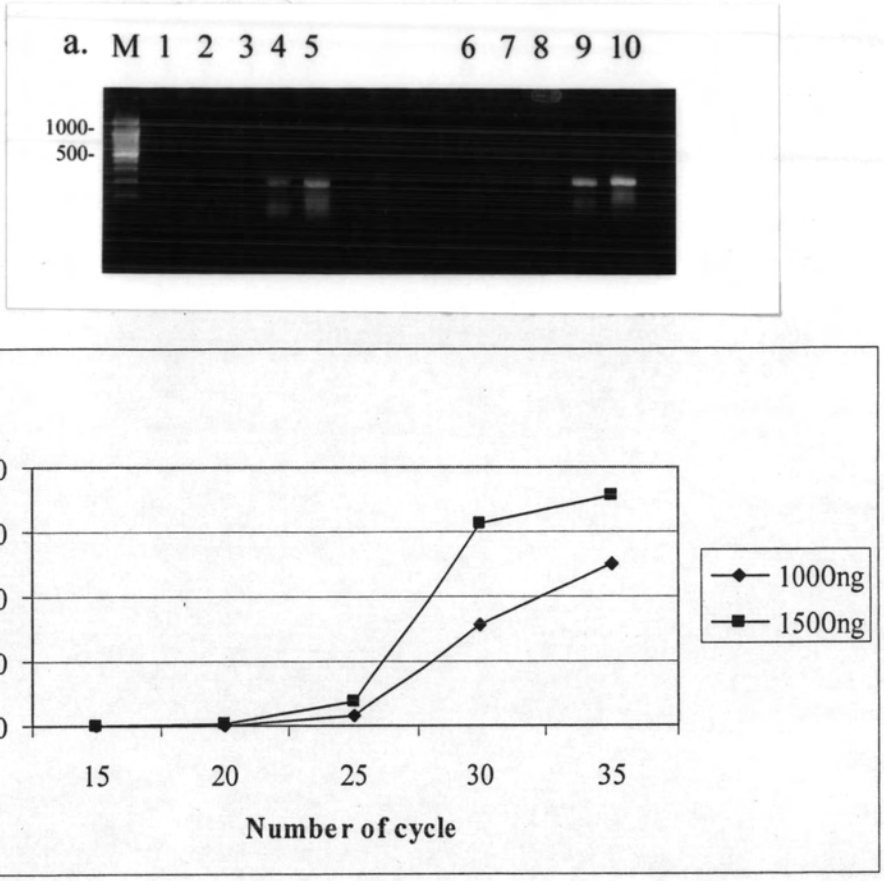


Figure 4.53 Optimization for suitable PCR condition of OPA18G-600-4-1 (Ubiquitin-like-7). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles for 1,000 ng (Lane a1-a5) and 1,500 ng (Lane a6-a10) of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (b.).

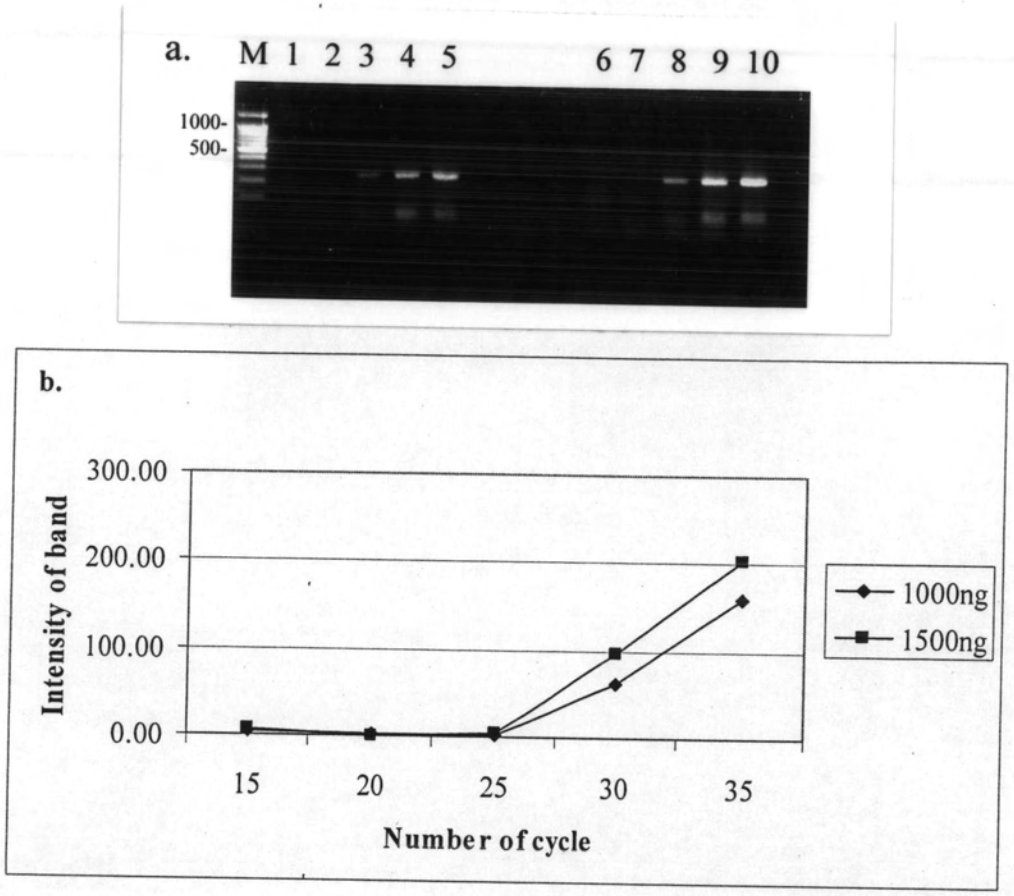


Figure 4.54 Optimization for suitable PCR condition of OPA01G-415-1 (Leucine zipper protein 5). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles for 1,000 ng (Lane a1-a5) and 1,500 ng (Lane a6-a10) of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (b.).

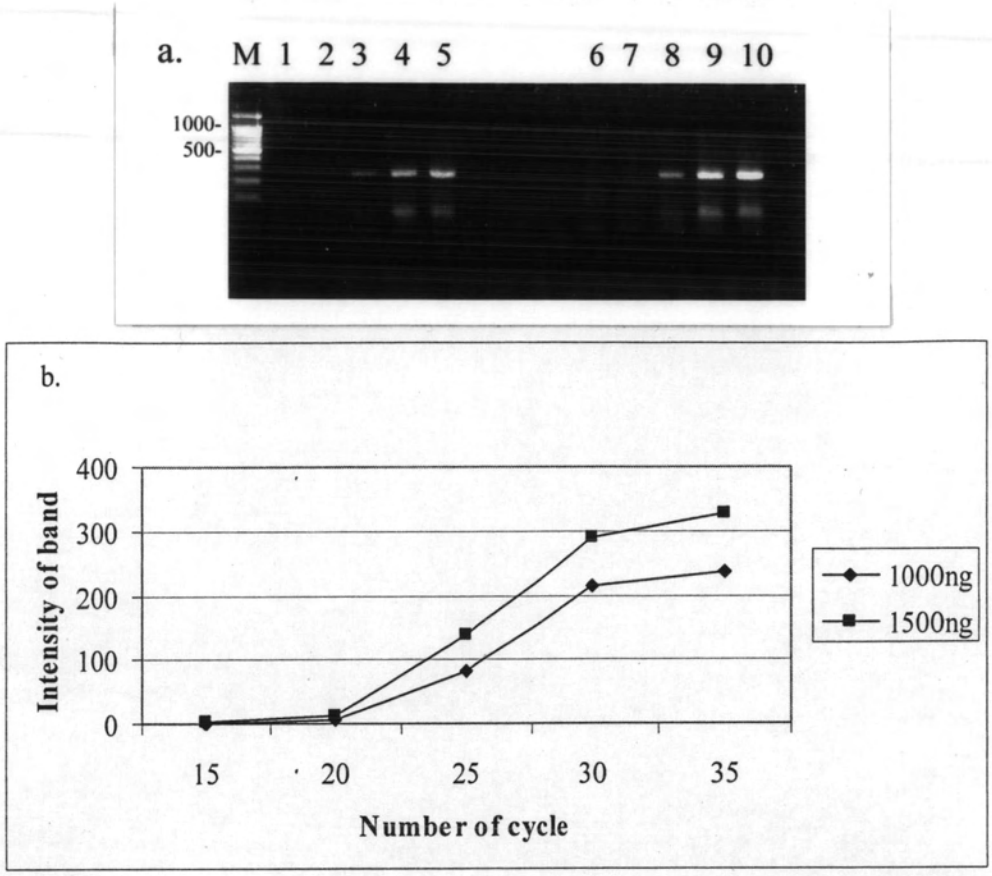


Figure 4.55 Optimization for suitable PCR condition of OPA02G-450-2 (sequence of unknown gene). Number of cycle was examined from the varied number of 15, 20, 25, 30, and 35 cycles for 1,000 ng (Lane a1-a5) and 1,500 ng (Lane a6-a10)of hepatopancreas first strand cDNA template. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (b.).

Table 4.20 Optimal condition for Semi-quantitative RT-PCR of **genes in hepatopancreas of chlorpyrifos-exposed shrimp**

Gene	Template (ng)	$MgCl_2$ (mM)	Primer (µM)	Annealing Temperature (°C)	PCR Cycle Number	PCR Product (bp)
1. Cytochrome P450 (*CYP4C39*)	1,000	1.5	0.1	65	23	355
2. Beta glucuronidase	750	1.0	0.15	60	28	196
3. Heat Shock Protein70	100	1.0	0.2	60	28	719
4. Heat Shock Protein90	750	1.5	0.1	50	30	612
5. Vitellogenin	1,000	1.0	0.15	50	30	416
6. OPA07G350-27-1 (LDL receptor member LR3)	1,000	1.0	0.1	50	30	232
7. UBC101C-1,000-D-3 (Esterase)	750	1.0	0.1	55	28	410
8. UBC119A-650-F-5 (*CYP330A1*)	750	1.5	0.1	55	28	349
9. Glutathione-s-transferase	750	1.5	0.1	55	28	255
10. OPA18G-600-4-1 (Ubiquitin-like-7)	750	1.5	0.1	65	28	255
11. OPA01G-415-1 (Leucine zipper protein 5)	1,000	1.5	0.1	65	30	217
12. OPA02G-450-2 (sequence of unknown gene)	1,000	1.5	0.2	55	30	200
13. Elongation factor -1 alpha	100	1.5	0.1	55	23	500

4.8.2 Semi-quantitative RT-PCR

Using the obtained optimal PCR condition for semi-quantitative analysis of gene expression level, results showed that none of the target genes, including cytochrome P450 (*CYP4C39*), beta glucuronidase, heat shock protein 70, heat shock protein 90, vitellogenin, , UBC101C-1,000-D-3 (esterase), UBC119A-650-F-5 (*CYP330A1*), glutathione-s-transferase, OPA18G-600-4-1 (Ubiquitin-like-7), OPA01G-415-1 (leucine zipper protein 5), and OPA02G-450-2 (sequence of unknown gene) showed significant systematic pattern in the difference of gene expression level among groups of shrimps exposed to 0-27.24 µg/l chlorpyrifos within 96 h (Figure 4.56-4.67, Table 4.21-4.30).

For vitellogenin, and OPA07G350-27-1 (LDL receptor member LR3), expression level of these genes could not be compared because most of the samples did not give the target product. Therefore, amplification was done in only one treatment period (24 h) and the results are shown in Figure 4.62- 4.63.

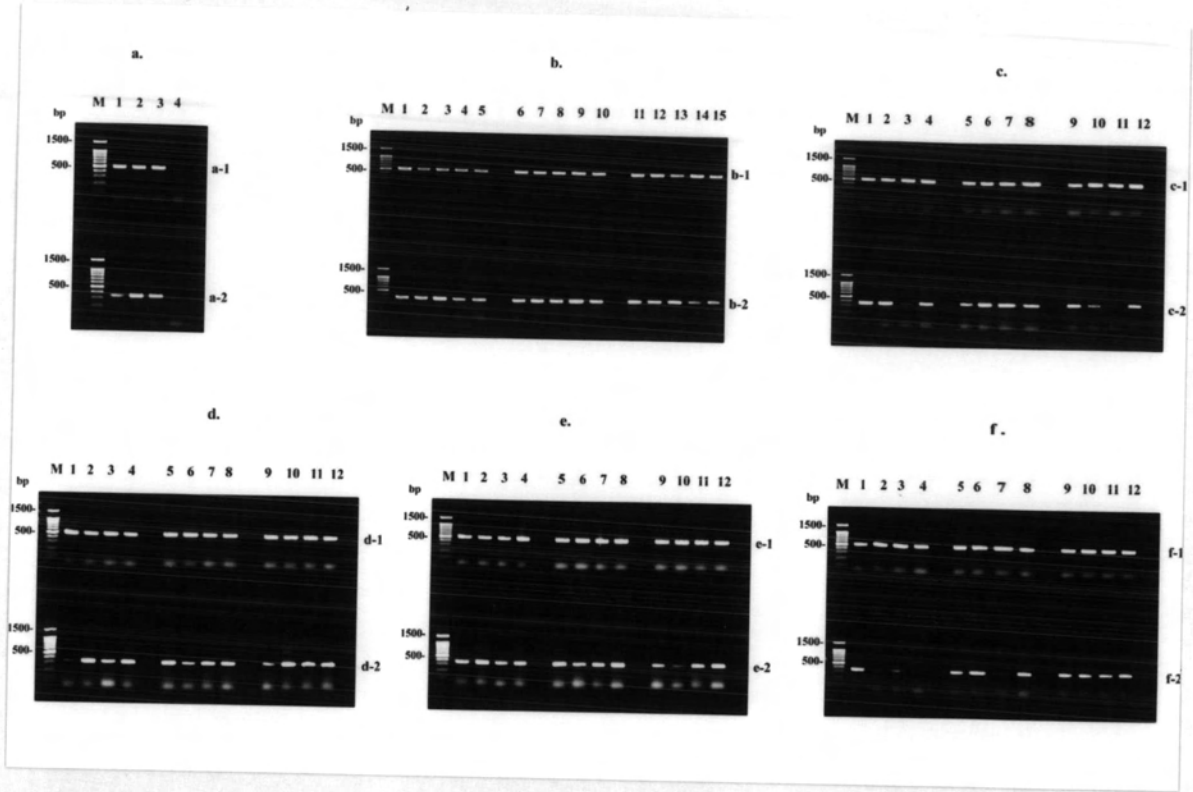


Figure 4.56 RT-PCR of cytochrome P450 (*CYP4C39*) in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0, 12, 24, 48, 72 and, 96 h (a-2- f-2). Elongation factor 1 alpha from the same template was used as internal control (a-1 – f-1).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 6, 11 = Control

Lane 2, 7, 12 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 8, 13 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 9, 14 = 13.62 µg/l chlorpyrifos exposure

Lane 5, 10, 15 = 27.24 µg/l chlorpyrifos exposure

For c-f:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

Table 4.21 Relative expression level of cytochrome P450 (*CYP4C39*) in hepatopancreas of *P. monodon*

Time of Exposure (h)	Chlorpyrifos Concentration (µg/l) (N=3)				
	0	**0.0681**	**6.81**	**13.62**	**27.24**
0	0.89±0.20	*NA**	*NA**	*NA**	*NA**
12	1.00±0.07	1.16±0.29	1.25±0.18	0.88±0.31	1.01±0.17
24	0.95±0.21	0.85±0.37	0.37±0.48	0.76±0.11	*NA***
48	0.58±0.48	0.99±0.34	0.89±0.10	1.04±0.08	*NA***
72	0.91±0.18	0.75±0.54	0.87±0.06	0.96±0.06	*NA***
96	0.89±0.15	0.58±0.44	0.34±0.34	0.59±0.49	*NA***

Remark: * data was not available according to 0 h exposure was specifically set for control group.

** data was not available according to mortality of shrimp exposed to 27.24 µg/l was 100% within 12 h.

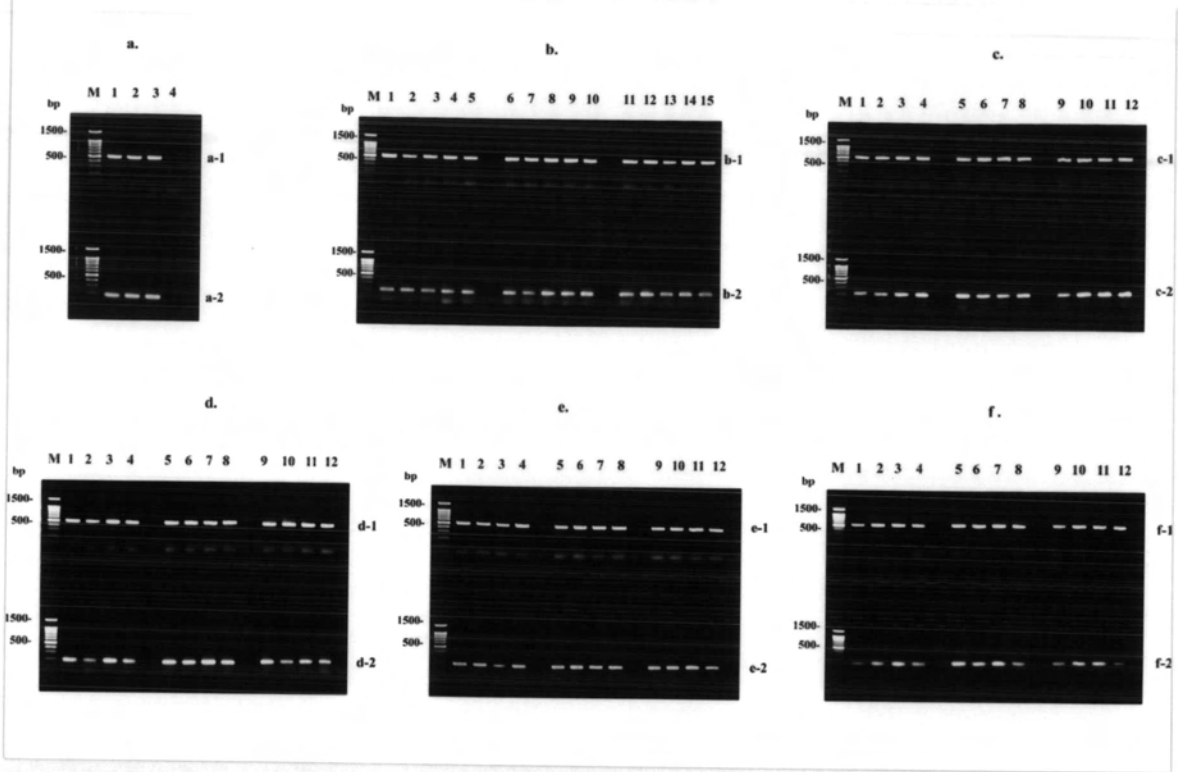


Figure 4.57 RT-PCR of beta glucuronidase in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0, 12, 24, 48, 72 and, 96 h (a-2- f-2). Elongation factor 1 alpha from the same template was used as internal control (a-1 – f-1).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 6, 11 = Control

Lane 2, 7, 12 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 8, 13 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 9, 14 = 13.62 µg/l chlorpyrifos exposure

Lane 5, 10, 15 = 27.24 µg/l chlorpyrifos exposure

For c-f:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

Table 4.22 Relative expression level of *beta* glucuronidase in hepatopancreas of *P. monodon*

Time of Exposure (h)	Chlorpyrifos Concentration (µg/l) (N=3)				
	0	**0.0681**	**6.81**	**13.62**	**27.24**
0	1.08±0.02	*NA**	*NA**	*NA**	*NA**
12	0.74±0.09	0.78±0.22	0.84±0.09	0.77±0.05	0.81±0.11
24	0.96±0.13	0.91±0.11	0.92±0.14	1.05±0.03	*NA***
48	1.03±0.04	0.85±0.16	1.01±0.19	0.87±0.07	*NA***
72	0.94±0.05	0.96±0.03	0.80±0.19	0.82±0.03	*NA***
96	0.78±0.32	0.90±0.11	1.00±0.01	0.65±0.26	*NA***

Remark: * data was not available according to 0 h exposure was specifically set for control group.

** data was not available according to mortality of shrimp exposed to 27.24 µg/l was 100% within 12 h.

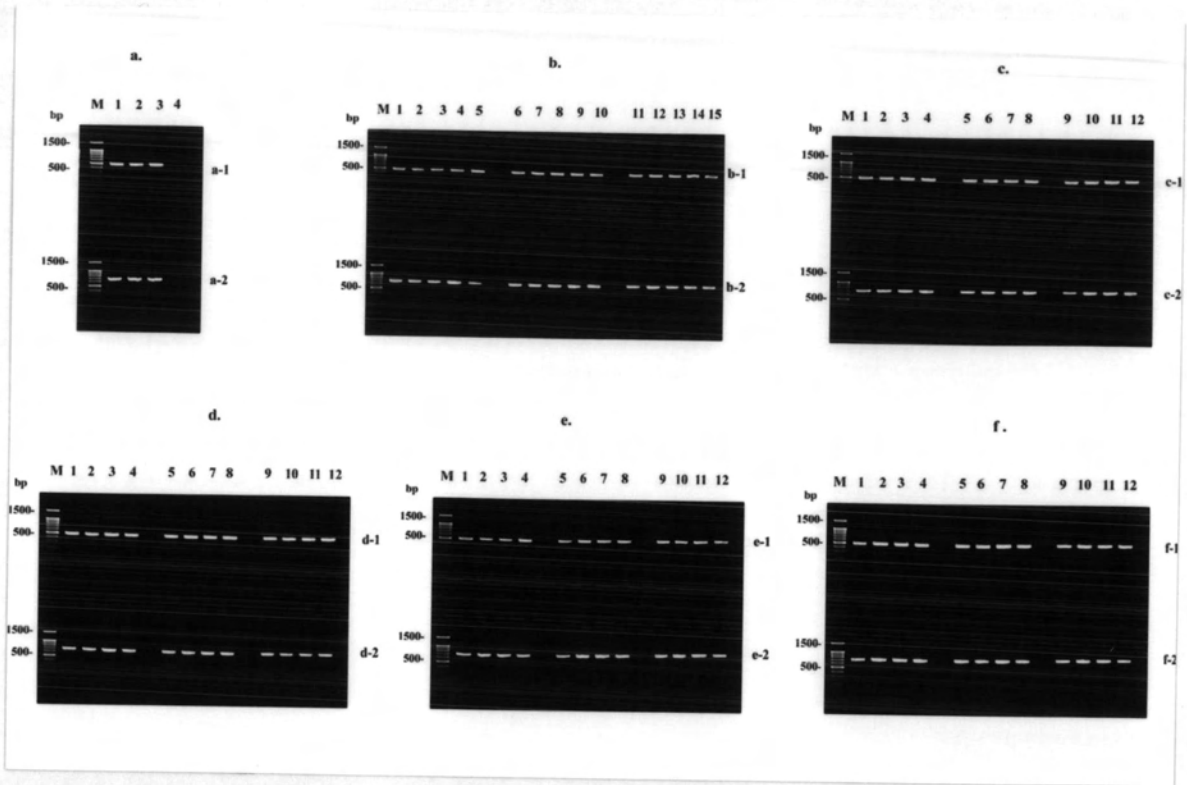


Figure 4.58 RT-PCR of heat shock protein 70 in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0, 12, 24, 48, 72 and, 96 h (a-1- f-1). Elongation factor 1 alpha from the same template was used as internal control (a-2 – f-2).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 6, 11 = Control

Lane 2, 7, 12 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 8, 13 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 9, 14 = 13.62 µg/l chlorpyrifos exposure

Lane 5, 10, 15 = 27.24 µg/l chlorpyrifos exposure

For c-f:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

Table 4.23 Relative expression level of heat shock protein 70 in hepatopancreas of *P. monodon*

Time of Exposure (h)	Chlorpyrifos Concentration (µg/l) (N=3)				
	0	**0.0681**	**6.81**	**13.62**	**27.24**
0	0.97±0.05	*NA**	*NA**	*NA**	*NA**
12	1.09±0.07	1.13±0.06	1.06±0.01	1.15±0.14	1.04±0.23
24	0.84±0.06	0.88±0.07	0.89±0.03	0.91±0.02	*NA***
48	0.88±0.05	0.84±0.09	0.86±0.10	0.84±0.04	*NA***
72	1.09±0.04	1.19±0.10	1.19±0.09	1.04±0.11	*NA***
96	0.94±0.03	0.91±0.03	0.92±0.03	0.89±0.07	*NA***

Remark: * data was not available according to 0 h exposure was specifically set for control group.

** data was not available according to mortality of shrimp exposed to 27.24 µg/l was 100% within 12 h.

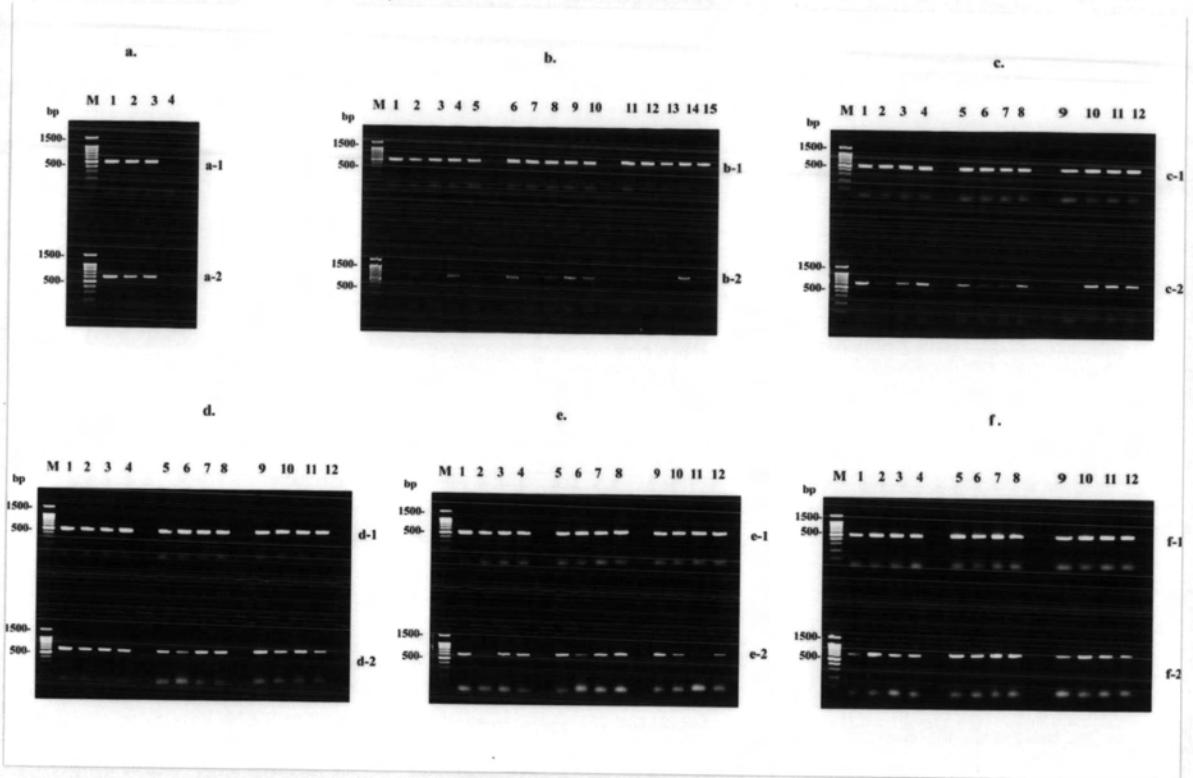


Figure 4.59 RT-PCR of heat shock protein 90 in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0, 12, 24, 48, 72 and, 96 h (a-2- f-2). Elongation factor 1 alpha from the same template was used as internal control (a-1 – f-1).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 6, 11 = Control

Lane 2, 7, 12 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 8, 13 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 9, 14 = 13.62 µg/l chlorpyrifos exposure

Lane 5, 10, 15 = 27.24 µg/l chlorpyrifos exposure

For c-f:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

Table 4.24 Relative expression level of heat shock protein 90 in hepatopancreas of *P. monodon*

Time of Exposure (h)	Chlorpyrifos Concentration (µg/l) (N=3)				
	0	**0.0681**	**6.81**	**13.62**	**27.24**
0	0.78±0.08	*NA**	*NA**	*NA**	*NA**
12	0.21±0.23	0.11±0.06	0.10±0.09	0.52±0.06	0.19±0.16
24	0.50±0.36	0.32±0.31	0.48±0.30	0.62±0.09	*NA***
48	0.80±0.11	0.61±0.16	0.77±0.04	0.63±0.12	*NA***
72	0.70±0.05	0.27±0.18	0.48±0.35	0.59±0.19	*NA***
96	0.59±0.17	0.85±0.07	0.73±0.11	0.75±0.20	*NA***

Remark: * data was not available according to 0 h exposure was specifically set for control group.

** data was not available according to mortality of shrimp exposed to 27.24 µg/l was 100% within 12 h.

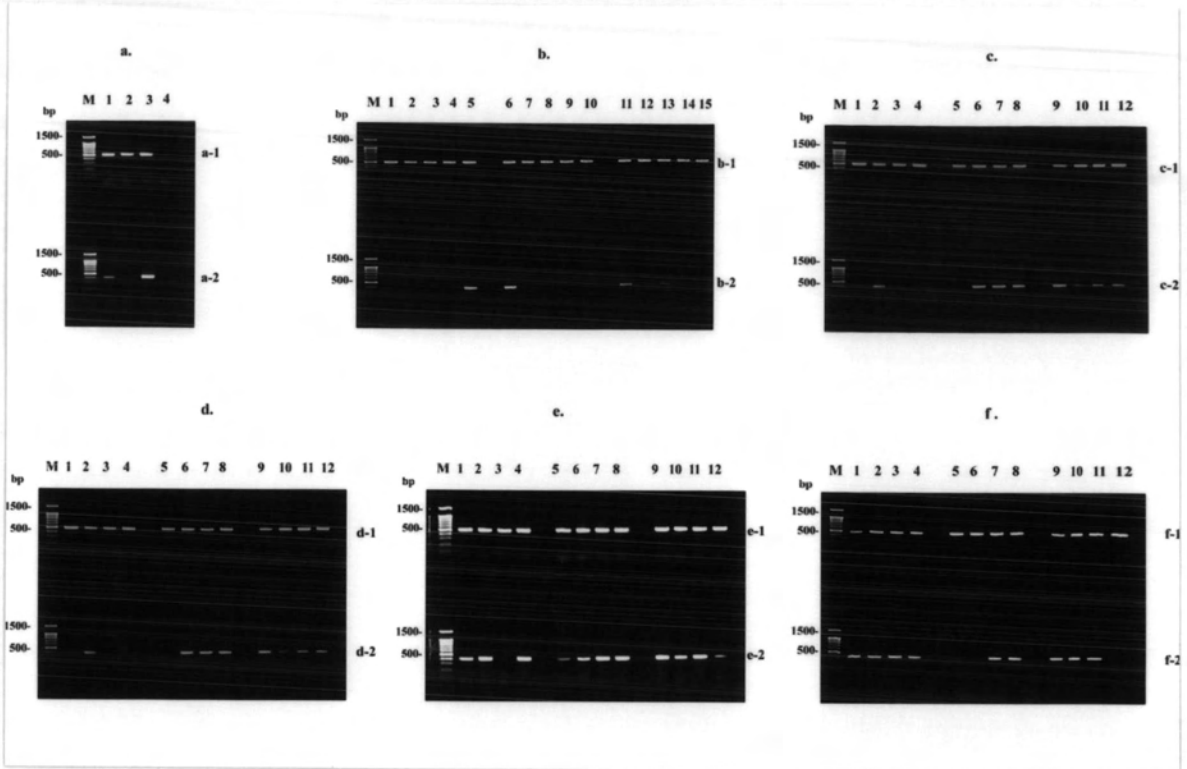


Figure 4.60 RT-PCR of UBC101C-1,000-D-3 in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0, 12, 24, 48, 72 and, 96 h (a-1- f-1). Elongation factor 1 alpha from the same template was used as internal control (a-2 – f-2).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 6, 11 = Control

Lane 2, 7, 12 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 8, 13 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 9, 14 = 13.62 µg/l chlorpyrifos exposure

Lane 5, 10, 15 = 27.24 µg/l chlorpyrifos exposure

For c-f:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

Table 4.25 Relative expression level of UBC101C-1,000-D-3 (Esterase) in hepatopancreas of *P. monodon*

Time of Exposure (h)	Chlorpyrifos Concentration (µg/l) (N=3)				
	0	**0.0681**	**6.81**	**13.62**	**27.24**
0	0.49±0.51	*NA**	*NA**	*NA**	*NA**
12	0.58±0.53	0.01±0.01	0.10±0.15	0.01±0.01	0.32±0.54
24	0.37±0.59	0.62±0.39	0.50±0.52	0.47±0.40	*NA***
48	0.79±0.19	0.28±0.45	0.35±0.51	0.30±0.49	*NA***
72	0.72±0.32	0.87±0.17	0.63±0.53	0.75±0.37	*NA***
96	0.75±0.65	0.61±0.53	0.91±0.13	0.60±0.51	*NA***

Remark: * data was not available according to 0 h exposure was specifically set for control group.

** data was not available according to mortality of shrimp exposed to 27.24 µg/l was 100% within 12 h.

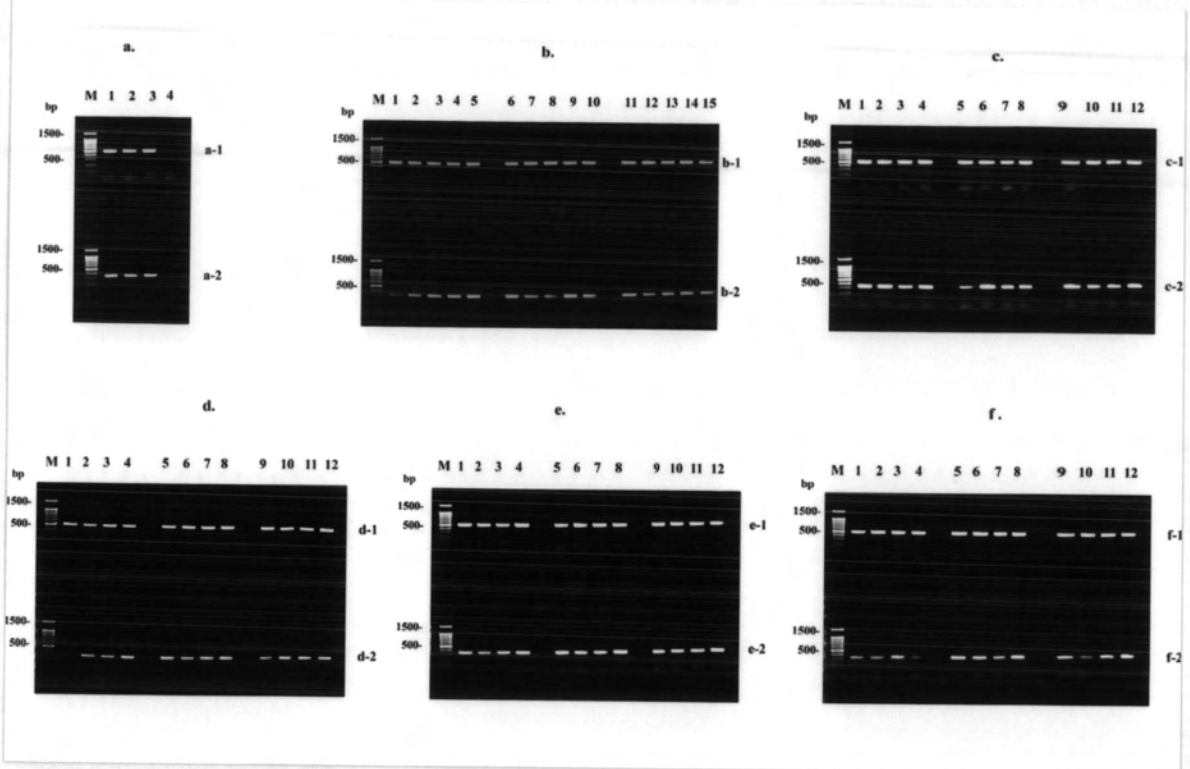


Figure 4.61 RT-PCR of UBC119A-650-F-5 in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0, 12, 24, 48, 72 and, 96 h (a-2- f-2). Elongation factor 1 alpha from the same template was used as internal control (a-1 – f-1).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 6, 11 = Control

Lane 2, 7, 12 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 8, 13 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 9, 14 = 13.62 µg/l chlorpyrifos exposure

Lane 5, 10, 15 = 27.24 µg/l chlorpyrifos exposure

For c-f:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

Table 4.26 Relative expression level of UBC119A-650-F-5 (*CYP330A1*) in hepatopancreas of *P. monodon*

Time of Exposure (h)	Chlorpyrifos Concentration (µg/l) (N=3)				
	0	**0.0681**	**6.81**	**13.62**	**27.24**
0	0.84±0.02	*NA**	*NA**	*NA**	*NA**
12	0.76±0.41	0.73±0.06	0.78±0.20	0.97±0.14	0.89±0.08
24	0.88±0.26	0.96±0.14	0.87±0.06	0.93±0.08	*NA***
48	0.52±0.45	0.83±0.06	0.82±0.08	0.86±0.13	*NA***
72	0.91±0.13	0.82±0.10	0.87±0.04	1.00±0.14	*NA***
96	0.76±0.12	0.56±0.20	0.75±0.08	0.68±0.42	*NA***

Remark: * data was not available according to 0 h exposure was specifically set for control group.

** data was not available according to mortality of shrimp exposed to 27.24 µg/l was 100% within 12 h.

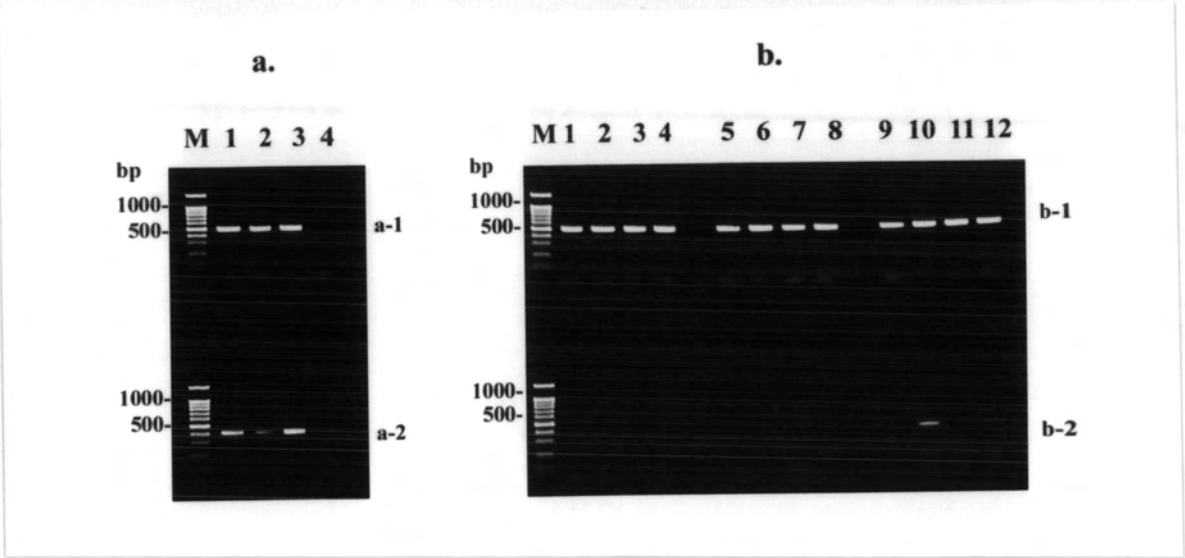


Figure 4.62 RT-PCR of vitellogenin in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0 and 24 h (a-2 and b-2). Elongation factor 1 alpha from the same template was used as internal control (a-1 – b-1).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

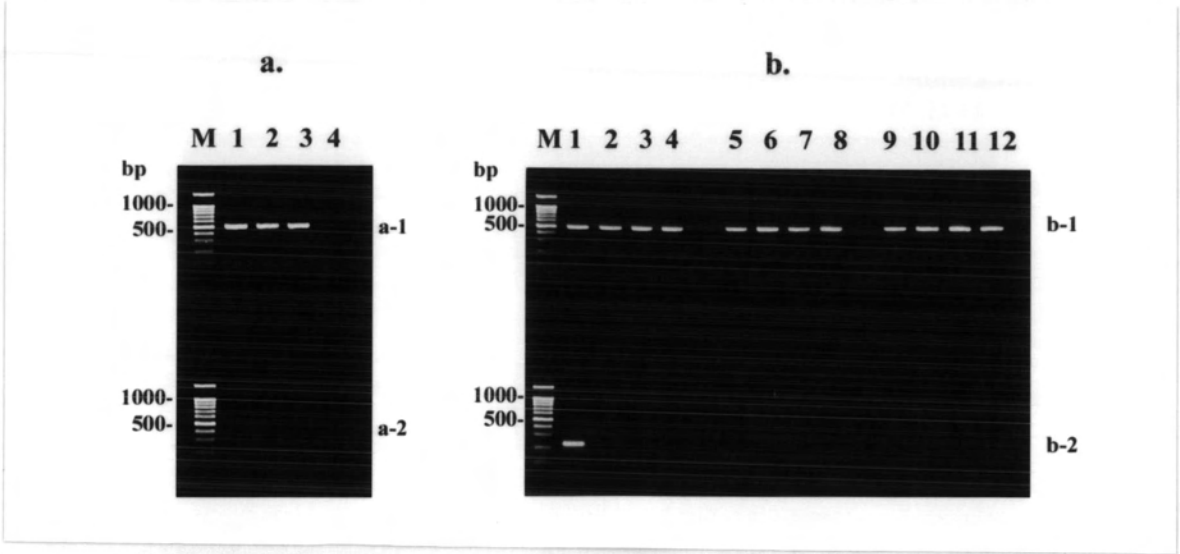


Figure 4.63 RT-PCR of OPA07G350-27-1 (LDL receptor member LR3) in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0 and 24 h (a-2 and b-2). Elongation factor 1 alpha from the same template was used as internal control (a-1 – b-1).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

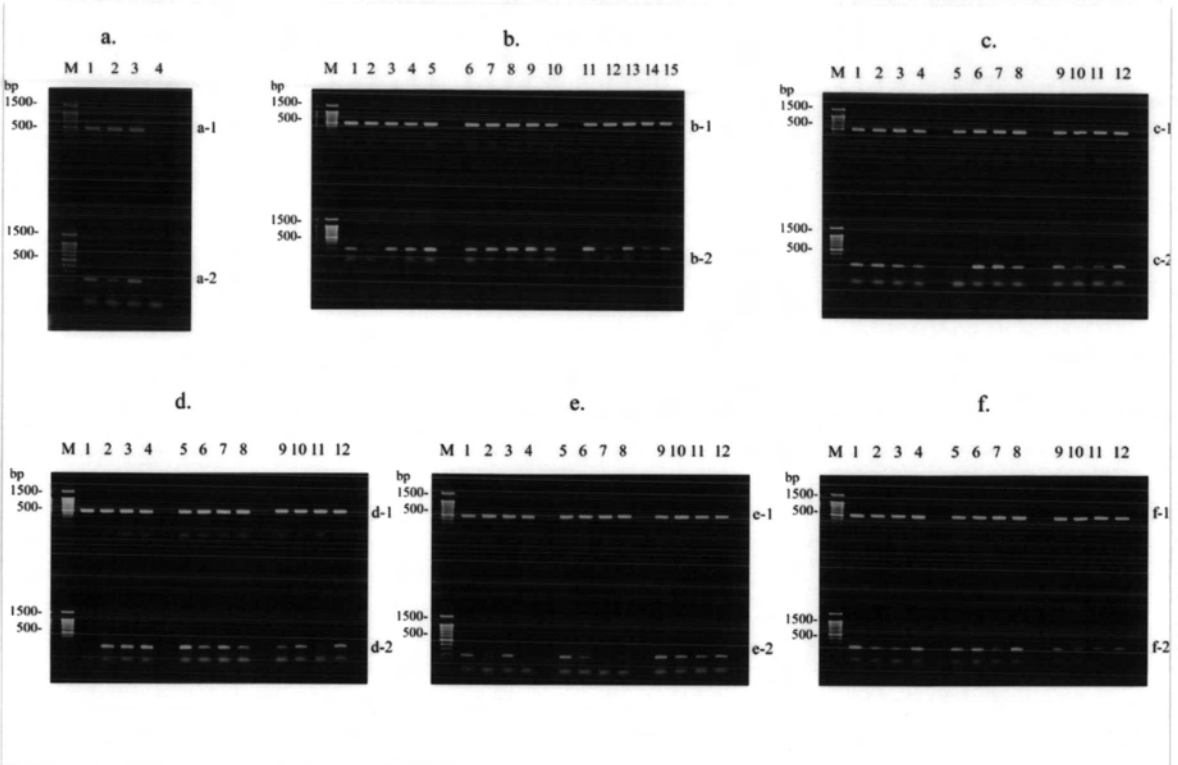


Figure 4.64 RT-PCR of glutathione-s-transferase in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0, 12, 24, 48, 72 and, 96 h (a-2- f-2). Elongation factor 1 alpha from the same template was used as internal control (a-1 – f-1).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 6, 11 = Control

Lane 2, 7, 12 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 8, 13 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 9, 14 = 13.62 µg/l chlorpyrifos exposure

Lane 5, 10, 15 = 27.24 µg/l chlorpyrifos exposure

For c-f:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

Table 4.27 Relative expression level of glutathione-s-transferase in hepatopancreas of *P. monodon*

Time of Exposure (h)	Chlorpyrifos Concentration (µg/l) (N=3)				
	0	**0.0681**	**6.81**	**13.62**	**27.24**
0	0.85±0.34	*NA**	*NA**	*NA**	*NA**
12	0.59±0.31	0.27±0.38	0.74±0.16	0.57±0.44	0.65±0.27
24	0.58±0.51	0.89±0.58	0.59±0.35	0.61±0.12	*NA***
48	0.35±0.42	0.53±0.13	0.42±0.28	0.51±0.30	*NA***
72	0.64±0.41	0.33±0.32	0.10±0.03	0.51±0.33	*NA***
96	0.61±0.29	0.18±0.21	0.27±0.22	0.28±0.41	*NA***

Remark: * data was not available according to 0 h exposure was specifically set for control group.

** data was not available according to mortality of shrimp exposed to 27.24 µg/l was 100% within 12 h.

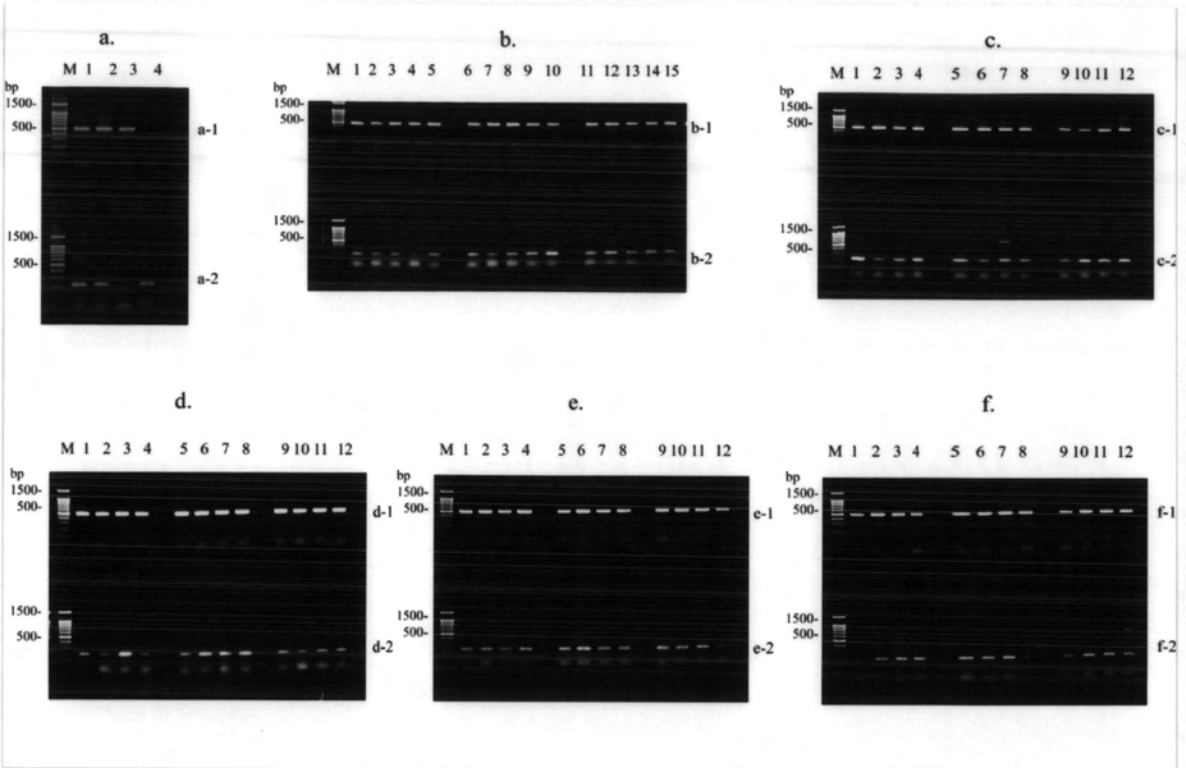


Figure 4.65 RT-PCR of OPA18G-600-4-1 (Ubiquitin-like-7) in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0, 12, 24, 48, 72 and, 96 h (a-2- f-2). Elongation factor 1 alpha from the same template was used as internal control (a-1 – f-1).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 6, 11 = Control

Lane 2, 7, 12 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 8, 13 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 9, 14 = 13.62 µg/l chlorpyrifos exposure

Lane 5, 10, 15 = 27.24 µg/l chlorpyrifos exposure

For c-f:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

Table 4.28 Relative expression level of OPA18G-600-4-1 (Ubiquitin-like-7) in hepatopancreas of *P. monodon*

Time of Exposure (h)	Chlorpyrifos Concentration (µg/l) (N=3)				
	0	**0.0681**	**6.81**	**13.62**	**27.24**
0	0.79±0.05	*NA**	*NA**	*NA**	*NA**
12	0.54±0.12	0.34±0.22	0.30±0.07	0.44±0.33	0.65±0.64
24	0.88±0.66	0.87±1.20	0.85±0.15	0.83±0.30	*NA***
48	0.24±0.02	0.33±0.36	0.59±0.38	0.29±0.28	*NA***
72	0.51±0.29	0.51±0.26	0.40±0.22	0.27±0.18	*NA***
96	0.19±0.33	0.38±0.08	0.56±0.04	0.39±0.35	*NA***

Remark: * data was not available according to 0 h exposure was specifically set for control group.

** data was not available according to mortality of shrimp exposed to 27.24 µg/l was 100% within 12 h.

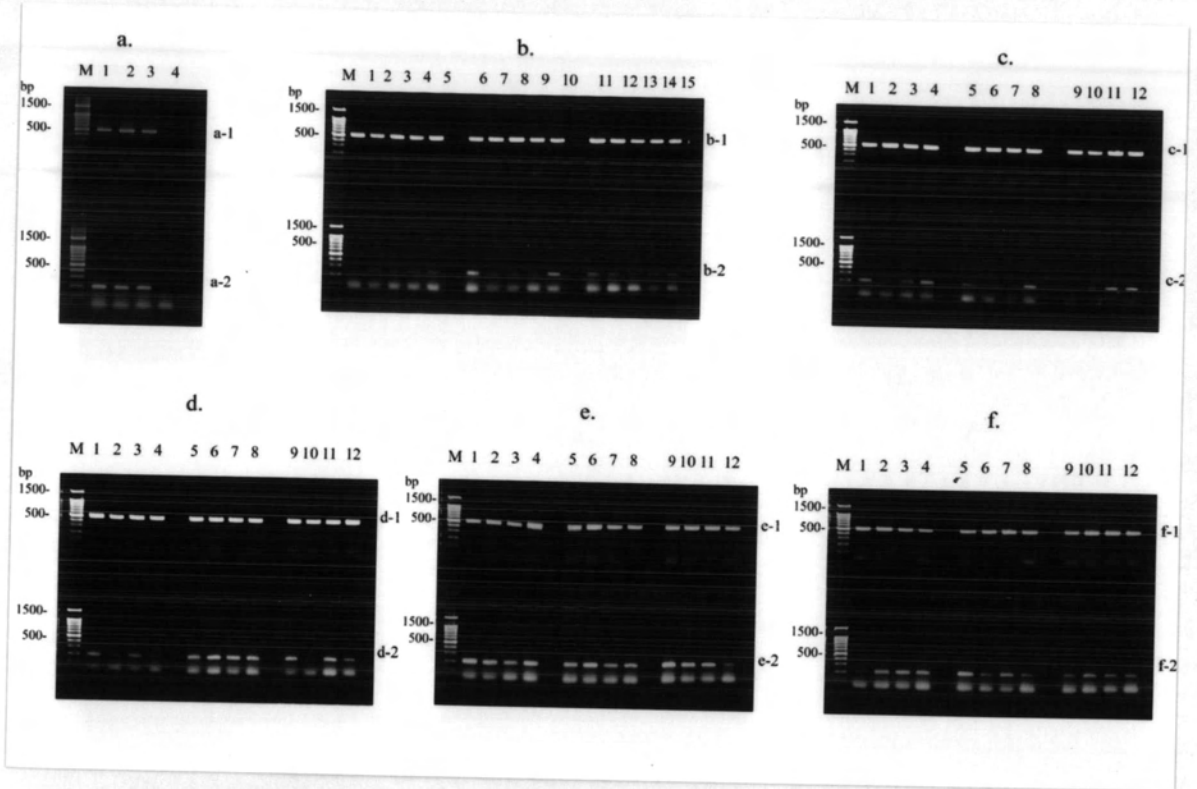


Figure 4.66 RT-PCR of OPA01G-415-1 (Leucine zipper protein 5) in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0, 12, 24, 48, 72 and, 96 h (a-2- f-2). Elongation factor 1 alpha from the same template was used as internal control (a-1 – f-1).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 6, 11 = Control

Lane 2, 7, 12 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 8, 13 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 9, 14 = 13.62 µg/l chlorpyrifos exposure

Lane 5, 10, 15 = 27.24 µg/l chlorpyrifos exposure

For c-f:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

Table 4.29 Relative expression level of OPA01G-415-1 (Leucine zipper protein 5) in hepatopancreas of *P. monodon*

Time of Exposure (h)	Chlorpyrifos Concentration (µg/l) (N=3)				
	0	**0.0681**	**6.81**	**13.62**	**27.24**
0	1.05±0.07	*NA**	*NA**	*NA**	*NA**
12	0.26±0.19	0.13±0.10	0.16±0.09	0.18±0.10	0.18±0.10
24	0.13±0.12	0.07±0.02	0.13±0.11	0.23±0.01	*NA***
48	0.35±0.12	0.21±0.34	0.34±0.17	0.26±0.28	*NA***
72	0.85±0.32	0.71±0.12	0.46±0.11	0.40±0.19	*NA***
96	0.45±0.48	0.38±0.18	0.56±0.34	0.53±0.50	*NA***

Remark: * data was not available according to 0 h exposure was specifically set for control group.

** data was not available according to mortality of shrimp exposed to 27.24 µg/l was 100% within 12 h.

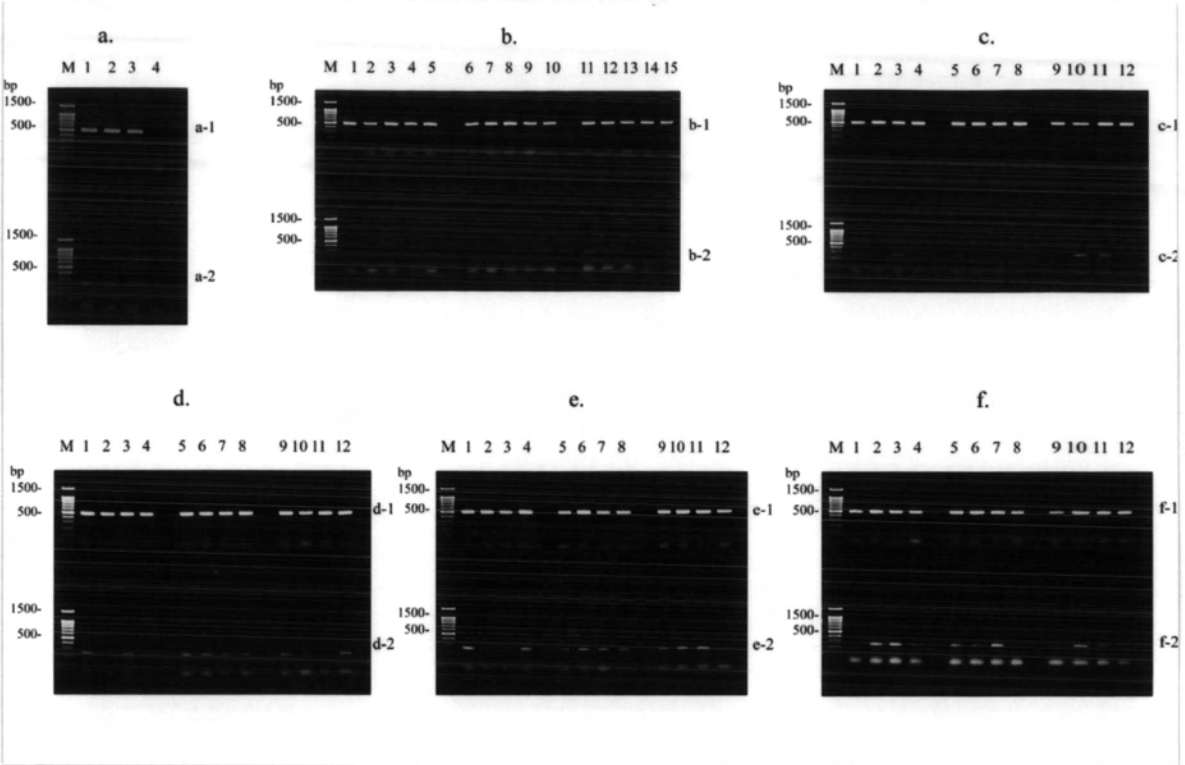


Figure 4.67 RT-PCR of OPA02G-450-2 (sequence of unknown gene) in hepatopancreas of *P. monodon* exposed to chlorpyrifos for 0, 12, 24, 48, 72 and, 96 h (a-2- f-2). Elongation factor 1 alpha from the same template was used as internal control (a-1 – f-1).

Lane M = DNA ladder

For a:

Lane 1, 2, 3 = Control

Lane 4 = Negative control for PCR reaction

For b:

Lane 1, 6, 11 = Control

Lane 2, 7, 12 = 0.0681 µg/l chlorpyrifos exposure

Lane 3, 8, 13 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 9, 14 = 13.62 µg/l chlorpyrifos exposure

Lane 5, 10, 15 = 27.24 µg/l chlorpyrifos exposure

For c-f:

Lane 1, 5, 9 = Control

Lane 2, 6, 10 = 0.0681µg/l chlorpyrifos exposure

Lane 3, 7, 11 = 6.81 µg/l chlorpyrifos exposure

Lane 4, 8, 12 = 13.62 µg/l chlorpyrifos exposure

Table 4.30 Relative expression level of OPA02G-450-2 (sequence of unknown gene) in hepatopancreas of *P. monodon*

Time of Exposure (h)	Chlorpyrifos Concentration (µg/l) (N=3)				
	0	**0.0681**	**6.81**	**13.62**	**27.24**
0	0.19±0.02	*NA**	*NA**	*NA**	*NA**
12	0.10±0.10	0.09±0.08	0.02±0.03	0.03±0.05	0.06±0.10
24	0.03±0.02	0.05±0.07	0.02±0.02	0.00±0.01	*NA***
48	0.09±0.02	0.05±0.04	0.03±0.05	0.05±0.05	*NA***
72	0.28±0.15	0.20±0.11	0.24±0.18	0.13±+0.12	*NA***
96	0.07±0.08	0.22±0.20	0.36±0.31	0.02±0.03	*NA***

Remark: * data was not available according to 0 h exposure was specifically set for control group.

** data was not available according to mortality of shrimp exposed to 27.24 µg/l was 100% within 12 h.