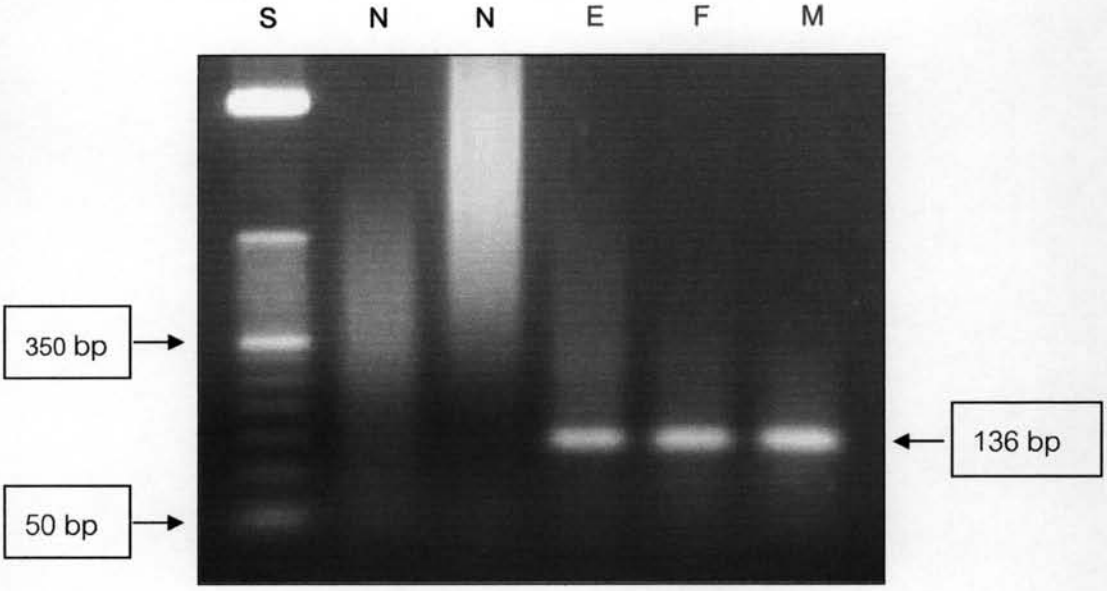
CHAPTER IV
RESULTS

In this study, *Wolbachia* F-supergroup from the naturally infected *C. hemipterus* was microinjected into newly emerged twenty-four-hour-old adult virgin females of naturally uninfected *Ae. aegypti* between the posterior pronotum and the sternopleuron region to genetic modification for suppression and replacement of the naturally population.

1. *Wolbachia* DNA extraction

Wolbachia DNA was extracted from the *C. hemipterus* eggs, adult males and females by using the modified salt procedure. DNA was screened for *Wolbachia* infection by using the *Wolbachia* specific 16S rDNA primers set (INTF2 and INTR2). We successful to amplify the *Wolbachia* specific 16S rDNA gene by PCR, the PCR product was performed an approximately 136 bp fragments as shown in Figure 4.1.



Lane	S	50 bp standard marker
Lane	N	Negative control (uninfected *Ae. aegypti*)
Lane	E, F and M	*Wolbachia* infected egg (E), female (F) and male (M)

Figure 4.1 Agarose gel electrophoresis of PCR amplifications from *Wolbachia* infected *C. hemipterus*

To confirm the *Wolbachia* infection, PCR product was inserted into pGEM-T Easy vector which contains SP6 and T7 RNA polymerase promote sequences and transform into *E. coli* DH5-α competent cell. The plasmid DNA was sequenced. The sequences displayed more than 98% (134/136) identity to *Wolbachia* of *C. hemipterus* reported in the GenBank (DQ399344.1) (Figure 4.2a and b). The sequences indicating that bed bugs used in this study were infected with *Wolbachia*.

>gi|89146855|gb|DQ399344.1| *Wolbachia* endosymbiont of *Cimex hemipterus* 16S ribosomal RNA gene, partial sequence

a.

GAAGGGATAGGGTCGGTTTGGCCGGATTTCACACAGGTGTTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGTAACCCTCATCCTTA
GTTACCATCAGATAATGCTGGGGACTTTAAGGAAACTGCTAGTGATAAACTGGAGGA
AGGTGGGGATGATGTCAAGTCATCATGGCCCTTATGGAGTGGGCTACACACGTGCT
ACAATGGTGGCTACAATGGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAG
CCATCTCAGTTCGGATTGTACTCTGCAACTCGAGTGCATGA**AGTTGGAATCGCTAGT**
AATCGTGGATCAGCATGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCC
GTCACGCCATGGGAATTGGTT

b.

AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAATG
GGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATTGT
ACTCTGCAACTCGAGTACATGA

Figure 4.2 Nucleotide bases of *Wolbachia* endosymbiont of *C. hemipterus*

a. The nucleotide bases of DQ399344.1 in the GenBank of *Wolbachia* endosymbiont of *C. hemipterus* based on 16S ribosomal RNA gene.

b. The nucleotide bases of *Wolbachia* endosymbiont of *C. hemipterus* which extracted from each stage used in this study.

There were two positions of the partial nucleotide sequence of the 16S rDNA gene of Wolbachia used in this study which difference from the previous report in the GenBank. The nucleotide sequence at position 14 has been changed from "T" to "C" and the position 131 has been changed from "A" to "G" (Figure 4.3).

```
>gb|DQ399344.1|  Wolbachia endosymbiont of Cimex hemipterus 16S ribosomal RNA
gene, partial sequence
Length=415

 Score =  241 bits (130),  Expect = 6e-61
 Identities = 134/136 (98%), Gaps = 0/136 (0%)
 Strand=Plus/Plus

Query  1    AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAATGGGC  60
            ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  AGTCATCATGGCCCTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAATGGGC  246

Query  61   TGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATTGTACTCTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  TGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATTGTACTCTG  306

Query  121  CAACTCGAGTACATGA  136
            |||||||||| |||||
Sbjct  307  CAACTCGAGTGCATGA  322
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Figure 4.3 Nucleotides sequences of the 16S rDNA gene from bed bug (*C. hemipterus*) used in this experiment compared with nucleotides sequences of the 16S rDNA gene from bed bug (*C. hemipterus*) reported in GenBank

2. Establishment of *Wolbachia* infected *Ae. aegypti*

The *Wolbachia* was extracted from 5 newly laid bed bug (*C. hemipterus*) eggs and immediately microinjected into 41 newly emerged adult females of naturally uninfected *Ae. aegypti* mosquitoes. 63% (26/41) of injected mosquitoes survived were designated as G_0. 46.1% (12/26) of surviving adults tested for *Wolbachia* transinfection by PCR technique based on *Wolbachia* specific protein (*wsp*)-16S rDNA gene.

3. Establishment of Isofemale lines

The isofemale lines were established by using surviving G_0 mosquitoes adults tested positive for *Wolbachia*. The G_0 mosquitoes were mated with naturally uninfected males to construct infected offspring. The only infected offspring were

chosen to start a new generation. The females from each generation were monitored for transmission of *Wolbachia* by mated with naturally uninfected males and the transmission efficiency of *Wolbachia* was determined by using a specific *wsp*-16S rDNA gene. From this study, transmission efficiency of *Wolbachia* in G_1 (55.55%, n=45), G_2 (40.9%, n=183), G_3 (37.80%, n=111), G_4 (54.71%, n=159), G_5 (28.43%, n=211), G_6 (6.0%, n=200), G_7 (48.1%, n=81), G_8 (21.7%, n=258) and G_9 (4.2%, n=189) as shown in Table 4.1 and Figure 4.4.

Table 4.1 Transmission efficiency of transinfected *Ae. aegypti* mosquitoes (n = number of mosquito tested)

Generation	Eggs		N	% Transinfection
	Total	Hatch		
G_0	-	-	26	46.1
G_1	356	314 (88.2%)	45	55.55
G_2	454	369 (81.2%)	183	40.9
G_3	375	300 (80.0%)	111	37.8
G_4	535	423 (79.0%)	159	54.71
G_5	711	533 (75.0%)	211	28.43
G_6	631	479 (75.9%)	200	6.0
G_7	271	166 (61.2%)	81	48.1
G_8	716	616 (86.0%)	258	21.7
G_9	433	382 (88.2%)	189	4.2
G_{10}	130	124 (95.3%)	Analyzing	Analyzing

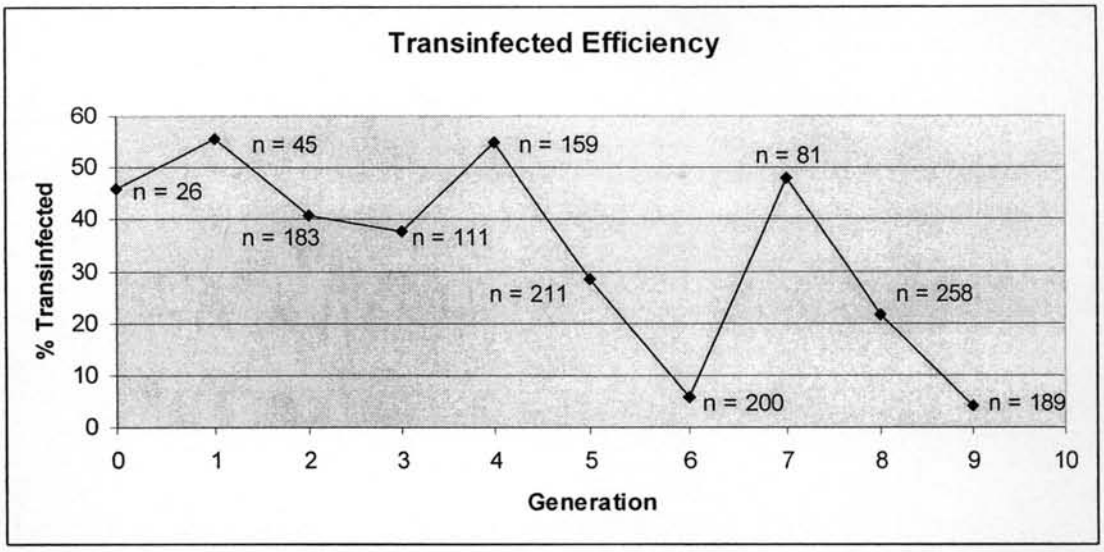


Figure 4.4 Graph demonstrates transmission efficiency of transinfected *Ae. aegypti*

4. Demonstration of the *Wolbachia* establishment

To confirmed the establishment of *Wolbachia* F-supergroup within transinfected *Ae. aegypti* populations. DNA from each generation was amplified, re-amplified, cloned and sequenced. In addition, to confirmed the *Ae. aegypti* DNA was extracted properly, the extracted mosquito DNA was amplified for the present of the Def A gene of the mosquito.

4.1 *Wolbachia* Transinfected *Ae. aegypti*

Isofemale lines DNA from all generation was extracted. DNA was screened for *Wolbachia* transinfection by using the *Wolbachia* specific 16S rDNA primers set (INTF2 and INTR2). We successful to amplify by using the PCR method described previously, the expected PCR product of approximately 136 bp was demonstrated by gel electrophoresis.

4.2 DNA sequencing

4.2.1 Colony selection

The *Wolbachia* transinfected line from each generation that *Wolbachia* positive by PCR technique were cloned and sequenced. In this study,

the PCR product size approximately 136 bp were prepared for DNA sequencing by ligated into the pGEM®-T Easy vector and transformed into *E. coli* DH5α competent cell. We successfully to insert the interest gene into the pGEM®-T Easy vector and transformed into *E. coli* DH5α competent cell. The white colonies from LB plate and ampicillin plus X-Gal and IPTG as Figure 4.5 were chosen to culture and plasmid extraction. And then, the plasmid was checked size of the *Wolbachia* DNA by PCR technique.

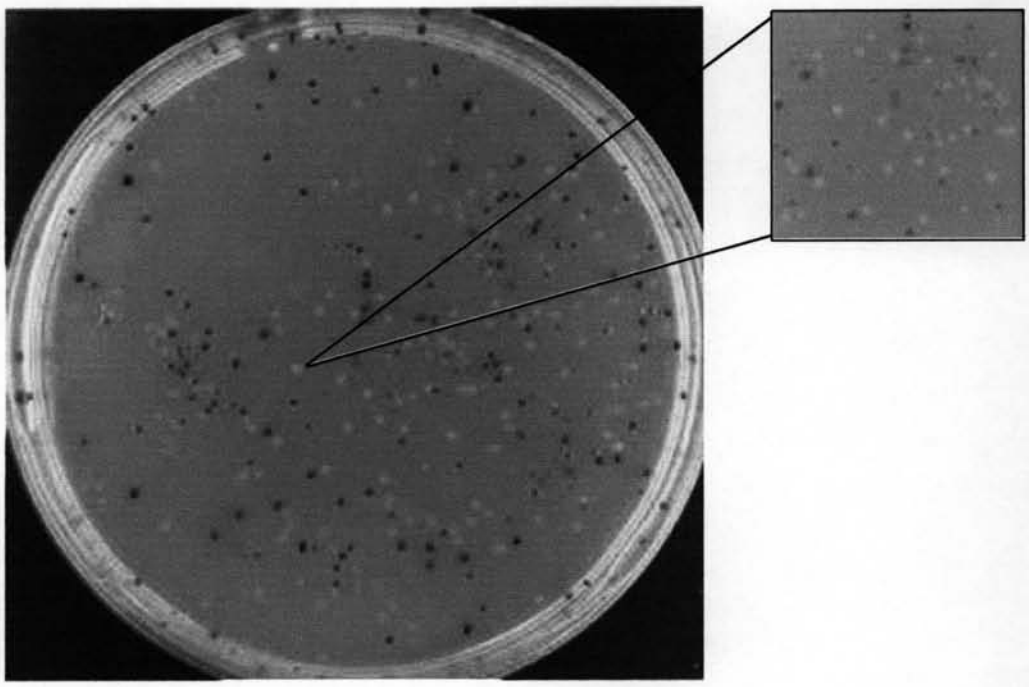
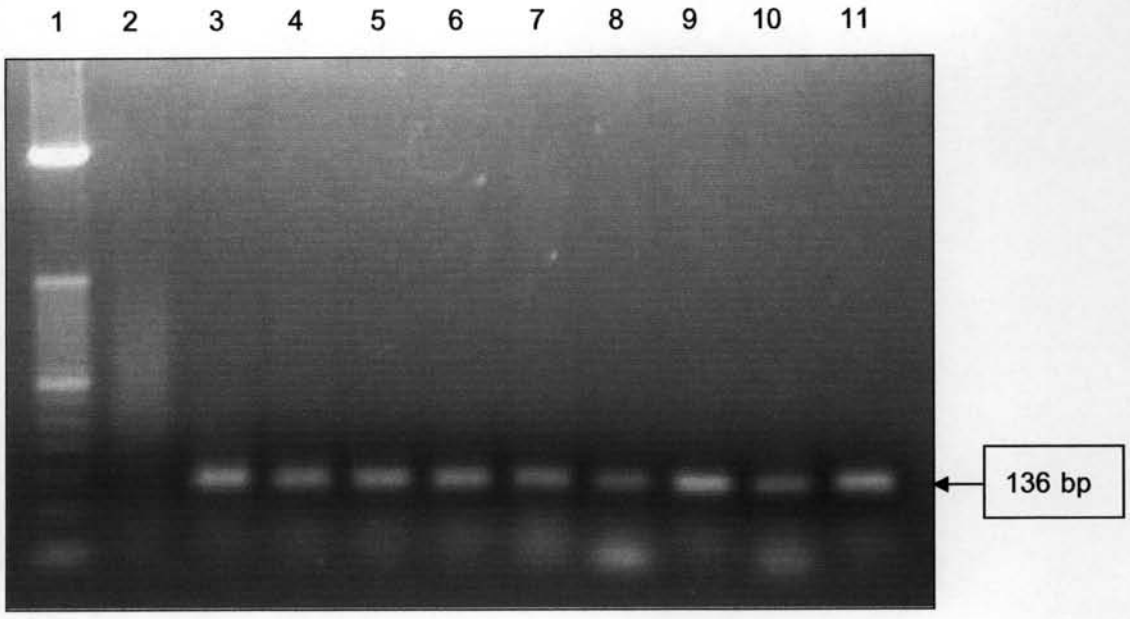


Figure 4.5 Colony screening from LB plate using ampicillin plus X-Gal and IPTG. The white colonies were selected for purified and sequenced.

4.2.2 Demonstration of inserted DNA

The plasmid was extracted by using the Fast Plasmid ™Mini prep. The inserted DNA was detected by PCR technique and resolved by 2% (wt / vol) agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light by using Gel Photodocumentation System (Bio-rad) compare with 50 bp of standard

marker. The product size of insert interest gene is an approximately 136 bp as show in Figure 4.6.



Lane 1	50 bp standard marker
Lane 2	Negative control
Lane 3	Positive control (*Wolbachia* from infected bed bug)
Lane 4-1	Samples from transformed colonies

Figure 4.6 PCR of 16S rDNA of *Wolbachia* DNA from cloned plasmids inserted with the 16S rDNA gene amplified from transinfected mosquitoes

4.1.3 DNA sequencing

The sequence of the 16S rDNA gene amplified from infected mosquitoes was shown more than 98% identity to *Wolbachia* endosymbiont of *C. hemipterus* with **DQ399344.1** in the GenBank. In addition, the DNA base occurred at the same sites and the same bases under different generations but slightly alteration was observed (Figure 4.7 and Figure 4.8). The changing in nucleotide sequence caused amino acid alteration at the position 81 of the 16S rDNA of *Wolbachia* microinjected in mosquitoes, "M (Methionine)" was changed to "V (Valine)" (Figure 4.9).

>DQ399344.1: *Wolbachia* endosymbiont of *Cimex hemipterus* 16S ribosomal RNA gene

GAAGGGATAGGGTCGGTTTGGCCGGATTTCACACAGGTGTTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGTAACCCTCATCCTTA
GTTACCATCAGATAATGCTGGGGACTTTAAGGAAACTGCTAGTGATAAACTGGAGGA
AGGTGGGGATGATGTCAAGTCATCATGGCCCTTATGGAGTGGGCTACACACGTGCT
ACAATGGTGGCTACAATGGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAG
CCATCTCAGTTCGGATTGTACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGT
AATCGTGGATCAGCATGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCC
GTCACGCCATGGGAATTGGTT

Bed bug egg (BB)

AGTCATCATGGCCCTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAAT
GGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATT
GTACTCTGCAACTCGAGTGCATGA

Generation 0

AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAATG
GGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATTGT
ACTCTGCAACTCGAGTACATGA

Generation 1

AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAGT
GGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATT
GTACTCTGCAACTCGAGTACATGA

Generation 2

AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAGT
GGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATT
GTACTCTGCAACTCGAGTACATGA

Generation 3

AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAGT
GGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATT
GTACTCTGCAACTCGAGTACATGA

Generation 4

AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAGT
GGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATT
GTACTCTGCAACTCGAGTACATGA

Generation 5

AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAGT
GGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATT
GTACTCTGCAACTCGAGTACATGA

Generation 6

AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAGT
GGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATT
GTACTCTGCAACTCGAGTACATGA

Generation 7

AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAGT
GGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATT
GTACTCTGCAACTCGAGTACATGA

Generation 8

AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAGT
GGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATT
GTACTCTGCAACTCGAGTACATGA

Generation 9

AGTCATCATGGCCTTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTACAGT
GGGCTGCAAAGTCGCGAGGCTGAGCTAATCTCTTAAAAGCCATCTCAGTTCGGATT
GTACTCTGCAACTCGAGTACATGA

Figure 4.7 Sequences of the 16S rDNA of *Wolbachia* infected mosquitoes

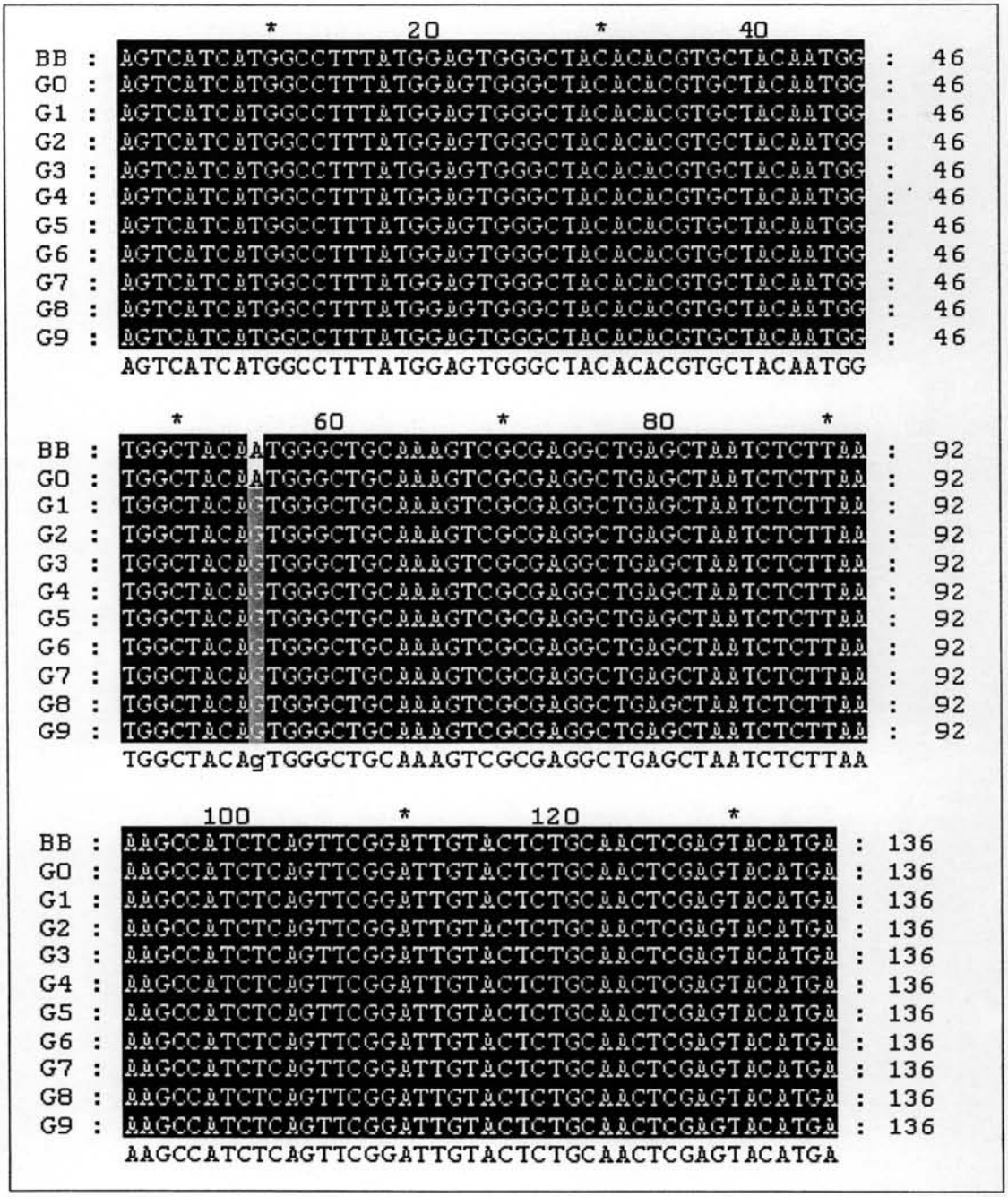


Figure 4.8 Comparison of the nucleotide bases from each generation with the nucleotide bases from transinfected.

BL = Bed bug from microinjection, G = Transinfected from each generation

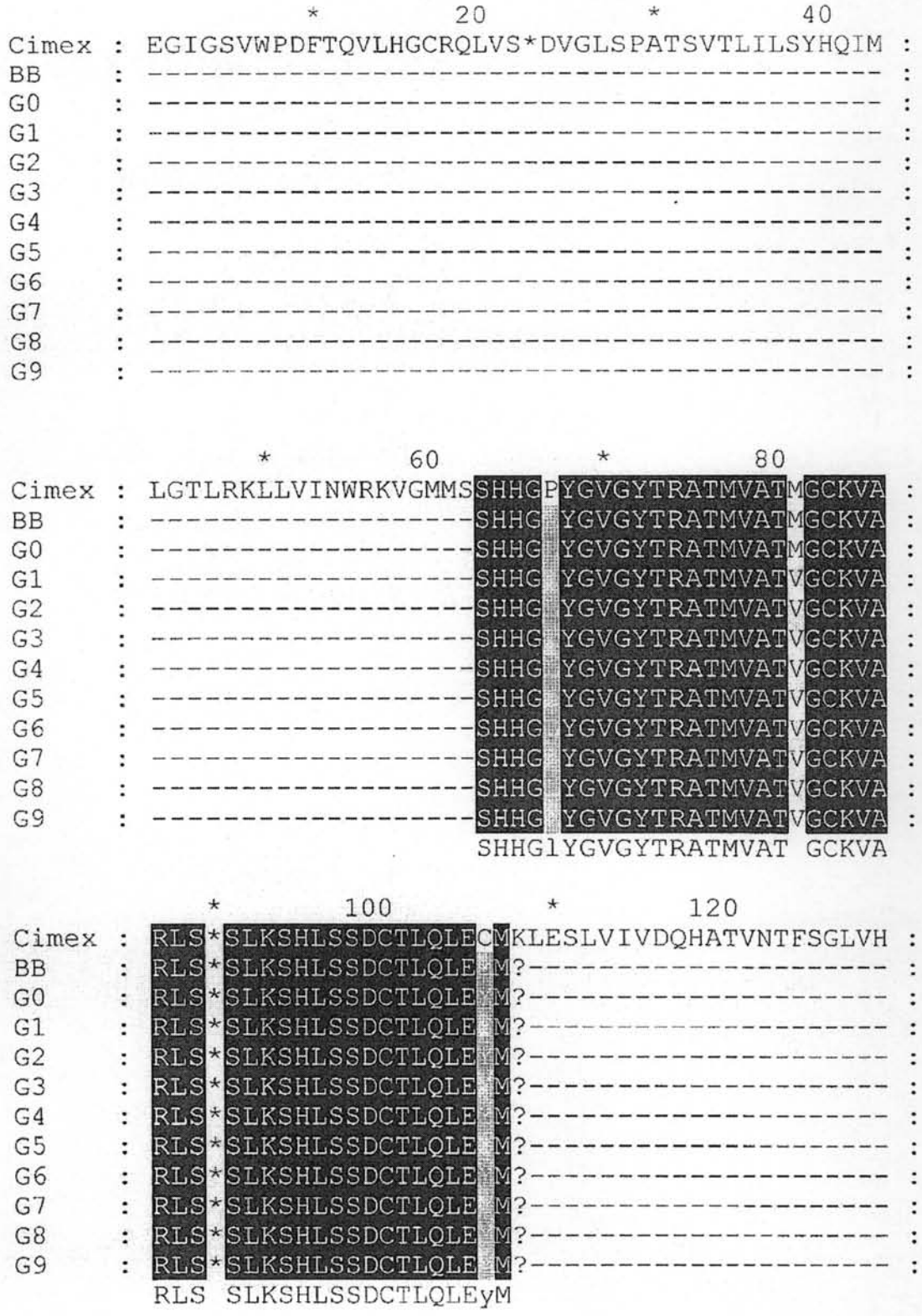


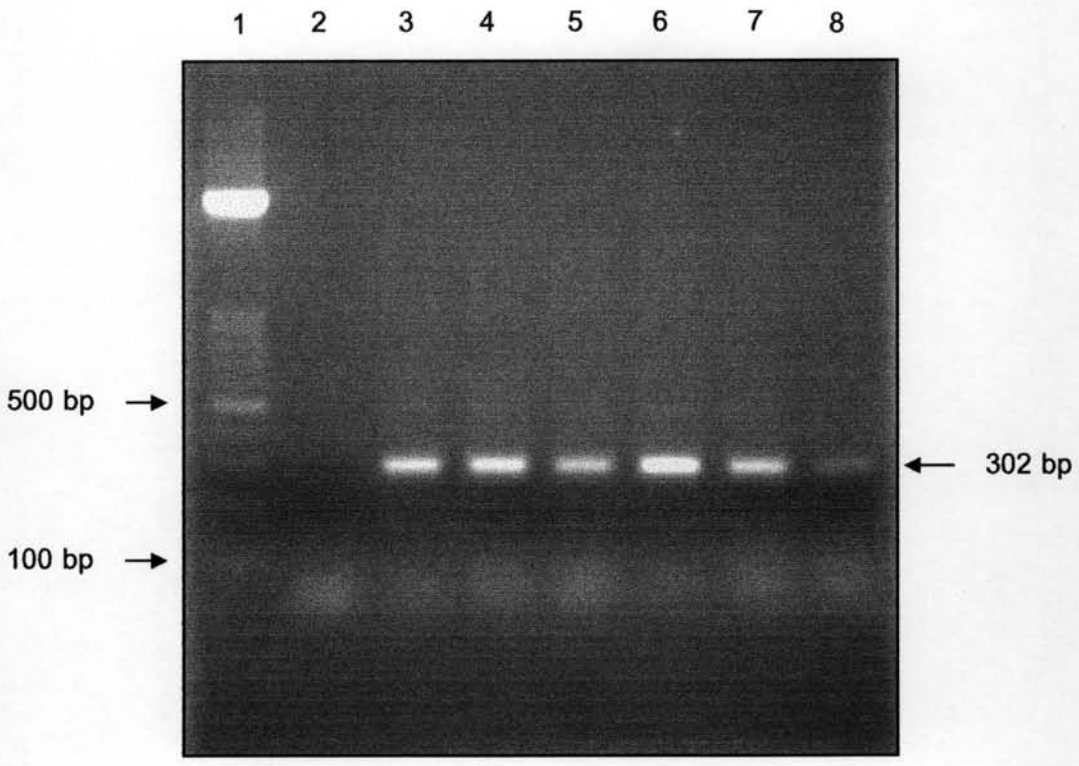
Figure 4.9 Comparison of the amino acid from each generation with the amino acid from *C. hemipterus* and bed bug from initially microinjection.

Cimex = *C. hemipterus*, BB = *C. hemiterus* from initially microinjection and

G = Transinfected from each generation

5. *Ae. aegypti* DNA

In order to determine that the DNA extraction method was performed properly, the extracted mosquito DNA was tested for presence of the defensin gene by using the primers specific to defensin A (Def-F and Def-R). We were successful to amplify the Def A gene by PCR method, and the PCR product was then inserted into pGEM-T Easy cloning vector which contains T7 RNA polymerase promote sequences as a previous described on 4.2.1, 4.2.2 and 4.2.3 consequently. The PCR product and plasmid DNA was performed an approximately 302 bp fragments as shown in Figure 4.10.



Lane 1	100 bp standard marker
Lane 2	Negative control
Lane 3	Positive control (naturally *Ae. aegypti*)
Lane 4–8	*Ae. aegypti* infected DNA

Figure 4.10 PCR of the Def-A gene of *Ae. aegypti* mosquito, the expected PCR products were approximately 302 bp.

The sequences of the Def A gene were shown more than 99% identity to the Def A of *Ae. aegypti* reported in the GenBank (**AF 387487.1)** as shown in Figure 4.11.

>AF387487 *Aedes aegypti* defensin A (DefA) mRNA, complete cds Length=478

a.

NNNNNCTGCCATGCTCCGGCCGCCATGGCGGCCGCGGGATTCGATTGACGCACAC
CTTCTTGGAGTTGCAGTAGCCTCCCCGATTGCCACGGGCAATGCAATGAGCAGCAC
AAGCACTATCACCAACGCCGAATCCGCTCAGCAGATCACAGGTGGCCCGTTTCAGG
CGGAAGTTCTCCACGGCGGCCTGATAGGTTTCCTCCGGCAGTTCATCGACTGTGAT
AAAGAGAAATTCTTAATAGAAAGATTTCTTCGATTACAAATATGTAAATTTACTTACAA
AGAGAGTTGGCAAAAGGGCGAGCTTCGTCCGCCAGCACCGGTTCCTGTGGGTAAG
CACCAGTGATA**ATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAG**
AGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCT
TGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC
ACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGA
GCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGT
CGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATT
GGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCG
GCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGG
ATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAA
AAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAA
AAATCGACGCTCAAGTCAAAGGGGGCGAAACCCGACAGGACTATAAAGATACCAGG
CGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACC
GGATACCTGTCCGCCTTTTCTCCCCTTTCGGGAAGCGTGGCGCTTTCTCATAGCTCA
CGCTGTAGGTATCTCAGTTCGCTTTGAGTCGTTCGCCTCCAGCTGGGCTTGTGTTGC
AAGAAACCCCCGTTTAAGCGGAACGGCGTGGGGCCTTATCGGGGTAACATAATTCC
NNTCTTGAAGGTCCACACCC

b.

GACGCACACCTTCTTGGAGTTGCAGTAGCCTCCCCGATTGCCACGGGCAATGCAAT
GAGCAGCACAAGCACTATCACCAACGCCGAATCCGCTCAGCAGATCACAGGTGGCC
CGTTTCAGGCGGAAGTTCTCCACGGCGGCCTGATAGGTTTCCTCCGGCAGTTCATC
GACTGTGATAAAGAGAAATTCTTAATAGAAAGATTTCTTCGATTACAAATATGTAAATT
TACTTACAAAGAGAGTTGGCAAAAGGGCGAGCTTCGTCCGCCAGCACCGGTTCCTG
TGGGTAAGCACCAGTGATA

Figure 4.11 Nucleotide bases of the Def A gene of *Ae. aegypti* mosquito used in this study

a. The nucleotide bases of AF 387487.1: *Ae. aegypti* defensin A (DefA) mRNA, complete cds Length=478

b. The nucleotide bases of *Ae. aegypti* from novel hosts population from each generation for negative control.

The nucleotide bases of the Def A of *Ae. aegypti* used in this study have been different from the *Ae. aegypti* defensin A (Def-A) in the GenBank, at the 28 regions from "G" to "A" as show in Figure 4.12.

```
>gb|AF387487.1|AF387487 Aedes aegypti defensin A (DefA) mRNA, complete cds
Length=478


 Score =  309 bits (167),  Expect = 4e-81
 Identities = 169/170 (99%), Gaps = 0/170 (0%)
 Strand=Plus/Minus

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Sbjct  236  GCGGAAGTTCTCCACGGCGGCCTGATAGGTTTCCTCCGGCAGTTCATCGA  187
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Figure 4.12 Nucleotide bases of the Def A of *Ae. aegypti* used in this study compared with the Def A of *Ae. aegypti* reported in the GenBank.

6. CI Expression in Transinfected *Ae. aegypti*

To determine the capability for CI expression and the effect of male and female mating on CI expression, test crosses were established between transinfected *Ae. aegypti* in G_2 and G_4 with naturally uninfected *Ae. aegypti,* the results are shown in Table 4.2a and b, which composed of single-pair copulations with one male and one female. We found that crosses between transinfected males and uninfected females produced 18.86% and 30.6% (mean = 24.73 ± 5.87 %) of egg hatch, compare with naturally uninfected crosses (77.37% and 87.57%, mean = 82.47 ± 5.10 %), which was significantly lower than for naturally uninfected crosses [P = 0.008]. In the other hand, transinfected crosses (77.33% and 73.30%, mean = 75.31 ± 2.01%) and transinfected female crosses with uninfected male (81.20% and 79.00%, mean = 80.10 ± 1.10 %) did not give significant in the mean hatch rate [P = 0.120] as shown in Table 4.2c.

Table 4.2 *Wolbachia* bacteria induced cytoplasmic incompatibility of the transinfected *Ae. aegypti* mosquitoes.

a. Tests crosses from G_2

b. Tests crosses from G_4

c. Mean T-test by SPSS version 11.5 program

a.

Cross (female x male)	Total No. of eggs count	Total No. of eggs hatch	% egg hatch
a.Transinfected x Transinfected	375	290	77.33
b.Transinfected x Uninfected	454	369	81.20
c.Uninfected x Transinfected	485	91	18.86
d.Uninfected x Uninfected	725	561	77.37

b.

Cross (female x male)	Total No. of eggs count	Total No. of eggs hatch	% egg hatch
a.Transinfected x Transinfected	405	290	77.30
b.Transinfected x Uninfected	535	423	79.00
c.Uninfected x Transinfected	490	91	30.60
d.Uninfected x Uninfected	837	733	87.57

c.

Cross (female x male)	Total No. of eggs count	Mean % egg hatch ± SE	Comparison	*P* value
a.Transinf x Transinf	780	75.31 ± 2.01		
b.Transinf x Uninf	989	80.10 ± 1.10	a, b	0.120
c.Uninf x Transinf	975	24.73 ± 5.87	c, d	0.008
d.Uninf x Uninf	1,562	82.47 ± 5.10		

7. The *Wolbachia* Density of Transinfected *Ae. aegypti*

To investigates the correlation between *Wolbachia* density and *Wolbachia* transmission in the novel hosts and CI expression. The *Wolbachia* densities were measured by using quantitative real-time PCR based on the SYBR green I. The number of Wolbachia copy was compared with the host cell copy number.

7.1 Analysis of *Wolbachia* density

7.1.1 Standard curve of *Wolbachia*

The slope of the standard curve indicates how quickly DNA concentration can be expected to increase with the amplification cycles. The standard curve is also referred to as the "efficiency" of the curve. A perfect amplification

reaction would produce a standard curve with an efficiency of 2.00 (range =1.80-2.20). In this study, reactions often have a lower efficiency of 1.896, as show in Figure 4.13.

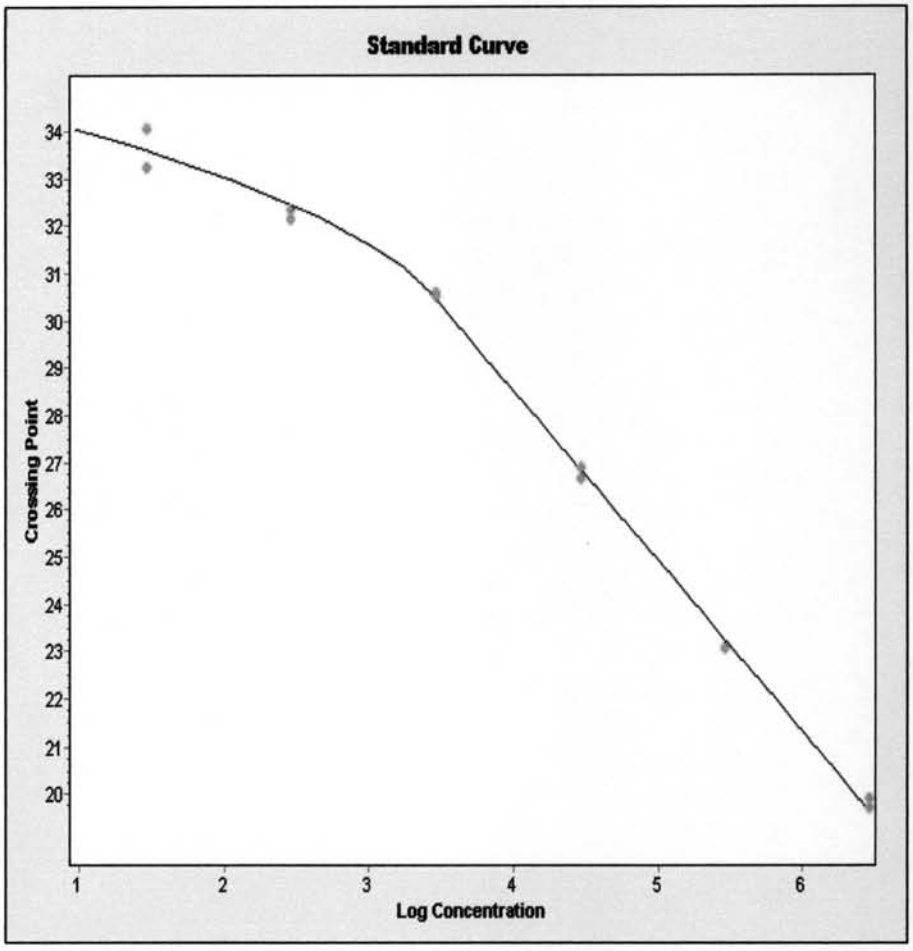

Figure 4.13 Standard curve of *Wolbachia* density.

7.1.2 *Wolbachia* copy Number

DNA from each generation that PCR positive were used. Replicate DNA was measured the copy number of *Wolbachia* transinfection.

7.1.2.1 Melting Temperature (Tm)

In this study, the specific primer was using to detect *Wolbachia* transinfected from *Ae. aegypti* mosquitoes using the LightCycler PCR assay combined with melting curve analysis of the PCR product. The Tm of *Wolbachia*

has found the melting peak curve for the standard and sample at approximately 86°C of the specific product and at approximately 82 °C of the non specific melting peak in negative product only. And then, the sized of the PCR product, 136 bp were separated by using 2% agarose gel electrophoresis compare with 50 or 100 bp standard marker.

7.1.2.2 Sample concentration

The standard curve was used to measure the concentration of sample. The concentration of each sample was represented in the Table 4.3.

7.2 Density of *Ae. aegypti*

7.2.1 Standard curve of *Ae. aegypti*

The slope of the standard curve indicates how quickly DNA concentration can be expected to increase with the amplification cycles. The standard curve is also referred to as the "efficiency" of the curve. A perfect amplification reaction would produce a standard curve with an efficiency of 2.00 (range =1.80-2.20). In this study, reactions often have a lower efficiency of 1.955, as show in Figure 4.14.

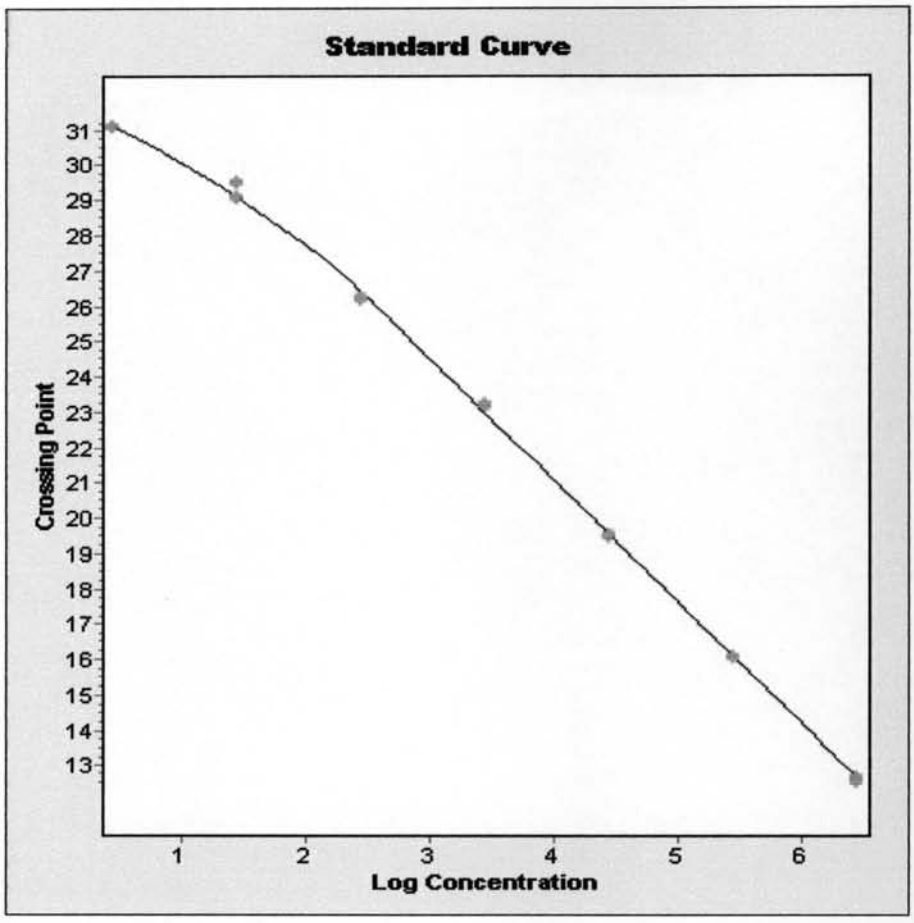

Figure 4.14 Standard curve of *Aedes aegypti* density.

7.2.2 *Ae. aegypti* copy Number

DNA from each generation that PCR positive and were used to established offspring as same on above. Replicate DNA was measured the copy number of *Ae. aegypti* hosts cell.

7.2.2.1 Melting Temperature (Tm)

In this study, the specific primer was using to detect the hosts cell of *Ae. aegypti* mosquitoes using the LightCycler PCR assay combined with melting curve analysis of the PCR product. The Tm of *Ae .aegypti* has found the melting peak curve for the standard and sample at approximately 89°C of the specific product and at approximately 82 °C of the non specific melting peak in negative product only. The PCR products, 302 bp were separated by using 2% agarose gel electrophoresis compare with the 50 or 100 bp standard marker.

7.2.2.2 Sample concentration

The standard curve was used to measurement the concentration of sample. The *Wolbachia* density of each generation was represented as in the Table 4.3 and Figure 4.15. The *Wolbachia* copy number from each generation was measured by using quantitative real-time PCR. The *Wolbachia* density in the host cell was calculated between the *Wolbachia* copy number and the *Ae. aegypti* copy number. From this study, *Wolbachia* copy number from microinject = 9.32×10^4. The *Wolbachia* copy number in $G_0 = 30.5 \times 10^{-1}$, $G_1 = 28.0 \times 10^{-1}$, $G_2 = 5.5 \times 10^{-1}$, $G_3 = 100.5 \times 10^{-1}$, $G_4 = 26.9 \times 10^{-1}$, $G_5 = 22.7 \times 10^{-1}$, $G_6 = 53.3 \times 10^{-1}$, $G_7 = 42.1 \times 10^{-1}$, $G_8 = 3.69 \times 10^{-1}$ and $G_9 = 35.6 \times 10^{-1}$.

Table 4.3 The *Wolbachia* density of the host cell in each generation.

Generation	*Wolbachia* copy number	*Ae. aegypti* copy number	*Wolbachia* density in host cell
Microinject	9.32×10^4	-	-
G_0	4.31×10^4	1.41×10^4	30.5×10^{-1}
G_1	7.33×10^4	2.00×10^4	28.0×10^{-1}
G_2	1.43×10^4	2.57×10^4	5.5×10^{-1}
G_3	17.7×10^4	1.76×10^4	100.5×10^{-1}
G_4	5.66×10^4	2.10×10^4	26.9×10^{-1}
G_5	4.48×10^4	1.97×10^4	22.7×10^{-1}
G_6	12.10×10^4	2.27×10^4	53.3×10^{-1}
G_7	15.1×10^4	3.58×10^4	42.1×10^{-1}
G_8	0.801×10^4	2.17×10^4	3.69×10^{-1}
G_9	6.88×10^4	1.93×10^4	35.6×10^{-1}

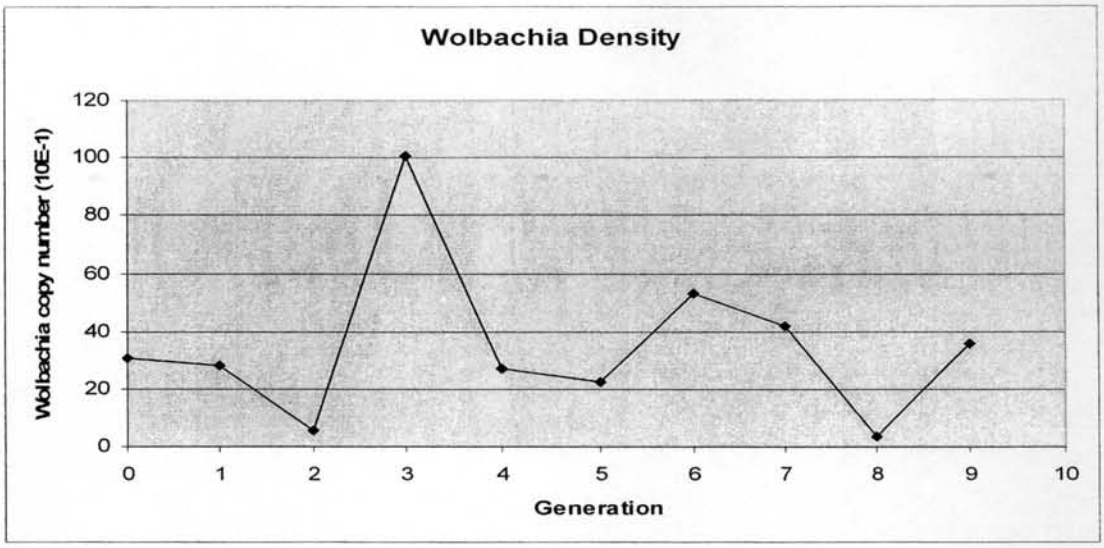

Figure 4.15 Correlation of the *Wolbachia* copy number in each generation