CHARACTERISATION OF *AEDES AEGYPTI* GLUTATHIONE *S*- TRANSFERASE ENZYME FAMILY

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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October 2005

DECLARATION

This work has not previously been accepted in substance for any degree and is not being currently submitted in candidature for any degree.

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DEDICATION

To my parents, Sak and Srida Promtet, My husband, Krisadakorn Lumjuan and my son, Natchanon Lumjuan with my deep appreciation for their patience, encouragement and love.

ABSTRACT

Glutathione S-transferases (GSTs) play a central role in the detoxification of xenobiotic compounds including insecticides. Overexpression of GST is an important mechanism of insecticide resistance. The aim of this study is to investigate GST-based resistance to DDT in *Aedes aegypti* from Thailand. Putative GST genes were retrieved from the *Aedes aegypti* genome and the gene sequences were confirmed.

Twenty seven GST genes were identified in *Aedes aegypti.* There were classified into at least 6 classes and their relationship to *Anopheles gambiae* GSTs investigated using phylogenetic analysis. Thirteen GSTs were further characterised; two were classified as Delta, two as Epsilon, one as Sigma, two as Theta class and three were unclassified. Both *GSTd1* and *GSTs1* are alternatively spliced.

The expression of four GST transcripts, *GSTe2*, *GSTe4*, *GSTu3* and *GSTt1* was quantified. Only *GSTe2* was up regulated in the DDT/permethrin resistant strain. Recombinant GSTE2-2 showed DDT dehydrochlorinase activity toward DDT. The expression of *GSTe4*, *GSTu3* and *GSTt1* were not correlated with insecticide resistance. GSTu3 has been previously studied and implicated in DDT/permethrin resistance in *Aedes aegypti* from South America [Grant and Hammock (1992) Mol Gen Genetics 234: 169-176]. However, this gene was not over expressed in the resistant strain from Thailand. Recombinant GSTU3-3 was not able to metabolise DDT.

Recombinant GSTE2-2 and GSTU3-3 showed an affinity to bind hematin, suggesting these enzymes act as binding protein, besides the enzymatic properties. The binding of hematin by GST may protect mosquitoes against heme toxicity during blood feeding.

PUBLICATION IN SUPPORT OF THIS THESIS

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LIST OF ABBREVIATIONS

Ah	Aryl hydrocarbon
BAC	Bacterial Artificial Chromosome
bp	Base pair
cDNA	Complementary Deoxyribonucleic acid
CDNB	1-choro-2, 4-dinitrobenzene
CHP	Cumene Hydroperoxide
CSPD	Disodium 3-(4-methyoxy-spirofl, 2-dioxetane-3,2'-(5-chloro) tricyclo[3.3.1.1] decan)4-yl) phenyl phosphate
DCNB	1, 2-dichloro-4-nitrobenzene
GSH	Reduced glutathione
GST	Glutathione *S*-transferase
DDE	1,1-dichloro-2,2-*bis*(p-chlorophenyl)ethylene
DDT	1,1,1-trichloro-2,2-*bis*(p-chlorophenyl)ethylene
dNTPs	2'-deoxyribonucleotide 5'-triphosphate
DTT	Dithriethrol
EDTA	Ethylene diamine tetraacetic acid
EST	Expressed sequence
IPTG	Isoproply-β-D-thiogalactopyranoside
kdr	Knock down resistance
MALDI-TOF	Matrix Assisted Laser Desorption/ Time of Flight
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
SDS-PAGE	Sodium Dodecyl Sulphate –Polyacrylamide Gel Electrophoresis
WGS	Whole Genome Shotgun
X-GAL	5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Chapter 1

GENERAL INTRODUCTION

1.1 *Aedes aegypti*

The mosquito *Aedes aegypti* is the major vector of urban arboviruses including yellow fever and dengue fever. Globally there are an estimated 50 million cases of dengue infection each year with 500,000 cases of dengue haemorrhagic fever (DHF) and over 24,000 deaths (WHO, 2002). Thailand suffers one of the highest rates of dengue and DHF infection in the world. In Thailand, DHF was first reported in 1950 and since then the incidence has fluctuated. There are currently approximately 60000 cases/ year (ranging from 18000 to over 90000 cases during 1985-2004) (http://w3.whosea.org/EN/Section10/section332_1100.htm). Epidemics of DHF have occurred annually depending on the temperature variation. The number of dengue cases increases during the rainy season and drops during the dry season (Nimmannitaya *et al.*, 1969). The increase in cases of DHF has been observed across different age groups but approximately 65% of reported cases in 1998 were observed in 5-14 years age group (Kantachuvessiri, 2002). The impact of DHF is leading to social and economic problems. The illness impacts socially on the patients and their families because of the duration of illness, the period of nursing care, the life disruption, and the psychological effects. The economic impact is caused by the financial cost of treatment, which is related to the economic status of the family, such as the absence from work and the increase in family expenses (Sornmani *et al.*, 1994).

1.2 Vector Control

There is no vaccine to protect against DHF. Therefore, the control of DHF should be directed at the vector, *Ae. aegypti* by focusing on habitat reduction and treatment to eliminate the breeding sites and the use of insecticides. Dichlorodiphenyltrichloroethane (DDT) was introduced globally in the 1950s as the primary means to control *Anopheline* vectors of malaria. The use of DDT for vector control has been prohibited or restricted in many countries because of its toxicity and persistence in the environment (Curtis & Lines, 2000). In Thailand, DDT use is still allowed for disease vector control only (WHO, 1995). However, the final DDT import was in 1993 for use against malaria only. Since 1992, synthetic pyrethroids have been employed for vector control in Thailand due to their low toxicity to mammals and high potency at low levels that quickly control insect populations. However, insecticide resistance to several insecticide classes has now been detected in mosquitoes and in Northern Thailand, populations of *Ae. aegypti* are resistant to both DDT and to pyrethroid insecticides (Somboon *et al.*, 2003).

1.3 Insecticide Resistance

According to WHO (WHO, 1957) resistance is defined as " the developed ability in a strain of insects to tolerate doses of toxicant, which would prove lethal to the majority of individuals in a normal population of the same species". Insecticide resistance can spread through a population very rapidly under strong selection pressure from insecticide use. Insects have developed three main physiological mechanisms by which they can protect themselves from the toxic effect of insecticides. These are cuticular resistance, alteration of target-sites and enhanced detoxification.

1.3.1 Cuticular Resistance

A change in the chemical composition of the insect cuticle can result in a reduction of the penetration of insecticides. This decreases the quantity of

insecticide in the insect. This mechanism has been reported in *Musca domestica* which is resistant to DDT and dieldrin (Plapp & Hoyer, 1968a) and in organophosphate resistant strains of *Culex quinquefasciatus* (Stone & Brown, 1969). However, this is a minor mechanism and accounts for little of the resistance observed in field populations.

1.3.2 Alteration of Target-Site

An alteration in the target site of the insecticide, sometimes involving the substitution of only a single amino acid, can reduce the rate of insecticide binding and lead to resistance. There are three major insecticide target-sites, acetylcholinesterase, the γ-aminobutyric acid (GABA) receptor and the sodium ion channel.

1.3.2.1 Acetylcholinesterase

Acetylcholinesterase (AChE) is an enzyme which terminates nerve impulses by hydrolysing the neurotransmitter acetylcholine at the nerve synapses. Organophosphates (OPs) and carbamates bind to AChE and inactivate the enzyme leading to the accumulation of acetylcholine in the synapse and a hyper excited central nervous system causing the death of insects. Decreased AChE sensitivity is a typical resistance mechanism for OPs and carbamates (Fournier & Mutero, 1994). Point mutations have been identified in the acetylcholinesterase (*ace*) gene, which are associated with resistance in OP resistant strains of houseflies (Mutero *et al.*, 1994; Walsh *et al.*, 2001). Two genes encoding AChE, namely *ace1* and *ace2* are found in insects including aphids (Li & Han, 2002b; Nabeshima *et al.*, 2003) and mosquitoes (N' Guessan *et al.*, 2003; Weill *et al.*, 2003), but only one ace gene, *ace2* is found in *D. melanogaster*. The *ace2* gene in *Culex pipiens* is linked to the sex locus and not associated with insecticide resistance (Malcolm *et al.*, 1998). However, mutations of *ace2* genes confer a low resistance to insecticides in *D. melanogaster* (Menozzi *et al.*, 2004). In contrast, mutations in the *ace1* gene are responsible for

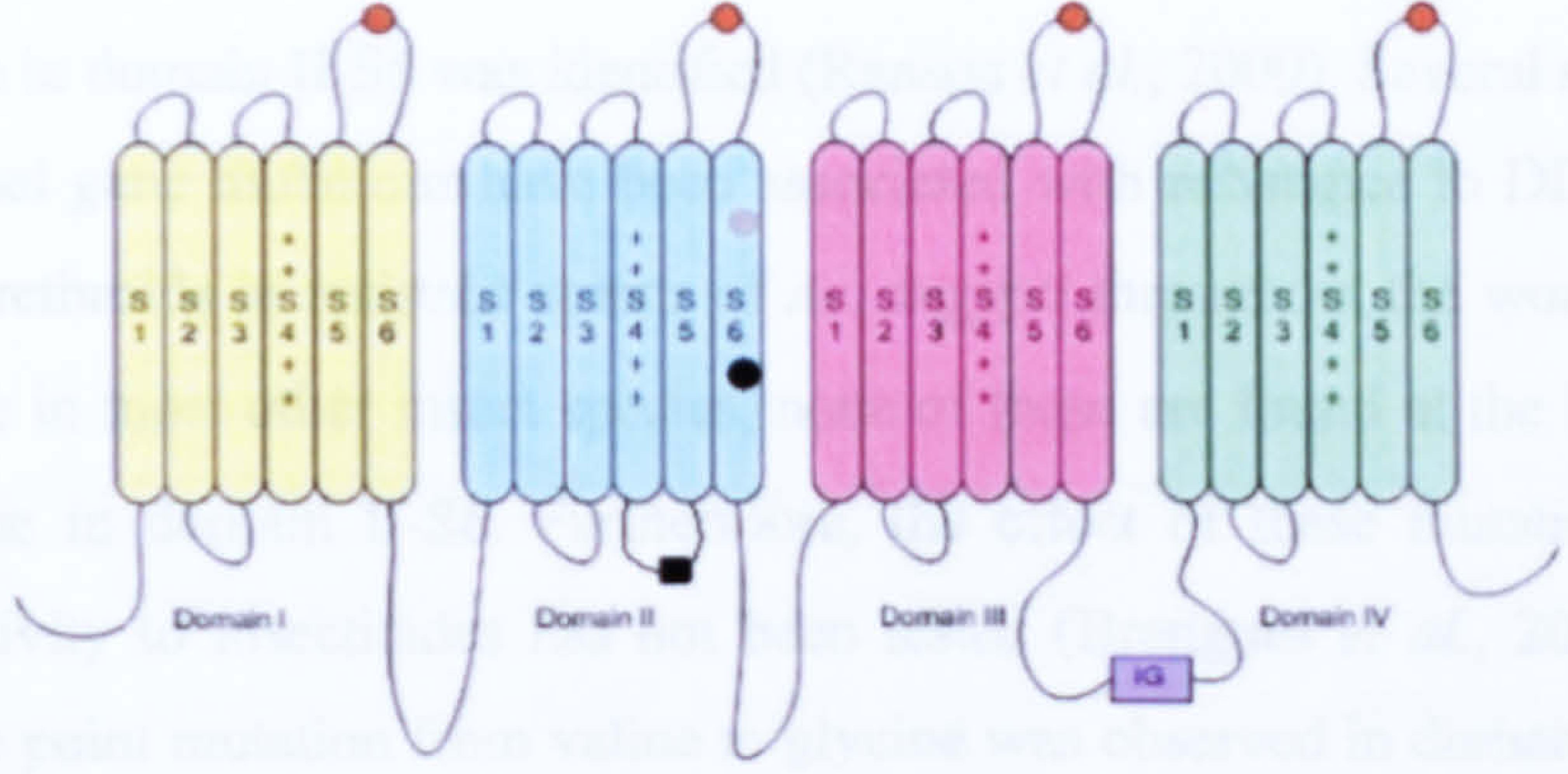
insecticide resistance in some species (Bourguet *et al.*, 1996a; Weill *et al.*, 2003). The high insensitivity of AChE due to a conserved glycine to serine substitution (G119S) in *ace1* has been reported in resistant strains of *Culex pipiens* and *An. gambiae* (Weill *et al.*, 2004). In *Ae. aegypti,* although carbamate and OPs has been extensively used, high levels of resistance to these chemicals has not been observed. It has been proposed that the silent base of glycine, encoded by GGA codon is immutable to serine in *Ae. aegypti*. Generally, the glycine at position 119 is encoded by GGC in other species (Weill *et al.*, 2004).

1.3.2.2 γ-Aminobutyric acid (GABA) receptor

The primary target of cyclodiene insecticides is the γ-Aminobutyric acid (GABA) receptor. The GABA receptor is the principal inhibitory neurotransmitter receptor in the central nervous system. Resistance to cyclodiene is conferred by the alteration of a single nucleotide in the GABA receptor resulting in the replacement of alanine 302 (located in the chloride ion channel pore of the protein) with a serine, or more rarely a glycine. This mutation has been observed in a wide range of different resistant insects, including *Ae. aegypti* (ffrench-Constant *et al.*, 2000; Thompson *et al.*, 1993).

1.3.2.3 The voltage-gated sodium ion channel

The voltage-gated sodium channel in the insect nervous system is the major target site for DDT and pyrethroids. Control of sodium permeability is essential for effective nerve function. The binding of DDT or pyrethroids to the sodium channel affects the closing of the channel after each action potential, resulting in an overall disruption in nerve transmission leading to nerve insensitivity (Narahashi, 1992; Soderlund & Bloomquist, 1989). The insect sodium channel consists of four domains (I-IV), and each of these domains consists of six transmembrane proteins (Figure 1.1). Mutations in the sodium channel gene have been associated with knock down resistance



L1014F (*kdr*)

V → G (mutation found in *A. aegypti* from Thailand)

M918T (*super kdr*)

Figure 1. 1 The diagram of voltage–gated sodium channel indicating four homologous domains (I-IV), each containing six transmembrane segments (SI-S6).

A high positive charged is observed in the fourth transmembrane segment (S4) of each domain. IG indicates an inactivation gate. The figure was adapted from http://www.omedon.co.uk/ionchan/channels/sodium.

(*kdr*). The term "knock down" has been used to indicate the paralysis of insects by chemicals, which interrupt the nerve sensitivity. The most common mutation in *kdr* insects is a leucine to phenylalanine substitution at amino acid residue 1014 in the S6 hydrophobic segment of domain II. This mutation was first reported in a pyrethroid resistant strain of housefly and has also been found in *An. gambiae* s.s. (Martinez-Torres *et al.*, 1998; Williamson *et al.*, 1996). A second resistant phenotype known as *superkdr* is caused by the combination of this mutation with a second, methionine to threonine, alteration at residue 918 between the linker S4-5 of domain II (Ingles *et al.*, 1996; Williamson *et al.*, 1993). This *superkdr* mutation has not been found in mosquitoes to date. In a pyrethroid resistant population of *An. gambiae* from Kenya an alternative substitution of leucine 1014 to

serine in domain II-S6 was identified (Ranson *et al.*, 2000). Several sodium channel gene mutations have been associated with resistance to DDT and or pyrethroids in resistant strains of *Ae. aegypti* throughout the world, but unlike in most other insect species, none of these are found at the leucine residue in domain II-S6. Furthermore, the effect of these mutations on sensitivity to insecticides has not been tested (Brengues *et al.*, 2003). A single point mutation from valine to glycine was observed in domain II-S6 in a DDT and pyrethriod resistant strain of *Ae. aegypti* from Thailand (Prapanthadara *et al.*, 2002).

1.3.3 Metabolic Resistance Mechanisms

In metabolic resistance, enhanced activity of enzymes in metabolic pathways of insects leads to insecticides being detoxified or sequestered before they reach the target-site. There are three major groups of enzymes involved in metabolic resistance, carboxylesterases, monooxygenases and *glutathione S-transferases* (GSTs). Metabolic resistance can be caused by either overproduction of these enzymes or increases in enzyme activity (Devonshire *et al.*, 1992).

1.3.3.1 Carboxylesterases

Carboxylesterases are enzymes that can hydrolyse ester bonds. OPs, carbamate and pyrethroids contain carboxylester and phosphotriester bonds that can be targeted by esterase enzymes. These esterases are members of the α/β hydrolase superfamily. Massive overproduction of nonspecific esterases, which sequester the insecticide before it reaches the target molecule acetylcholinesterase, is a common mechanism of OP resistance in insects (Devonshire *et al.*, 1998). In *Culex quinquefasciatus,* this overproduction is due to gene amplification (Vaughan *et al.*, 1995). Elevated esterase activity associated with pyrethroid resistance has also been reported (Mourya *et al.*, 1993; Vulule *et al.*, 1999). Less frequently, changes in the amino acid sequence of a specific esterase can cause

resistance to OPs e.g. a single point mutation in the carboxylesterase gene is observed in an OP resistance strain of blowfly (Newcomb *et al.*, 1997).

1.3.3.2 Monooxygenases

Monooxygenases, mixed function oxidases or cytochrome P450s are a complex family of enzymes which are involved in a NADPH-requiring oxidation system of a wide variety of xenobiotic compounds and endogenous compounds. There are over 100 different cytochrome P450 enzymes in insects, *An. gambiae* and *D. melanogaster* (Ranson *et al.*, 2002; Tijet *et al.*, 2001). A subset of these enzymes can metabolise insecticides, including cyclodiene and organophosphate insecticides, leading to detoxification and this process is enhanced in insect strains with metabolic resistance to insecticides (Feyereisen, 1999). Insect P450s have been assigned to 28 families (http://p450.antibes.inra.fr). Resistance based on cytochrome P450 oxidases has been observed both in the structural changes in specific cytochrome P450 genes, and more commonly in the overproduction of specific cytochrome P450s (Brandt *et al.*, 2002). P450s from families CYP4, CYP6 and CYP12 are overproduced in several insecticide resistance strains of insects (Scott, 1999). In *An. gambiae*, 111 P450 genes have been identified (Ranson *et al.*, 2002). A large cluster of CYP6 genes is localised on chromosome 3R division 30 within the boundaries of a major locus responsible for pyrethroid resistance in *An. gambiae* (Ranson *et al.*, 2002). The over expression of an adult-specific *CYP6Z1* gene in this region has been identified in a pyrethroid resistant strain of *An. gambiae* relative to the susceptible strain (Nikou *et al.*, 2003). Daborn *et al.* (Daborn *et al.*, 2002) demonstrated that the *Cyp6g1* gene was over expressed in strains of DDT-resistant *D. melanogaster* in the field populations around the world. This *Cyp6g1* is also found over expressed in DDT-resistant strain of *D. simulans* (Schlenke & Begun, 2004). However, recent microarray experiments show that not only *Cyp6g1* but also *Cyp12d1* are over expressed in laboratory-selected strains of *D.*

melanogaster (Festucci-Buselli *et al.*, 2005). Point mutations in the structural gene of *Cyp6a2* are responsible for DDT resistance in *Drosophila*. The altered protein found in the resistant strain able to metabolise DDT to dicofol, dichlorodiphenyl dichloroethane and dichlorodiphenyl acetic acid (Amichot *et al.*, 2004).

1.3.3.3 Glutathione S-transferases (GSTs)

Glutathione S-transferases (GSTs; EC 2.5.1.18) are major phase II detoxification enzymes found in most organisms. They detoxify a wide range of hydrophobic toxic compounds such as drugs, insecticides and toxic endogenous substrates by catalysing the conjugation of glutathione to the hydrophilic centre of the toxic substances (Hayes & Pulford, 1995). Exogenous substrates of GSTs include natural toxic compounds (toxins) and man-made chemicals (e.g. carcinogens, drugs and insecticides). Endogenous substrates include metabolites produced under oxidative stress, e.g. 4-Hydroxynonenal (4-HNE), an endogenous substrate produced from lipid peroxidation (Alin *et al.*, 1985) which is toxic at high concentrations. High concentration of HNE can induce apoptosis, differentiation and other signalling pathways (Awasthi *et al.*, 2004). GSTs therefore are involved indirectly in signal transduction by reducing the intracellular concentration of HNE.

Direct signalling by mammalian GSTs has also been shown (Alder *et al.*, 1999; Cho *et al.*, 2001; Elsby *et al.*, 2003; Yin *et al.*, 2000). Monomeric GST Pi acts as a Jun N-terminal kinase (JNK) inhibitor to form a complex with JNK and c-Jun under non-stressed conditions. Free GST Pi can be released and inactivated by GST Pi dimer formation under condition of oxidative stress, leading to the accumulation of active JNK and c-Jun followed by the activation of the transcription of specific genes that are involved in the JNK pathway (Alder *et al.*, 1999). In *Diptera*, *Anopheles*

dirus alternatively spliced GSTs has been involved in the JNK pathway (Udomsinprasert *et al.*, 2004).

Some GSTs play an important role in metabolic pathways such as aromatic amino acid degradation (Fernandaz-Canon & Penalva, 1998), steroid hormone (Johansson & Mannervik, 2001) and eicosanoid synthesis (Beuckmann *et al.*, 2000; Jowsey *et al.*, 2001; Kanaoka *et al.*, 2000). GSTs can also bind hydrophobic compounds that are not their substrates. This nonsubstrate binding is possibly associated with the sequestration, storage and transportation of drugs, hormones and other metabolites, such as bilirubin, fatty acids and heme (Hayes & Pulford, 1995).

1.4 Structure of Glutathione *S*-transferases

1.4.1 Cytosolic GSTs

Most GSTs are cytosolic, comprising two subunits forming homodimers or heterodimers, each subunit ranging from 17-28 KD. It has been proposed that the GST fold originated from the thioredoxin fold, βαβαββα (Armstrong, 1997). The cytosolic GST fold consists of the thioredoxin fold with an addition of α–helices at the C-terminal as shown in Figure 1.2. Each GST subunit contains a specific glutathione (GSH)-binding site (G-site) next to a nonspecific hydrophobic ligand- binding site (H-site). The G-site is found at the N-terminal of the protein. In general, the G-site consists of a highly conserved tyrosine residue, which is an essential component of the active site of GSTs. However, in some classes of GSTs, the active site tyrosine is replaced with either a serine (Wilce *et al.*, 1995) or a cysteine (Board *et al.*, 2000) residue. Moreover, it has been reported that the *Drosophila* GST-3 lacks the 15 amino acid residues including the tyrosine residue at the N-terminal but it still has catalytic activity (Sawicki *et al.*, 2003). The H-site, which interacts with the hydrophobic substrate, is found in the C-terminal. The high level of diversity in this region is responsible for the differences in substrate specificities (Mannervik &

Danielson, 1988). The tripeptide glutathione (GSH) binds to the G-site where it is ionised to the thiolate ion (GS^-). Structure of adGSTD5-5 from *An. dirus* species B is shown in Figure 1.3 (Udomsinprasert *et al.*, 2005). The enzyme then catalyses the conjugation between GS^- and the substrate by acting as a potent nucleophile acting on electrophilic carbon, nitrogen, sulfur, or oxygen atoms contained in the substrate bound to the H-site. The consequence of this reaction is to increase the solubility of the compounds, thus aid excretion (Hayes & Pulford, 1995). GSTs also catalyse the metabolism of insecticides in the absence of the conjugation reaction. For example, the dehydrochlorination of DDT, which requires GSH as a cofactor without the formation of thio ether intermediates (Clark & Shamaan, 1984).

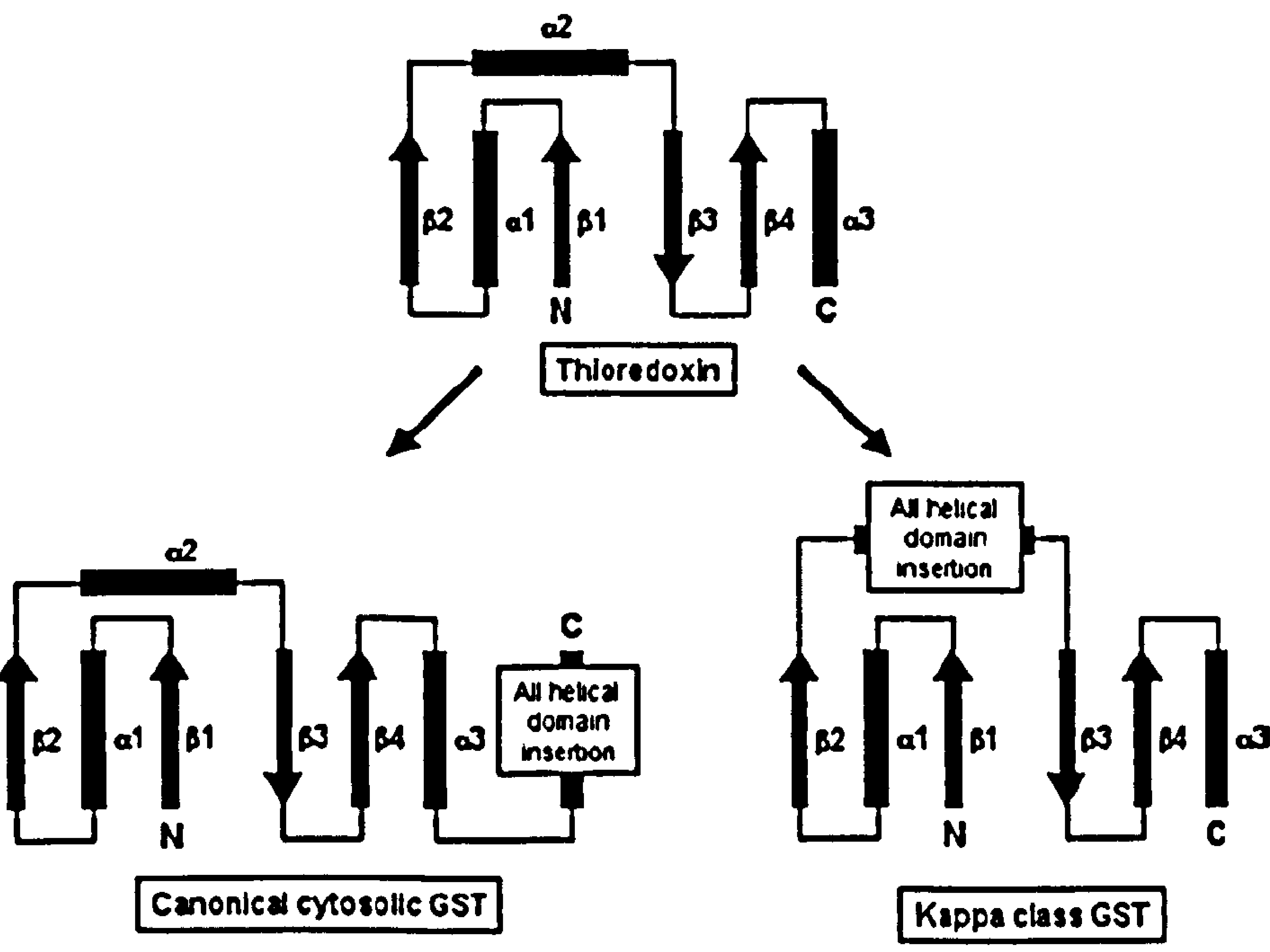


Figure 1. 2 The differences of GST fold between cytosolic GST and mitochondrial GST (Kappa class GST).

Both GST folds are very similar to thioredoxin fold (βαβαββα). The cytosolic GST fold contains the thioredoxin fold before the insertion of all helical domains at C-terminal, but in mitochondrial, the helical domains are located instead of the second α-helix (Hayes *et al.*, 2005).

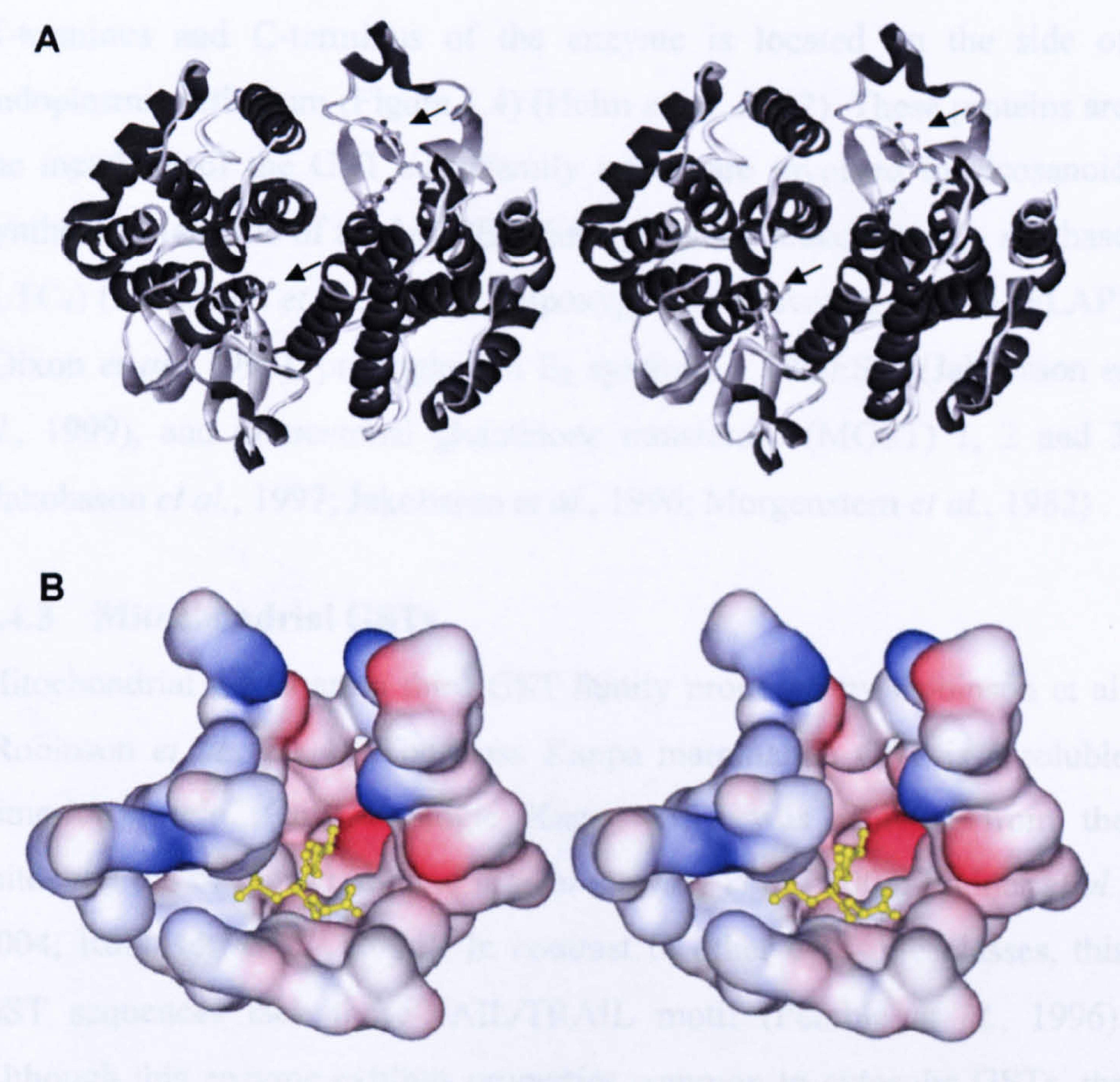


Figure 1. 3 Structure of adGSTD5-5 from *Anopheles dirus* (Udomsinprasert *et al.*, 2005).

(A) Two ribbon representations of adGSTD5-5 indicate the active site for GSH. Glutathione sulphonic acids are shown in ball-and stick (arrow). **(B)** The two three-dimensional structures of adGSTD5-5 show the pockets of G-site, which conjugate to GSH (yellow).

1.4.2 Membrane-Associated Proteins in Eicosanoid and Glutathione Metabolism (MAPEG)

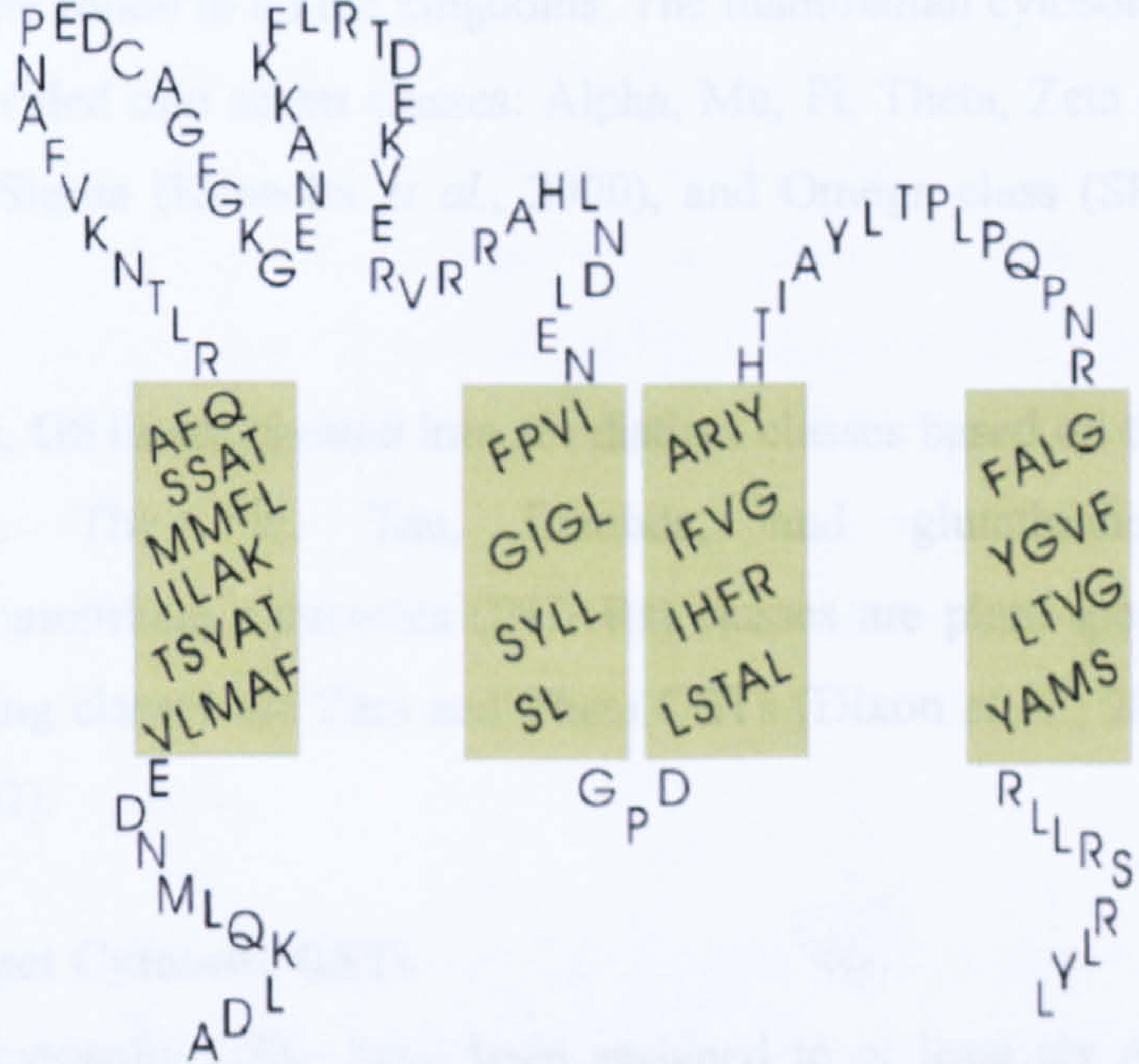
The second group of GSTs are the membrane-bound GSTs which are trimeric proteins and very different from cytosolic enzymes (Jakobsson *et al.*, 1999). Each subunit comprises four membrane-spanning regions. The N-terminus and C-terminus of the enzyme is located on the side of endoplasmic reticulum (Figure 1.4) (Holm *et al.*, 2002). These proteins are the members of the GST superfamily which are involved in eicosanoid synthesis. Members of the MAPEG family include leukotriene C_4 synthase (LTC_4) (Nicholson *et al.*, 1993), 5-lipoxygenase activating protein (FLAP) (Dixon *et al.*, 1990), prostaglandin E_2 synthase 1 (PGES1) (Jakobsson *et al.*, 1999), and microsomal glutathione transferase (MGST) 1, 2 and 3 (Jakobsson *et al.*, 1997; Jakobsson *et al.*, 1996; Morgenstern *et al.*, 1982) .

1.4.3 Mitochondrial GSTs

Mitochondrial GSTs are a third GST family proposed by Robinson et al. (Robinson *et al.*, 2004). The class Kappa mammalian GST is a soluble dimeric protein. Only a single Kappa GST was isolated from the mitochondria of mouse, rat and human (Jowsey *et al.*, 2003; Ladner *et al.*, 2004; Robinson *et al.*, 2004). In contrast to other cytosolic classes, this GST sequences lack the SNAIL/TRAIL motif (Pemble *et al.*, 1996). Although this enzyme exhibits properties common to cytosolic GSTs, the secondary structure of the Kappa GST suggested that this enzyme is distinct from other classes of soluble cytosolic GSTs which also posses a thioredoxin fold with an insertion of α-helices at $\alpha 2$ after $\beta\alpha\beta$ motif (Figure 1.2) (Robinson *et al.*, 2004).

1.5 Classification of Cytosolic Glutathione *S*-transferases

The major criteria by which GSTs are assigned into classes are sequence identity and phylogenetic relationship. In general, GSTs that share sequence identity greater than 30 % are assigned to the same class



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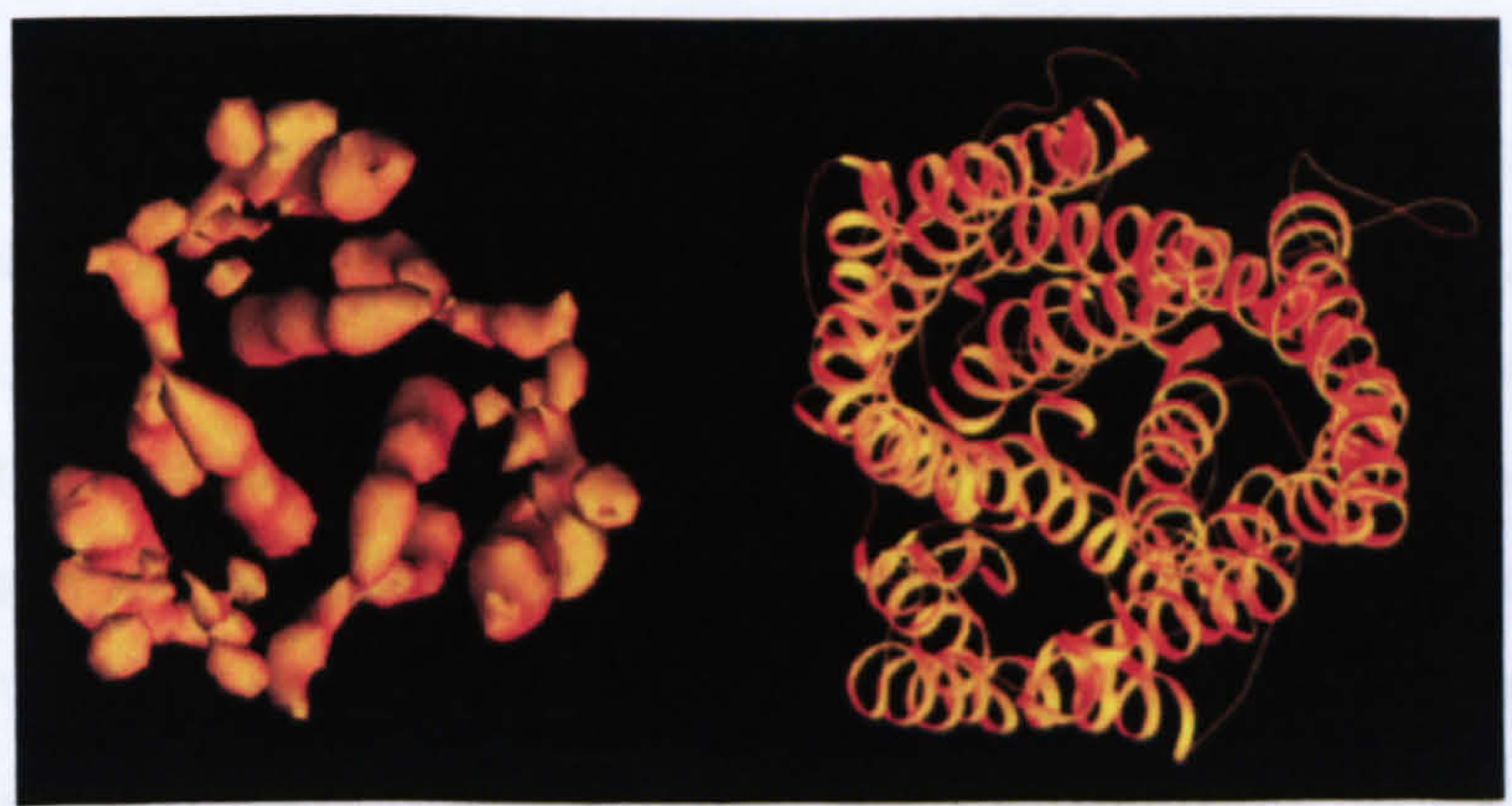


Figure 1. 4 The structure of mitochondrial GST1 (MGST1) from rat.

(A) The model of MGST1 shows four transmembrane regions with the N- and C- terminal at the luminal side of the endoplasmic reticulum (bottom), whereas the active of MGST1 is expected to be in the cytosolic side (Top). (B) The structure of MGST1, contains 3 subunits (left) is viewed from the cytosolic side compared with subunit 1 of cytochrome c oxidase (right) (Holm et al., 2002).

(Sheehan *et al.*, 2001). Substrate specificity is unsuitable for assigning GSTs to classes because of the broad and overlapping activity of multiple GSTs (Ranson *et al.*, 2002). Some GST classes are taxa-specific, but others are found in all the kingdoms. The mammalian cytosolic GSTs have been divided into seven classes: Alpha, Mu, Pi, Theta, Zeta (Board *et al.*, 1997), Sigma (Kanaoka *et al.*, 2000), and Omega class (Sheehan *et al.*, 2001).

In plant, GSTs are divided into six distinct classes based on their sequence identity. The Phi, Tau, Lambda, and glutathione dependent dehydroascorbate reductases (DHARs) classes are plant-specific; the two remaining classes are Zeta and Theta GSTs (Dixon *et al.*, 2002; Dixon *et al.*, 2002).

1.6 Insect Cytosolic GSTs

Insect cytosolic GSTs have been assigned to at least six classes: Delta, Epsilon, Omega, Sigma, Theta and Zeta (Ranson *et al.*, 2001). Members of these six classes have been identified in *An. gambiae* (Ranson *et al.*, 2002). In addition some GSTs in this species are currently still unclassified, namely *GSTu1*, *GSTu2* and *GSTu3* (Ding *et al.*, 2003). The Delta and Epsilon classes are both specific to insects whereas the remaining classes are also found in other organisms. There are fifteen and eleven Delta GST genes in *An. gambiae* and *D. melanogaster*, respectively (Ranson *et al.*, 2002). In *D. melanogaster,* the coding region of the Delta genes are intronless (Sawicki *et al.*, 2003) but in *An. gambiae*, the majority of the Delta genes contain a single intron. One *An. gambiae* Delta gene is alternatively spliced to produce four different transcripts that share the same N-terminal region but differ at their 3' ends (Ranson *et al.*, 1998; Ranson *et al.*, 1997). The Delta class is tightly clustered in *D. melanogaster* on chromosome 3R (Sawicki *et al.*, 2003), division 87B,

whereas two clusters of six Delta GST genes are located on chromosome 2R divisions 18B and 19B in *An. gambiae* (Ding *et al.*, 2003).

There are 10 Epsilon GST genes (DmGSTE1-DmGSTE10) in *D. melanogaster* which are intronless (Sawicki *et al.*, 2003) whereas the eight Epsilon class genes in *An. gambiae* contain one or more introns. These GST genes are sequentially organised on chromosome 3R division 33B of *An. gambiae* genome (Ding *et al.*, 2003). Clustering of Epsilon GST genes also occurs in the genome of *D. melanogaster* (Sawicki *et al.*, 2003). The phylogenetic tree shows the Delta and Epsilon classes of GSTs evolved independently in *An. gambiae* and *D. melanogaster* (Figure 1.5). This is probably due to the adaptation in these two species in their different ecological systems.

The Sigma class of GSTs contains a single gene in both species. In *An. gambiae* two different transcripts are produced by alternative splicing, whereas only one transcript of the Sigma GST was found in *D. melanogaster* (Ding *et al.*, 2003).

The Omega class of GSTs includes enzymes from mammals, nematodes and insects (Board *et al.*, 2000; Ranson *et al.*, 2002). Zeta GSTs have been found across kingdoms ranging from mammals (Polekhina *et al.*, 2001) to plants (Edwards *et al.*, 2000). Zeta GSTs have a maleylacetoacetate isomerase activity and are involving in degradation of tyrosine (Blackburn *et al.*, 1998; Dixon *et al.*, 2000; Fernandaz-Canon & Penalva, 1998). In *An. gambiae*, the Omega and Zeta GST class each consists of a single gene (Ranson *et al.*, 2002).

Pemble and Taylor (Pemble & Taylor, 1992) demonstrated that Theta GSTs are widely distributed in organisms including mammals, bacteria, insects and plants. Two Theta GSTs have been identified in *An. gambiae* (Ranson *et al.*, 2002).

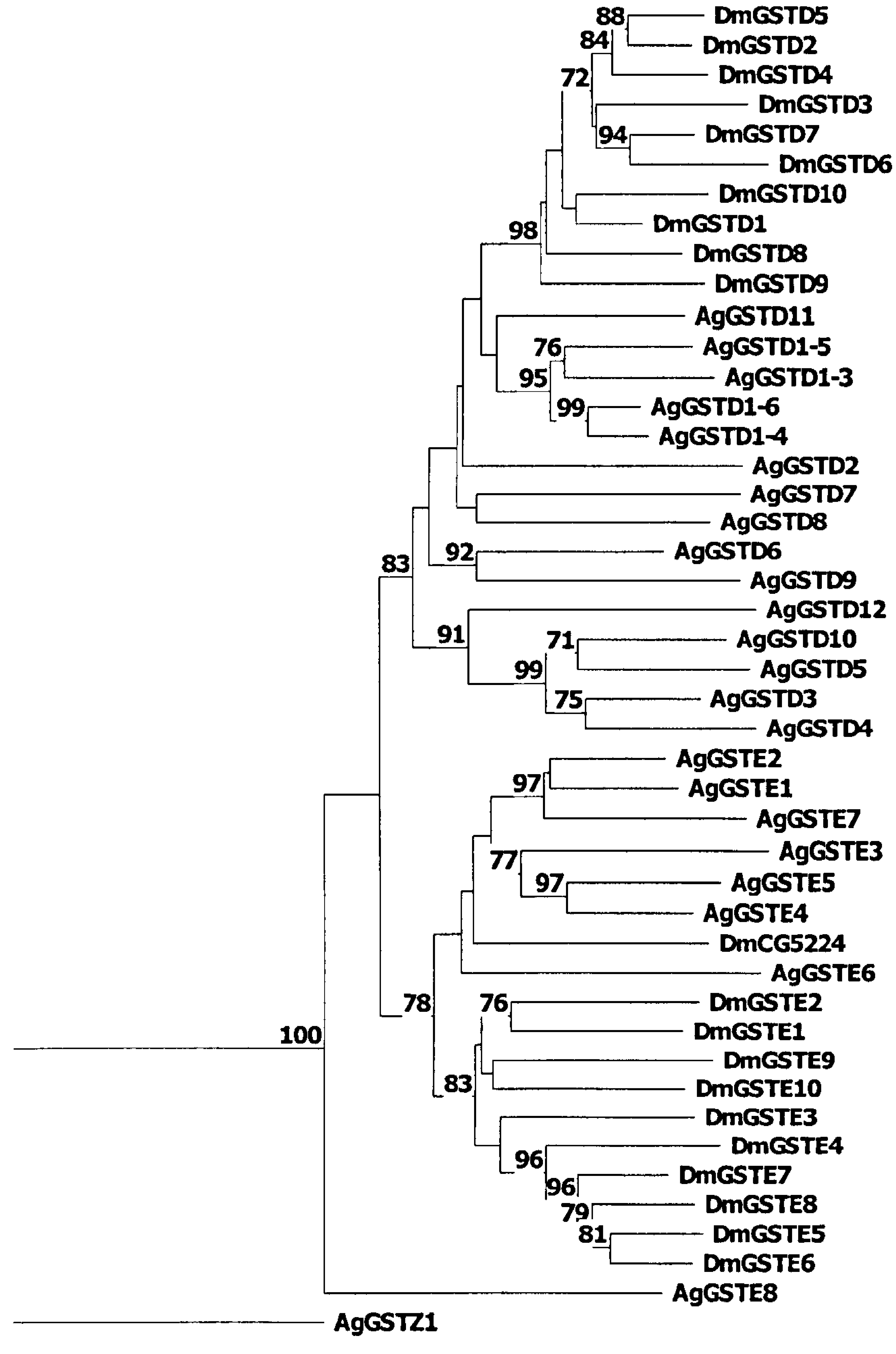


Figure 1. 5 Phylogenetic relationships of Delta (D) and Epsilon (E) GSTs in *An. gambiae* and *D. melanogaster.*

Amino acid sequences were aligned using ClustalW and a distance neighbour-joining tree was generated using TREECON. Nodes with distance bootstrap values (1000 replicates) of >70% are shown. Ag = *An. gambiae* and Dm = *D. melanogaster*.

1.7 Glutathione S-transferase-Mediated Insecticide Resistance

Increased GST activity against the model substrate, 1-choro-2, 4-dinitrobenzene (CDNB) has been found in insecticide resistant strains (Prapanthadara & Ketterman, 1993; Vontas *et al.*, 2001). GSTs are implicated in resistance to insecticides through direct metabolism of the insecticide (Chiang & Sun, 1993), sequestration (Kostaropoulos *et al.*, 2001) or by protecting against secondary toxic effects, such as increases in lipid peroxidation, induced by insecticide exposure (Vontas *et al.*, 2001). GST-mediated detoxification has been reported for both OPs (Motoyama & Dauterman, 1975) and DDT (Prapanthadara *et al.*, 2002; Ranson *et al.*, 1997).

The major role of GSTs in OP resistance is the detoxification of the insecticide by a conjugation reaction with GSH. The transformation of OPs mediated by GSTs are *O*-dealkylation (Oppenoorth *et al.*, 1979) and *O*-dearylation (Chiang & Sun, 1993). The overproduction of GSTs in an OP resistant strain of the housefly *Musca domestica* is associated with an increase in insecticide-conjugating activity (Wei *et al.*, 2001).

GSTs can catalyse the metabolism of DDT to non-toxic DDE. DDT dehydrochlorination occurs by the removal of a chlorine atom from DDT molecule and a hydrogen atom from the nearby carbon (Figure 1.6) (Clark & Shamaan, 1984; Matsumura, 1975). However, not all GSTs exhibit DDT dehydrochlorinase activity. Tang and Tu (Tang & Tu, 1994) demonstrated that the over expression of GSTD1 was associated with DDT resistance in *Drosophila* by increasing DDT dehydrochlorinase activity in this protein, whereas this activity was not found in recombinant GSTD21. Five of eight of the Epsilon GSTs genes in *An. gambiae* are over expressed in a DDT resistant strain relative to the susceptible strain (Ding *et al.*, 2003). Three of these recombinant proteins, GSTE1-1, GSTE2-2, and GSTE4-4 were assayed for DDT dehydrochlorinase activity, however only one of these,

GSTe2 encodes an enzyme which shows DDT dehydrochlorinase activity (Ortelli *et al.*, 2003; Ranson *et al.*, 2001).

1,1,1-Trichloro-2,2-bis(4'-chlorophenyl)ethane (DDT)

DDT dehydrochlorinase

HCl

1,1-Dichloro-2,2-bis(4'-chlorophenyl)ethylene (DDE)

Figure 1. 6 DDT dehydrochlorination reaction.

The reaction requires GSH as a cofactor to remove a chlorine and hydrogen atom from DDT.

There is no evidence that GSTs catalyse the detoxification of pyrethroids. However, it has been proposed that GSTs may act indirectly by protecting tissues from oxidative damage caused by the metabolism of pyrethroid insecticides (Vontas *et al.*, 2001). The over-expression of *nlGST1-1 in* pyrethroid resistant strains from the rice brown planthopper, *Nilaparvata lugens* is associated with peroxidase activity and lipid peroxidation (Vontas *et al.*, 2002). Peroxidase activity of GSTs using cumene hydroperoxide as a model substrate has been observed in insect GSTs (Prapanthadara & Ketterman, 1993; Vontas *et al.*, 2002). GSTs have also been proposed to protect against pyrethroid insecticides by sequestering the insecticide and reducing the amount of chemical reaching the target site (Kostaropoulos *et al.*, 2001).

1.8 GST Expression, Induction and Regulation

Many studies have shown that GST activity varies during the development of insects (Grant & Matsamura, 1988; Kostaropoulos & Papadopoulos, 1998; Prapanthadara & Ketterman, 1993). GST activity in *Ae. aegypti* was found to be age-dependent, maximum in the pupae stage and lower in adults (Hazelton & Lang, 1983). In a separate study, a minimum GST activity was observed at days 2 and 3 and a maximum at day 5 after hatching in *Ae. aegypti* (Grant & Matsamura, 1988). Tissue specific expression has also been demonstrated in some GST isozymes in *Ae. aegypti*, the over expression of GST-2 was found in head, thorax and abdomen, but not in ovaries (Grant *et al.*, 1991). In the housefly, Sigma class GST is mainly localised on the thin filaments of the indirect flight muscle (Franciosa & Berge, 1995), where it interacts with troponin-H and presumably plays a role in flight muscle (Clayton *et al.*, 1998).

Plapp (Plapp, 1984) demonstrated that the induction of detoxifying enzymes in response to chemicals is coordinated. This study showed that the level of monooxygenase and GST activity increased in response to the same stimulus in the housefly. The induction of GST activity has been reported in many insects after OP and organochlorine exposure (Clark, 1989; Hayaoka & Dauterman, 1982; Lagadic *et al.*, 1993), and pyrethroid exposure (Punzo, 1993; Yu *et al.*, 1984). Le Goff *et al.* (Le Goff *et al.*, 2001) demonstrated that the expression level of GSTD1 in *Drosophila* increased approximately 3-fold after 72 hours phenobarbital treatment.

The induction of GSTs in response to environmental stimuli, such as xenobiotics is mediated via several different transcriptional mechanisms. Extensive studies in GSTA1 (GST Ya) gene from the rat indicated that the structural gene contained at least 5 *cis*-acting elements located in the 5'-DNA flanking the GST A1 gene promoter involved in the transcriptional regulation (Rushmore & Pickett, 1993). One of these is the xenobiotic

responsive element (XRE), which is recognized by Ah (Aryl hydrocarbon) receptor complex. The Ah receptor forms a complex with aromatic compounds and binds to XRE sequences leading to transcriptional activation. Another *cis*-acting element is antioxidant-responsive element (ARE) (also called electrophile-responsive element). The affinity of *trans*-acting element to *cis*-acting elements on regulatory region of structural gene affects transcription in different ways; some *trans*-acting element increases the transcription, but others repress transcription.

The regulation of GST by endogenous electrophiles is also mediated through the keap1/Nrf2 pathway (Itoh *et al.*, 2004). The nuclear factor-erythroid2 p45-related factor 2 (Nrf2) bind to a small Maf protein and this complex activates transcription of genes, including GSTA1 and glutamate cysteine ligase via the ARE (Hayes & McMahon, 2001; Zhu & Fahl, 2001). It is hypothesised that the metabolites from oxidative stress such as 4-HNE, hydroperoxides and H_2O_2 can modify the actin-binding protein Keap1, generally bound to Nrf2, leading to the accumulation of Nrf2 following the activation of the transcription (Ishii *et al.*, 2004).

In GST-mediated insecticide resistance, increased GST activity is not a result of induction but rather due to a constitutive over expression of one or more GST genes (Ding *et al.*, 2003). In *Aedes aegypti*, Grant and Hammock (Grant & Hammock, 1992) demonstrated that the up-regulation of *GST-2* is due to a mutation in a *trans*-acting repressor element. The over expression of *GST-2* was dominant in an insecticide resistant strain, whereas no expression of *GST-2* was observed in the F1 progeny from crosses between the susceptible and resistant strain. This suggested that the expression of *GST-2* in F1 hybrids and susceptible strain was suppressed by a *trans*-acting element rather than the influence of regulatory gene (*cis*-acting regulatory gene). A mutation in this *trans*-acting element was proposed to cause the over expression of *GST-2* in the resistant strain.

In contrast, up-regulation of *GSTe2* in a DDT resistant strain of *An. gambiae* is due to mutations in the *cis*-acting regulatory regions (Ding *et al.*, 2003). A two adenosine indel (insertion/ deletion) was observed in the promoter region of *GSTe2* from the resistant strain. Site-directed mutagenesis indicated this 2bp deletion increased the GSTe2 promoter activity.

The expression of genes may also be regulated via RNA stability (Sachs, 1993). The instability of mRNA suppressed the expression of GSTD21 in *D. melanogaster* (Tang & Tu, 1995). GSTD21 is transcribed at a higher rate than GSTD1 but expression of GSTD21 at mRNA and protein level was lower than GSTD1. In the presence of pentobarbital treatment, GSTD21 mRNA increased 20-fold, but the induction of GSTD21 only resulted in a 2-fold increased in the transcription rate and no elevation GSTD21 protein was observed. It is proposed that the increased mRNA stability by pentobarbital is regulated via the alternative polyadenylation of GSTD21 mRNA (Akgul & Tu, 2004).

1.9 Background and Aims of the Study

In Thailand, DDT resistance was first reported in *Ae. aegypti* in 1966 (Neely, 1966) and has also been reported in the malaria vectors, *An. dirus*, *An. minimus* and *An. maculatus* (Chareonviriyaphap *et al.*, 1999). *Ae. aegypti* collected from Bangkok, Thailand was found to be highly resistant to both DDT and pyrethroid insecticides when compared with a laboratory susceptible strain (Chadwick *et al.*, 1977). High resistance to DDT was also reported in *Ae. aegypti* from many areas in northern Thailand (Somboon *et al.*, 2003). Elevation in DDT dehydrochorinase activity in two strains of *Ae. aegypti* (DDT resistant and DDT/ permethrin cross-resistant strains from Chiang Mai, Thailand) relative to the insecticide susceptible strains indicated that GSTs play a major role in DDT resistance (Prapanthadara *et al.*, 2002).

At the beginning of this study, only one molecular study on *Ae. aegypti* GSTs had been reported (Grant *et al.*, 1991; Grant & Hammock, 1992; Grant & Matsamura, 1988; Grant & Mutsumura, 1989). Three different GST isozymes had been purified and characterised in *Ae. aegypti* from South America, GST-1a, GST-1b and GST-2 (Grant & Matsamura, 1988; Grant & Mutsumura, 1989). Both GST-1a and GST-2 are over expressed in the DDT resistant strain approximately three and fifty fold respectively compare to the susceptible strain. The GST-2 gene was identified and sequenced and shown to be over expressed in the DDT resistant strain. The aim of this study is to characterise GST-based DDT resistance in *Ae. aegypti* mosquito from Thailand and to investigate the involvement of GST-2 in DDT metabolism.

Chapter 2

SUSCEPTIBILITY ASSAY AND GLUTATHIONE TRANSFERASE –BASED RESISTANCE IN *Aedes aegypti* FROM THAILAND

2.1 INTRODUCTION

DDT resistance has been reported in many insect vectors. Two main mechanisms are involved: alterations in the sodium channel and increased production of GSTs. Mutations in the *para*-sodium channel, the target site of DDT and pyrethroids are associated with knockdown resistance (*kdr*) by reducing the sensitivity of this target to insecticides (Martinez-Torres *et al.*, 1998). A common substitution (L1014F) in the domain II segment 6 in the sodium channel gene has been found in many insects (see review in (Soderlund & Knipple, 2003)), but not in *Ae. aegypti*. GSTs confers DDT resistance by increasing DDT dehydrochlorinase activity to eliminate chloride ion from DDT leading to the production of non-toxic DDE (Ortelli *et al.*, 2003; Prapanthadara *et al.*, 2002; Ranson *et al.*, 1997; Tang & Tu, 1994). In Thailand, resistance to DDT and pyrethroids has been reported in *Ae. aegypti* populations (Somboon *et al.*, 2003). The aim of this chapter is to investigate the role of GST-mediated DDT resistance in *Ae. aegypti*.

The susceptibility of *Ae. aegypti* mosquitoes from northern Thailand to DDT and pyrethroid insecticides was determined using standard WHO susceptibility kits (WHO, 1981). The level of DDT dehydrochlorinase activity was measured in resistant and susceptible populations to predict the importance of this enzyme family in conferring insecticide resistance.

2.2 MATERIALS AND METHODS

2.2.1 Mosquito strains

The Pang Mai Dang (PMD) strain of *Ae. aegypti* was colonised from field caught material originating from Ban Pang Mai Dang, Mae Tang district, Chiang Mai Province in northern Thailand. A highly resistant line (PMD-R) was generated from the parental PMD colony by selection of one-day adults from alternate generations with either 4% DDT or 0.75% permethrin using standard WHO susceptibility kits (WHO, 1981) by Dr. Pradya Somboon from Parasitology department, Medicine faculty, Chiang Mai University, Thailand. The resistance ratios of the parental PMD colony and the selected line were determined by comparison to a laboratory insecticide susceptible strain of *Ae. aegypti* originally collected from New Orleans, USA and kindly provided by Centre for Disease Control (CDC), Atlanta, USA.

The three colonies were reared in separate insectary rooms in the Liverpool School of Tropical Medicine, UK. All life stages were reared at a controlled environment of a 12/12 light-dark cycle, 25 ± 3°C and 80 ± 10% RH. Filter paper containing eggs was laid in the tray with trap water. Hatched larvae were reared for 5 days with ground fish food. Pupae were transferred into a small cup and kept in a new cage. Emerging adults were fed on 10% glucose soaked cotton pads. Female mosquitoes (aged 3-5 days) were starved for 24h, and then fed on blood using membrane-feeding device. Three days after blood feeding, eggs were laid on water-soaked filter paper. Dried eggs were then used to produce another generations.

2.2.2 Diagnostic susceptibility assay

Adult bioassays were performed by exposure to paper impregnated with 4% DDT or 0.75% permethrin. Female *Ae. aegypti* mosquitoes (15-20 per tube, three replicates per test) were exposed for different periods of time to insecticide impregnated papers. For each exposure time, a control tube was

set up in which mosquitoes were exposed to filter paper treated with olive oil alone. After exposure, the mosquitoes were transferred to a recovery tube for 24 hours and provided with a source of sugar liquid. The mortality was recorded and plotted against exposure time to obtain log-probit mortality lines using LdP Line software according to Finney (Finney, 1971). The lethal time at 50% mortality (LT_{50}) was obtained and the resistance ratio was calculated. If mortality between 5 and 20% was observed in the controls, the % mortality was recalculated using Abbott's formula (Abbott, 1925).

2.2.3 Mosquito crosses

Genetic crosses between the susceptible New Orleans and PMD-R resistant strains were established to determine the level of resistance in the F1 progeny. Approximately 100 females from PMD-R strain and 100 males from New Orleans strain were mass mated. Reciprocal crosses were also carried out between male PMD-R and female New Orleans. Female from F1 populations were collected for susceptibility assays as described above.

2.2.4 Determination of GST activity

Twenty mosquitoes from each strain and life stage were homogenized in 200μl 0.1 M phosphate buffer pH 6.5 containing 10 mM DTT. The supernatants were used for determining protein concentration and GST activity. GST activity against 1-choro-2, 4-dinitrobenzene (CDNB) was determined according to the method of Habig *et al.* (Habig *et al.*, 1974). GSTs catalyse the conjugation between GSH and CDNB to produce the yellow intermediate, dinitrophenyl thioether. This reaction can be measured spectrophotometricaly at 340 nm. One unit of GST activity is defined as the amount of enzyme catalysing the conjugation of 1 μmol of CDNB with GSH per minute at 25°C. The substrate mixture contains 1.0 ml of 10.5 mM GSH, prepared in 0.1 M phosphate buffer pH 6.5 and 50 μl of 21 mM CDNB (prepared in absolute ethanol). The reaction was started

by addition 200 μl of substrate mixture into 10 μl of sample and the optical density was measured kinetically for 2 minutes at 25°C in a VERSAMax microplate reader (Molecular Devices). The final concentrations of GSH and CDNB were 10 mM and 1 mM, respectively. The extinction coefficient of GSH-CDNB conjugation is 9.6 mM^{-1} cm^{-1} and the light path of the solution in the microtitre plate is 0.6 cm.

2.2.5 DDT dehydrochlorinase assay

DDT-dehydrochlorinase activity was determined by measuring conversion of DDT to DDE as described previously (Prapanthadara *et al.*, 2002). The enzyme in 0.1 M phosphate buffer pH 6.5 was incubated with 10 mM GSH and 0.1 mM DDT (prepared in ethanol) at 28°C for 2 hours. The reaction mixture was then extracted with 3 x 1.5 ml of chloroform and the chloroform extracts was transferred and pooled into a new glass tube. After drying at room temperature, the extract was stored at –20°C until used.

DDT metabolism was detected by HPLC using a Beckman analytical ultrasphere ODS column (Beckman). The extract was resuspended in 100 μl of isopropanol and 100 μl of mobile phase (methanol: acetonitrile: water; 72.5: 12.5: 15) was added. Fifty microlitres of the extract was injected into the column with a flow rate of 0.8 ml /min. Peaks were integrated into peak area at 236 nm by the Chromelon software version 6.10 (Dionex company). *p,p*'-DDT (Chem Service, 91.1% purity) and *p,p*'-DDE (Chem Service, 99.5% purity) at concentrations ranging from 0.1-200 μM were used and standard curves were plotted relating DDT or DDE concentration to peak area. The chromatograms of the analysis are shown in Figure 2.1. DDT dehydrochlorinase activity was calculated as the amount of DDE production relative to the amount of protein. Controls were carried out in the absence of GSH or using denatured enzyme in the reaction mixture.

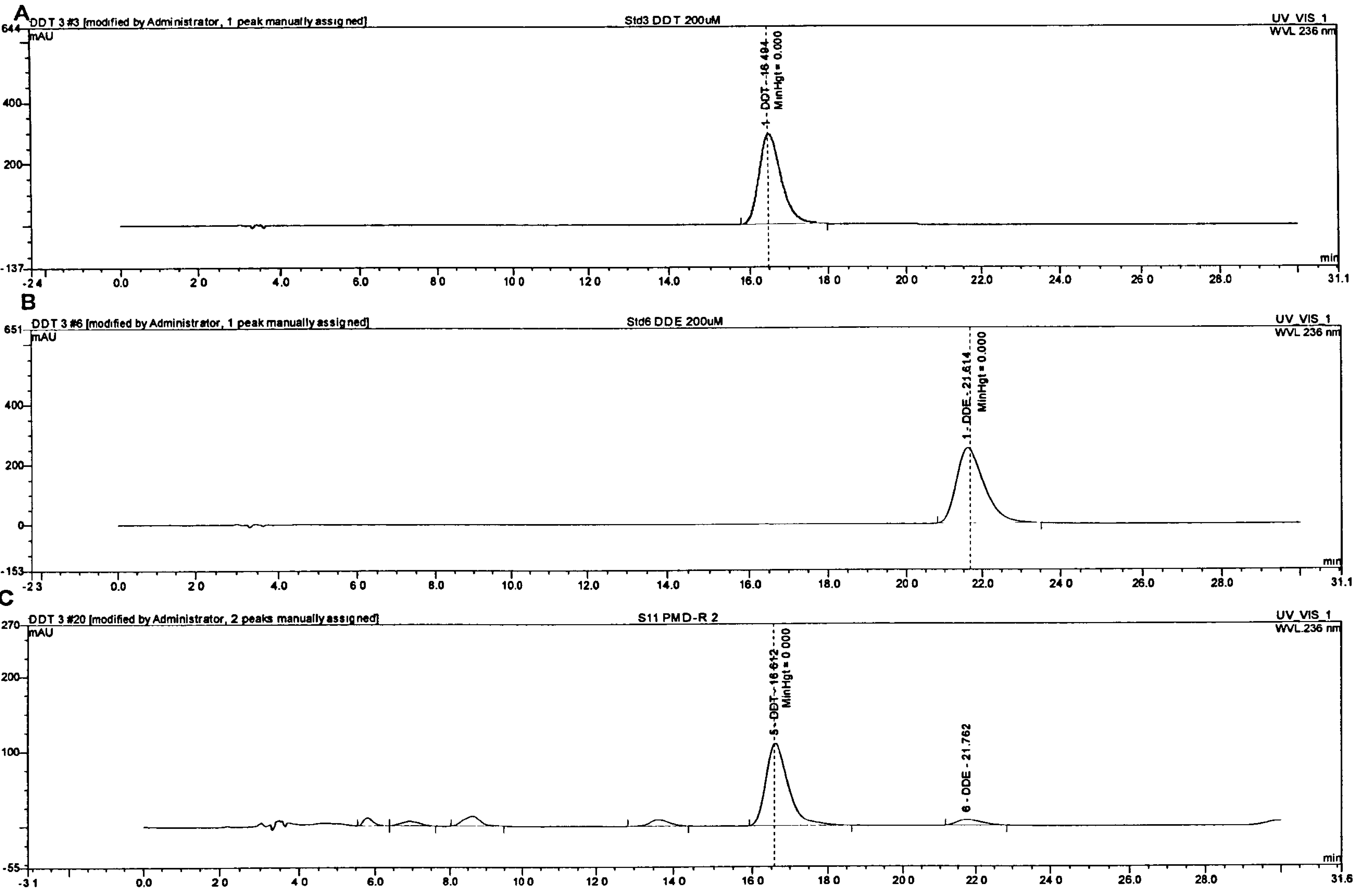


Figure 2. 1 DDT metabolism analysis by HPLC (A) standard DDT 200 μM, (B) standard DDE 200 μM and (C) Crude homogenate from PMD-R. strain.

2.2.6 Protein concentration determination

Protein concentration was determined using the Bio-Rad Protein Reagent. This reagent contains an acidic coomassie blue dye that binds to the primarily basic and aromatic amino acid residues of proteins (Bradford, 1976). The absorbance of the dye complex was measured at 595 nm. Bovine serum albumin, at concentrations ranging from 0.1 to 0.5 mg/ml, was used to produce a standard curve. The working dye reagent was prepared by diluting the dye reagent concentrate 1: 4 in deionised, distilled water and filtered through Whatman #1 to eliminate the particulates. Ten microlitres of standard or sample was incubated with 200 μl of working dye reagent for 5 minutes at room temperature. After reading the absorbance at 595 nm using VERSAMax microplate reader (Molecular Devices), the standard curve was plotted and the linear regression line was calculated to enable the protein concentration of the samples to be determined.

2.3 RESULTS

2.3.1 WHO susceptibility assays

The New Orleans susceptible strain and the parental PMD strain from *Ae. aegypti* mosquitoes were exposed to 4% DDT or 0.75% permethrin for different time periods. After recovering for 24 hour, the % mortality was recorded and plotted against exposure time (Figure 2.2). The LT_{50} values for DDT were 24.2 min and 127.8 min in New Orleans susceptible strain and parental PMD strain, respectively (resistance ratio (RR) of 5.3) (Table 2.1). The slopes of the regression lines in both strains were similar, which is an indication of the genetic homogeneity of the strains (Table 2.1). The LT_{50} values for 0.75% permethrin were 8.1 min and 18.7 min in susceptible and parental PMD strain, respectively (RR = 2.3). As with DDT, the slopes were similar in both strains. It can be concluded that the parental PMD strain is slightly resistant to both DDT and permethrin. PMD-R strain has been selected from parental PMD for many generations with 4% DDT and 0.75% permethrin. After selection, the susceptibility test was repeated. One hundred

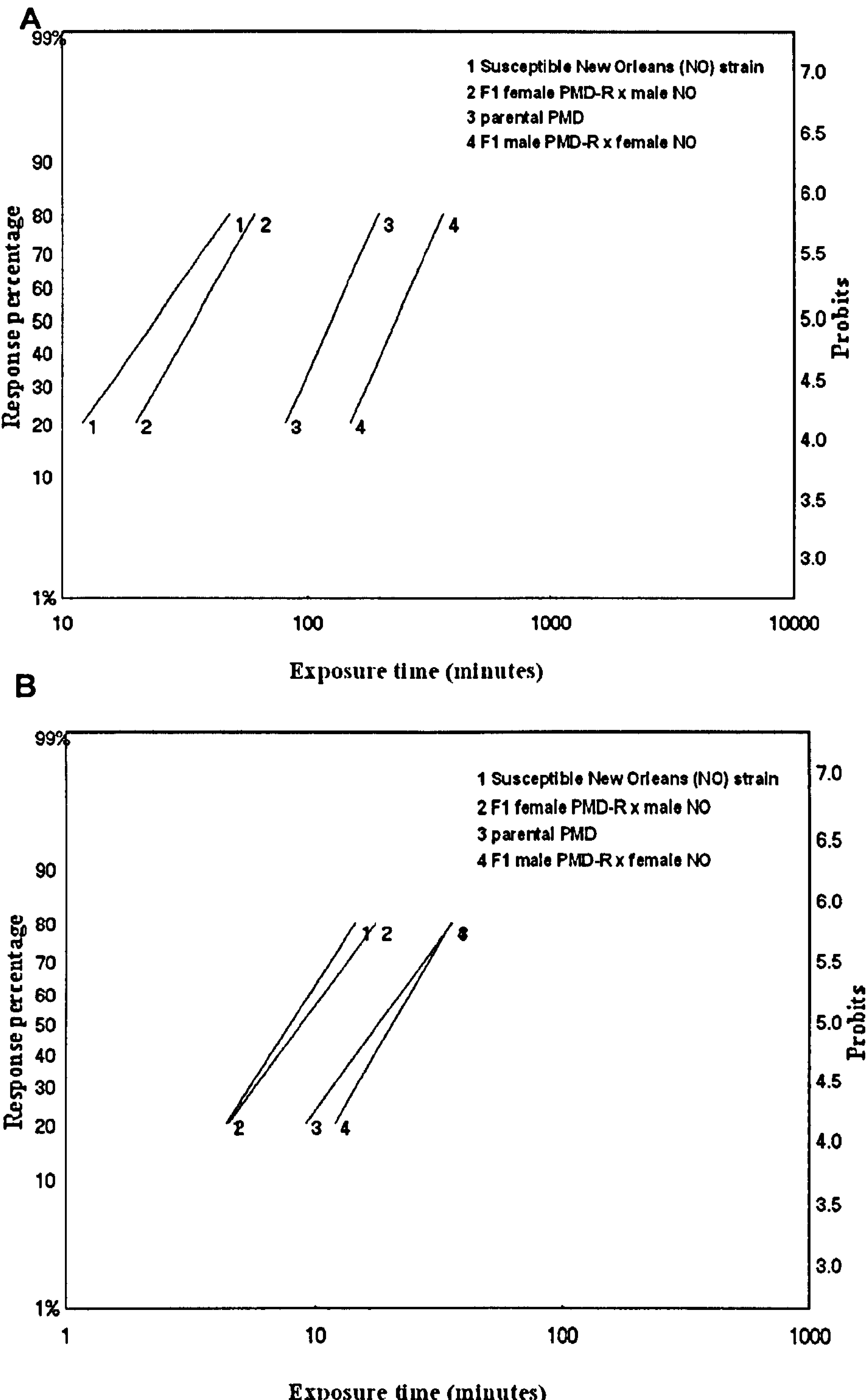


Figure 2. 2 Time-response curves for the New Orleans (NO), PMD and the F1 progeny from crosses between the PMD-R and NO strains of *Ae. aegypti* to 4%DDT (A) and 0.75% Permethrin (B).

Probits is an alternative to logistic regression analysis.

Table 2. 1 LT_{50} to 4% DDT and 0.75% Permethrin. RR indicates resistance ratio compared to New Orleans strain.

	4% DDT			0.75% Permethrin		
	LT_{50} (min)	RR	Slope	LT_{50} (min)	RR	Slope
New Orleans (NO)	24.2	1	2.8 ± 0.4	8.1	1	3.2 ± 0.3
F1 PMD-R♀ X NO♂	35.1	1.4	3.4 ± 0.4	9.0	1.1	2.8 ± 0.2
PMD	127.8	5.3	4.3 ± 0.4	18.4	2.3	2.8 ± 0.2
F1 NO♀ X PMD-R♂	236.1	9.7	4.4 ± 0.3	21.0	2.6	3.6 ± 0.3

percent survived exposure to 4% DDT or 0.75% permethrin for 7 hours. Due to the extremely high levels of resistance in this strain it was not possible to obtain an accurate value for LT_{50}.

2.3.2 GST activity

Four life-stages of *Ae. aegypti* were used; 4th instar larvae, pupae, one day old adult male and female. GST activity was significantly increased in all life stages in PMD-R strain compared with New Orleans strain ($p<0.001$ in adult male and female, $p< 0.01$ in pupae and $p< 0.05$ in 4th instar larvae) as shown in Table 2.2. GST activity in adult male and female from parental PMD were significantly higher than GST activity from susceptible New Orleans ($p< 0.001$ in both stages). In contrast, no significant differences were observed between parental PMD and New Orleans in 4th instar larvae and pupae. Significant increases in GST activity were also observed in immature stages of the PMD-R relative to parental PMD strains. In contrast, in the adults, GST activity was lower in the selected compared to the parental PMD strain.

2.3.3 DDT dehydrochlorinase activity

Twenty 4th instar larvae from New Orleans susceptible, parental PMD and PMD-R strains were used. The DDTase activity results are shown in Table 2.3. Significantly increased DDTase activity was observed in DDT/ permethrin resistant PMD-R strain relative to the parental PMD ($p<0.05$). Whereas, in the parental PMD strain the DDTase activity was significantly decreased compared to the susceptible strain ($p<0.05$).

2.3.4 Genetics of resistance to DDT and permethrin

Since it was not possible to obtain reliable time-response data for the PMD-R strain, it is difficult to interpret the genetics of resistance in this strain. Resistance in the F1 progeny from crosses between New Orleans and PMD-R populations is intermediate between the two parental strains indicating partially dominant

resistance. The F1 progeny from crosses between NO♀ X PMD-R♂ was more tolerant to both DDT and permethrin than the F1 progeny from the reciprocal cross and also more tolerant to insecticides than the parental PMD strain. (Figure 2.2A and B).

GST activities are significantly decreased in both F1 crosses relative to the PMD-R strain in mature stages shown in Table 2.2. In contrast, there are no differences in GST activity in F1 crosses when compared with New Orleans strain. In immature stages, however, GST activities are lower in the F1 progeny than in either parental strain.

The PMD-R strain had the highest level of DDT dehydrochlorinase activity and resistance to DDT (Table 2.3 and Table 2.1). The F1 progeny from crosses between NO♀ X PMD-R♂ showed higher DDT dehydrochlorinase activity than from the reciprocal cross (Table 2.3), resulting in greater resistance in this population (Figure 2.2A). Hence these results show a positive correlation between DDT resistance and DDT dehydrochlorinase activity, although, since a small number of samples were used in this study, it was not possible to obtain an accurate R^2 of this correlation. However, it should be noted that different life stages were used in these assays as DDT dehydrochlorinase assay was done in larvae stage, but the bioassay was tested in adult mosquitoes.

2.4 CONCLUSIONS

The parental PMD strain from Thailand was resistant to both DDT and permethrin. Further selection increased the resistance to both these insecticides to produce a highly resistant strain, PMD-R. Elevated GST activity implicated in conferring resistance to DDT and pyrethroid insecticides in other insects (Grant & Mutsumura, 1989; Prapanthadara & Ketterman, 1993; Vontas *et al.*, 2001), was detected in the resistant PMD-R strain in all life-stages relative to the susceptible strain. However GST activity was lower in adult from the selected line compared

Table 2. 2 GST activity against CDNB substrate.

	New Orleans (NO)[1]	PMD[1]	PMD-R[1]	F1PMD-R♀ X NO♂[2]	F1 NO♀ X PMD-R♂ [2]
Larvae	0.466 ± 0.011	0.463 ± 0.014	0.541 ± 0.034[c,c]	0.285 ± 0.024[a,a,b]	0.339 ± 0.028[b,b,c]
Pupae	0.339 ± 0.012	0.350 ± 0.010	0.396 ± 0.016[b,c]	0.267 ± 0.016[c,c,b]	0.278 ± 0.008[-,c,c]
Male	0.230 ± 0.009	0.513 ± 0.029[a]	0.354 ± 0.014[a,a]	0.202 ± 0.004[-,a,a]	0.227 ± 0.010[-,a,b]
Female	0.238 ± 0.011	0.460 ± 0.014[a]	0.422 ± 0.012[a,c]	0.212 ± 0.007[-,a,a]	0.242 ± 0.007[-,a,a]

Results represented in Mean (μmol/min/mg protein) ± Standard Error. [1]N =20. [2]N = three different batches of 10 mosquitoes in each stages. Statistically significant differences were evaluated with ANOVA test ($p < 0.001$ indicates by [a], $p < 0.01$ illustrates as [b] and $p < 0.05$ illustrates as [c] relative to New Orleans strain (1st letter) , PMD (2nd letter) or PMD-R (3rd letter).

Table 2. 3 DDT dehydrochlorinase activity.

Strains	DDT dehydrochlorinase activity (nmol DDE formation / g protein)
New Orleans (NO)	330 ± 113
PMD	103 ± 48[c_NO]
PMD-R	619 ± 209[c_PMD]
F1PMD-R♀ X NO♂	232 ± 132
F1 NO♀ X PMD-R♂	517 ± 61[a_PMD, c_F1 PMD-R♀]

Three different batches were used in the experiment. Each batches contained 20 4th instar larvae of each strain. The data are mean ± SD. Statistically significant differences were evaluated with ANOVA test ($p< 0.001$ indicates by [a] and $p < 0.05$ illustrates as [c]).

to adults from the parental PMD strain. In contrast, bioassays all conducted on adult mosquitoes indicated that the parental and selected lines are resistant to both DDT and permethrin. These results suggest that GST activity as measured with CDNB is not an accurate measure of DDT resistance.

DDT dehydrochlorinase activity was higher in the selected line (PMD-R strain) compared to the parental PMD. The susceptible New Orleans strain also had higher DDTase activity than the parental PMD strain. This may be partially explained by the different geographical origins of the two populations. Variations in DDT dehydrochlorinase activity from different geographic regions have been observed previously and DDT dehydrochlorinase activity was higher in *Ae. aegypti* from America, but lower in the Asiatic strain (Kimura & Brown, 1964).

The F1 progeny from crosses between the susceptible New Orleans strain and the PMD-R strain show different resistant phenotypes depending on the crossing

direction. Unfortunately, it was not possible to obtain an accurate probit line from the PMD-R strain and therefore the dominance of the trait could not be accurately determined. However, it is clear that both F1 progeny showed intermediate dominance. In contrast, GST activity was decreased in the F1 progeny in all development stages, to below the levels in the susceptible strain. The DDT dehydrochlorinase activity showed a stronger correlation with the bioassay data. The genetics of the trait is discussed further in Chapter 5.

Some of the discrepancies observed between GST and DDT dehydrochlorinase activity and the bioassay data in this chapter may be due to the presence of additional non GST resistance mechanisms in the Thai strain. Increased cytochrome P450 has been reported in DDT resistant strain in insects including *Ae. aegypti* (Amichot *et al.*, 2004; Daborn *et al.*, 2002; Prapanthadara *et al.*, 2002). In addition, although the classical *kdr* mutation is not present in the PMD strain, a valine to glycine substitution within domain II-S6 of the sodium ion channel has been observed in the PMD-R strain (Prapanthadara *et al.*, 2002). Its role, if any in conferring resistance to DDT is unknown.

Cross-resistance to DDT and pyrethroids has been reported in insects (Chadwick *et al.*, 1977; Plapp & Hoyer, 1968). These two insecticides share the same target site, and mutations in the sodium channel can lead to resistance to both insecticides (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). Elevated monooxygenase was also found in DDT/pyrethroid cross-resistant strains (Brogdon *et al.*, 1999). In addition, microarray study indicates constitutive over expression of GST and monooxygenase has been found in DDT and pyrethroid resistant strains of *An. gambiae* (David *et al.*, 2005). Only GST-based resistance was investigated in this study. However, other mechanisms involved in DDT/permethrin cross-resistance in PMD-R strain need to be investigated further.

Chapter 3

IDENTIFICATION AND CLONING OF *Aedes aegypti* GSTS

3.1 INTRODUCTION

GST activity is elevated in the insecticide-resistant PMD strain relative to susceptible strain, suggesting that GSTs may be important in DDT resistance in *Ae. aegypti*. Previously, only one *Aedes* GST had been sequenced completely. This gene is expressed at elevated levels in a DDT resistant strain of *Ae. aegypti* from South America (Grant & Hammock, 1992). However, given that there are 28 cytosolic GSTs in *An. gambiae* (Ding *et al.*, 2003), it is expected that many additional GST genes are present in *Aedes*. The aim of this chapter is to begin to identify and clone these *Ae. aegypti* GSTs. This was facilitated by the large scale EST sequence database for *Ae. aegypti* that has been compiled by The Institute of Genome Research (TIGR) and the availability of a large insert *Ae. aegypti* BAC library (Jimenez *et al.*, 2004).

Anopheles gambiae GST sequences and the keyword 'GST' were used to search for *Ae. aegypti* GSTs in the TIGR EST database. GST sequences from *Ae. aegypti* were amplified using specific forward and reverse primers and the GST genes were characterised in terms of intron/exon sizes, phylogenic relationship and amino acid similarity. GST gene organisation was investigated by screening the BAC library.

3.2 MATERIALS AND METHODS

3.2.1 Identification of *Ae. aegypti* GST Genes Using the EST Database

GST genes from *Ae. aegypti* mosquitoes were identified by searching the sequences in EST database (http://www.tigr.org) by keyword (GST) or by using the BLAST algorithm to search with all *An. gambiae* GST sequences.

3.2.2 Extraction of Genomic DNA

Genomic DNA was extracted from one-day adults of the parental PMD strain of *Ae. aegypti* using the LIVAK buffer extraction method (Collins *et al.*, 1987). Individual mosquitoes were homogenized in 100μl LIVAK buffer (0.13 M Tris-HCl containing 80 mM NaCl, 0.16 M sucrose, 0.05 M EDTA, 0.5% v/v SDS, pH 8.0) and the solution incubated at 60°C for 30 minutes. Potassium acetate (8M) was added to a final concentration of 1 M and the homogenate was mixed and incubated on ice for 10 minutes. The sample was centrifuged at 14,000g, 4°C for 10 minutes and the supernatant was transferred to a new tube. After precipitating DNA with cold absolute ethanol and washing twice with 70% (v/v) ethanol to remove the salt, the pellet was resuspended in 100 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the genomic DNA stored at 4°C.

3.2.3 Extraction of Total RNA

Individual mosquitoes were homogenized in 150 μl TRI reagent (Sigma). Samples were centrifuged at 14,000g for 10 minutes at 4°C to remove the mosquito debris and the supernatant was transferred to 0.5 ml tube and allowed to stand at room temperature for 5 minutes. Thirty microlitres of chloroform were added and the tube was shaken vigorously for 15 seconds and allowed to stand at room temperature for 10 minutes. The sample was centrifuged at 14,000g for 15 minutes at 4°C. The upper layer was transferred to a 1.5 ml fresh tube and RNA was precipitated with one volume of isopropanol. The sample was mixed and incubated at room temperature for 5 minutes. After centrifugation at 14,000g for 10 minutes at 4°C, the pellet was washed with two volume of cold 75% (v/v)

ethanol. The pellet was resuspended in 20 μl of nuclease-free water. Total RNA was treated with RQI DNAse (Promega) to degrade any remaining DNA, in the presence of RNasein (Promega) (to inhibit any contaminating RNAse activity). The total RNA was then re-extracted as above and resuspended in 20 μl nuclease-free water and stored at -80°C.

3.2.4 Synthesis of 1st Strand cDNA

First strand cDNA was synthesised from total RNA using SuperScript™ III Rnase H⁻ Reverse Transcriptase (Gibco, BRC) and an oligo dT adaptor primer (5′-GACTCGAGTCGACATCGA $(dT)_{17}$-3′). One microgram of RNA was incubated with 1 μg of primer at 70°C for 10 minutes to denature the RNA. After chilling the mixture on ice for 1 minute, the RNA was pre-warmed to 42°C for 2 minutes with 4 μl of 5x first Strand Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 μl of 10 mM of each dNTPs and 1 μl of 0.1 M DTT. One microlitre of SuperScript™ III Rnase H⁻ Reverse Transcriptase was added and the reaction was incubated at 42°C for 50 minutes. The reaction was heated to 70°C for 15 minute to inactivate the SuperScript™ III Rnase H⁻ Reverse Transcriptase. First strand cDNA was collected by brief centrifugation and stored at –20°C.

3.2.5 Amplification of GST Genes in *Ae. aegypti*

Primers were designed based on the EST sequence (Table 3.1). PCR products were amplified using genomic DNA (gDNA) and complementary DNA (cDNA) as templates in the presence of 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.5 μM of each primers and 2.5 unit of Taq DNA polymerase in the reaction buffer (Bioline, UK). PCRs were performed using a DNA Engine PTC-200 (MJ research), The cycling parameters were 95°C for 5 min, followed by 35 cycles of 95°C for 30s, 55°C for 30s and 72°C for 45s, and a final extension of 72°C for 7 min. The PCR products were separated on a 1% (w/v) agarose gel in the presence of ethidium

bromide (0.5 μg/ml) and then visualised using the Gene Genius Bio Imaging System (SYNGENE).

Table 3. 1 Sequences of oligonucleotide primers used to amplify full-length *Ae. aegypti* GSTs.

Gene	Primer name	Primer sequence (5'-3')
GSTe2	GSTe2F	CTGCTCCACAATGACGAAGC
	GSTe2F2	TATGTGCAAAAGGCTTACCAACTG
	GSTe2R	TGCCTTTTGAGCATTCTTCTCC
GSTe4	GSTe4F	GTTAATCTTCACCACTAGAAATG
	GSTe4R	CGAACAATGCAATTACTTCTT
GSTt1	GSTt1F	ATGTCGAAGCTACGGTACTTTTAC
	GSTt1R	CAACTTCGGCTTCGGAACG
GSTt2	GSTt2F	ATGGCAAACGGTCGCAAC
	GSTt2R	CGTCGCAGTAGGCGCA

3.2.6 Subcloning of PCR Products into pGEM-T Easy Vector

3.2.6.1 Ligation of PCR products into the pGEM-T easy vector

The PCR products were ligated into the pGEM-T easy vector using pGEM-T easy vector ligation system (Promega). To obtain a 3:1 (insert: vector molar ratio), the following equation was used to determine the amount of PCR product that was required in the ligation reaction. Each reaction contained 25 ng of pGEM-T easy vector and 3 units of T4 DNA ligase. The reactions were incubated at room temperature for 2 hours and the ligations were stored at 4°C.

$$\frac{25\text{ ng of vector x kb size of insert}}{\text{kb size of vector}} \text{ x insert : vector molar ratio = ng of insert}$$

pGEM Teasy vector = 3.0 kb

Insert : vector molar ratio = 3:1

3.2.6.2 Transformation of E. coli XL1-Blue subcloning-grade competent cells with plasmid DNA

The XL1-Blue subcloning-grade competent cells (Stratagene) were thawed on ice. Fifty microlitres of cells were added into pre-chilled 15 ml Falcon 2059 polypropylene tube (Becton Dickinson). Two microlitres of the ligation reaction were added to the cells and the tube was incubated on ice for 20 minutes. The plasmid was introduced into the XL1-Blue subcloning-grade competent cells by heat-pulse in a 42°C water bath for 45 seconds. After incubating the tube on ice for 2 minutes, 0.9 ml of preheated SOC medium at 42°C (2% (w/v) bacto-tryptone, 0.5% (w/v) yeast, 0.05% (w/v) NaCl and 20 mM Glucose), was added into each tube, which was then incubated at 37°C for 30 minutes with shaking at 250 rpm. One hundred microlitres of transformed bacteria were plated on agar plates containing 100 µl 2% (w/v) X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, Bioline) and 10 µl of 0.1 M IPTG (isopropyl-beta-D-thiogalactopyranoside, Sigma). The plates were incubated overnight at 37°C.

3.2.6.3 Identification of positive colonies by PCR screening

PCR amplification was used to identify positive colonies. White colonies were inoculated, using sterile toothpicks, onto fresh LB-agar plate containing 50 µg/ml ampicillin, and then a small proportion was deposited in a sterile PCR tube. The

LB-agar plate was incubated overnight at 37°C. PCR reactions each contained 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.5 μM of M13 forward [5'-GTAAAACGACGGCCAGT-3'] and M13 reverse [5'-GGAAACAAGCTATGACCATG-3'] primer and 2.5 unit of DNA polymerase in the reaction buffer from Bioline. The PCR reaction was carried out at 95°C for 5 min followed by 35 cycles of 95°C for 30s, 52°C for 30s and 72°C for 1 min, and finally 72°C for 7 min. PCR products were separated on a 1% (w/v) agarose gel and sized by comparing with DNA ladder standard.

3.2.6.4 Culture of the selected transformation colonies

Single colonies containing a PCR insert of the expected size were inoculated using a yellow sterile pipette tip into 2 ml of LB-broth-containing 50 μg/ml ampicillin. The cultures were incubated overnight at 37°C, 250 rpm in an orbital incubator.

3.2.6.5 Plasmid purification

Bacterial culture (1.5 ml) was centrifuged at 14,000g for 5 minutes at room temperature. Plasmid DNA was then isolated using the QIAprep miniprep kit (Qiagen). The pelleted bacterial cells were resuspended in 250 μl buffer P1 (Resuspension buffer containing 0.1 mg/ml RNase A). Alkaline lysis was performed by adding 250 μl of buffer P2 (Lysis buffer) and mixing gently by inverting the tube. Neutralisation buffer, Buffer N3 (350 μl) was added to neutralise the alkali and the tube was inverted immediately, but gently 4-6 times. The solution was centrifuged for 10 minutes at 14,000g to separate cell debris from the plasmid supernatant. The supernatant was transferred to the QIAprep column and the plasmid DNA was bound to the column because of the high-salt binding condition from buffer N3. The column was centrifuged for 1 minute at 14,000g and the flow-through discarded. After washing the column with 0.75 ml buffer PE (Binding buffer) to get rid of contaminants, plasmid DNA was eluted with 30 μl Elution buffer (10 mM Tris-HCl, pH 8.5) and allowed to stand for 1

minute. The column was centrifuged at 14,000g for 1 minute to collect the plasmid DNA.

3.2.7 DNA Sequencing

Plasmid DNA was sequenced in both directions using the vector M13 forward primer and M13 reverse primer. DNA sequencing was performed using the dye terminator sequencing method and the products were electrophoresed on a Beckman Coulter CEQ8000. The sequences were analysed using DNAStar software (Lasergene). The DNA sequences were then searched against the database of the National Institute of Health, USA (http://www.ncbi.nlm.nih.gov/Blast) using the BLAST program in order to confirm their identity with related sequences.

3.2.8 Phylogenetic analysis

Deduced amino acid sequences of GSTs were aligned using ClustalW (Thompson *et al.*, 1994). The alignment was converted to PHYLIP file using TREECON software (Van de Peer & De Wachter, 1993). Phylogenetic trees were generated by TREECON using the distance neighbour-joining method (Saitou & Nei, 1987).

3.2.9 Identification of GST Sequences in the *Aedes aegypti* Bacterial Artificial Chromosome (BAC) Library

3.2.9.1 BAC library construction

The *Aedes aegypti* (Liverpool strain) BAC library was kindly provided by Severson, D.W. from Department of Biological Sciences, University of Notre Dame, Indiana, USA. BAC library construction is described in Jiménez *et al.* (Jimenez *et al.*, 2004). The BAC library was plated as individual colonies in 384 well plates.

3.2.9.2 BAC DNA preparation

BAC DNA was prepared as described previously (Hong *et al.*, 2003). Briefly, the bacterial cells from all wells of a 384 well plate were transferred to a 2x YT agar

plate containing 12.5 μg/ml of chloramphenicol using a 384-pin colony picker. Colonies were grown at 37°C overnight and then BAC colonies from the whole plate pools were transferred into 10 ml LB medium using a flame-sterilised spatula. For row and column pools, BAC colonies were collected by scraping blocks of rows or columns from the agar plate into 1.4 ml LB medium as shown in Figure 3.1 After centrifugation, the pelleted cells were collected and kept at-80°C for DNA extraction.

The BAC DNA was extracted by a modified plasmid purification method using Qiagen purification buffer kit as described in section 3.2.6.5. Plasmid DNA was precipitated by adding 0.7 volume of isopropanol and frozen at –80°C for 1 hour. After centrifugation, the pellets were washed with 70% (v/v) ethanol. The pellets were air dried at room temperature for 5 minutes and then resuspended in 40 μl of TE buffer. The plasmid concentration was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies).

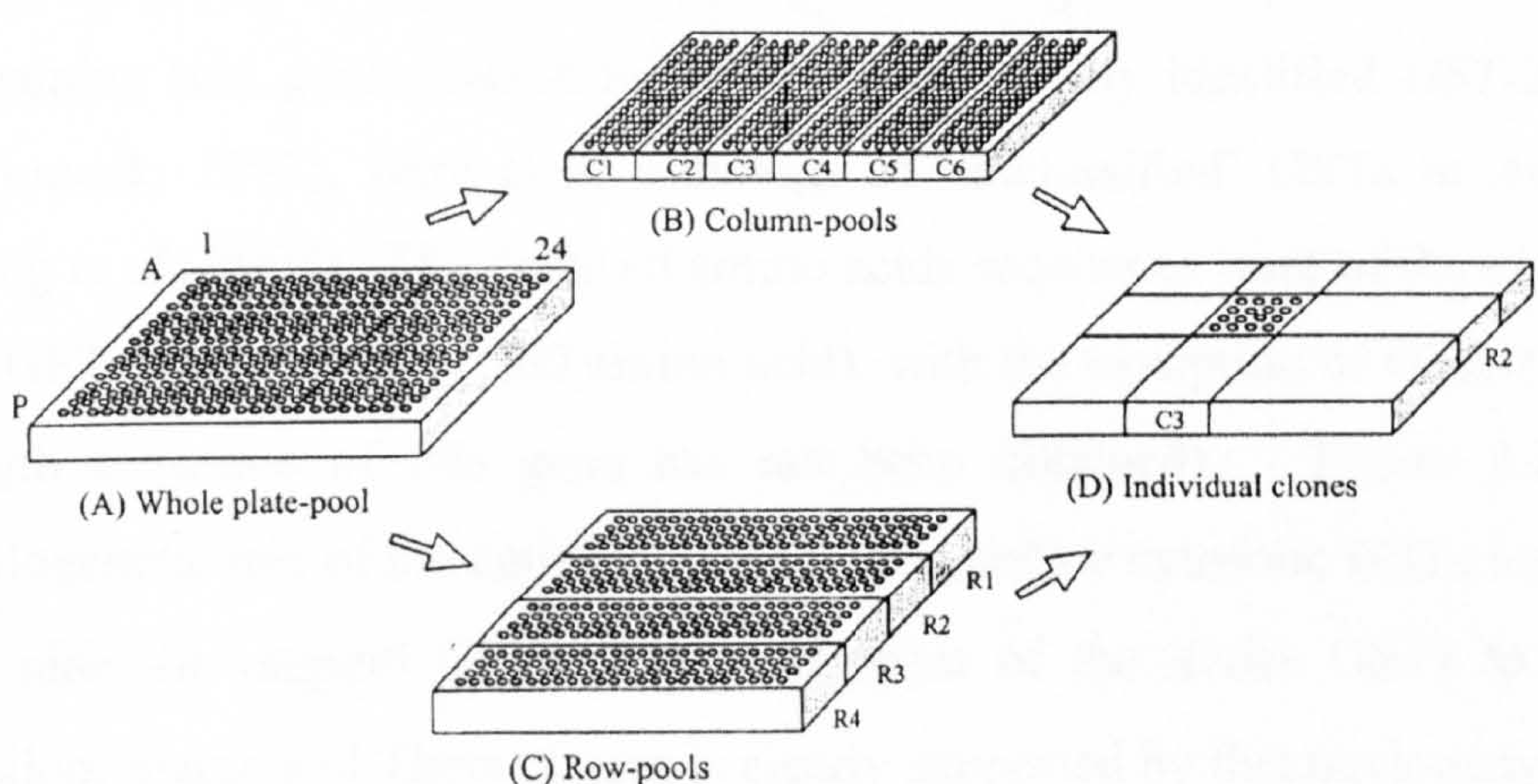


Figure 3. 1 Column and row-pools of BAC DNAs for PCR screening.
Figure is from Hong, *et al.* (Hong *et al.*, 2003).

3.2.9.3 PCR screening of the BAC library

A PCR-based plate pool screening approach, followed by column and row pools and individual colony PCR (Hong *et al.*, 2003), was used to isolate BAC clones containing GST genes. Fifty nanograms of purified BAC DNA was used as a template in PCR reactions using primer pairs to amplify full length or partial GSTs. If positive clones were obtained from the whole plate pools, the column and row pools were screened to further localise the positive colony. Finally individual clones were used as PCR template to obtain a single positive colony.

3.3 RESULTS

3.3.1 Identification of *Ae. aegypti* GST genes

Nine putative GST sequences were retrieved from the TIGR database and each of these was then searched against the non-redundant database at NCBI, using BLASTX, to confirm their identity as putative GST genes. Of the nine *Ae. aegypti* GSTs, two were classified as Delta, two as Epsilon, one as Sigma and two as Theta class, according to sequence similarities with *An. gambiae* GSTs (Table 3.2). The remaining two genes, which included the previously identified *GST-2* (Grant & Hammock, 1992), were clear orthologs of 'unclassified' GSTs in *An. gambiae* (Ding *et al.*, 2003). The deduced amino acids sequences were of the expected size for GST (approximately 200 amino acid), with the exception of *GSTd11* (the full-length sequence of this gene has not been obtained). Figure 3.2 shows a phylogenetic tree of the entire family of *An. gambiae* cytosolic GSTs together with the nine *Ae. aegypti* GSTs. The assignment of the *Aedes* GSTs to the Delta, Epsilon, Sigma and Theta classes is clearly supported by this phylogeny. There are secure orthologs between the *Ae. aegypti* GSTs and *An. gambiae* GSTs, supported by bootstrap values of >90 %, except for *GSTt2* (Figure 3.2).

Table 3. 2 The *Aedes aegypti* GST EST sequences from TIGR database.

TC number	TC Length (bp)	Length of Coding sequence	Similarity with *An. gambiae* GSTs	GST class	Proposed name of *Ae. aegypti* GSTs
TC5882	1377	1-218	GSTu3, complete (65%)	unclassified	GSTu3*
TC13554	970	1-229	GSTt1, *partial* (51%)	Theta	GSTt2
TC7575	857	1-227	GSTu1, *partial* (96%)	unclassified	GSTu1
TC9454	990	1-195	GSTs1-2, *complete*	Sigma	GSTs1
TC10552	730	1-222	GSTe2, *partial* (90%)	Epsilon	GSTe2
TC11673	1509	1-224	GSTe4, *partial* (94%)	Epsilon	GSTe4
TC11721	905	1-229	GSTt1, *partial* (91%)	Theta	GSTt1
TC12233	642	34-209	GSTd11, *partial* (69%)	Delta	GSTd11
TC9051	942	1-209	GSTd1-5, *complete*	Delta	GSTd1

*indicates this gene has been identified previously and named GST-2. TCs indicate tentative concensus sequences, which were created by TIGR by assembling ESTs into virtual transcripts.

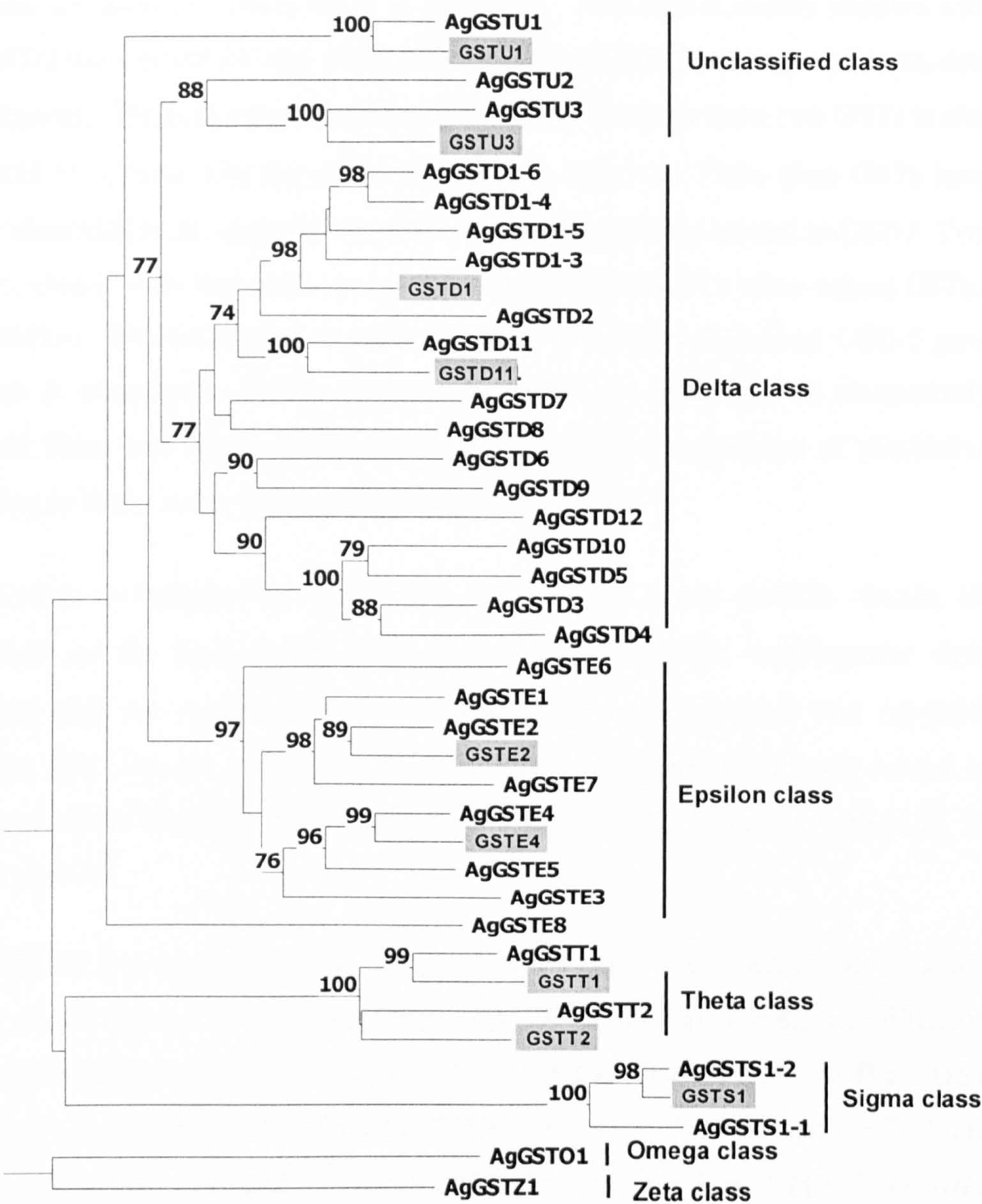


Figure 3. 2 Phylogenetic relationships of nine GSTs in *Ae. aegypti*, with all *An. gambiae* GSTs

Amino acid sequences were aligned using ClustalW (www.ebi.ac.uk/clustalW) and a distance neighbour-joining tree was generated using TREECON (Van de Peer & De Wachter, 1993). Nodes with distance bootstrap values (1000 replicates) of >70% are shown. GSTs from *Ae. aegypti* are highlighted in grey.

The phylogenetic relationship between this second *Ae. aegypti* Theta class GST and the *An. gambiae* Theta GSTs is less clear. Although it clearly clusters with *AgGSTt2* the support for this clade is low (61 % of 1000 bootstrap replicates, data not shown). The percentage amino acid similarity between these two GSTs is also low (51.1% (Table 3.3) but given that, to date, only two Theta class GSTs have been identified in *Ae. aegypti*, this GST has been tentatively named as *GSTt2.* Two GSTs, clearly orthologs with *An. gambiae* unclassified GSTs were named *GSTu1* and *GSTu3*. The latter gene is identical to the previously described GST-2 gene (Grant & Hammock, 1992). *GSTd1* and *GSTs1* are orthologs of alternatively spliced Delta and Sigma GSTs in *An. gambiae* and the presence of alternative splicing in these *Aedes* genes is investigated in Chapter 7.

To further investigate the relationship between the insect specific classes, all members of the Epsilon and Delta class from *Drosophila melanogaster* were aligned with the mosquito GSTs and the phylogenetic analysis was repeated. (Figure 3.3). The *Ae. aegypti* Epsilon and Delta GST genes are closely related to the equivalent families in *An. gambiae* but distantly related to those in *D. melanogaster*.

To confirm the sequence of the ESTs and to determine the structure of the genes from which they were derived, primers were designed to enable the amplification of the full coding region of each gene. Only the two Epsilon and two Theta GST genes were amplified in this CHAPTER. However, *GSTu1* and *GSTu3* are described later in CHAPTER 6 and *GSTd1* and *GSTs1* in CHAPTER 7. *GSTd11* was not studied further as a full length sequence was not available to this gene.

The percentage similarities of *Aedes* GSTs and their orthologs in *An. gambiae* range from 51.1 - 71.5 % (Table 3.3). The deduced amino acid sequences of the four *Ae. aegypti* GSTs are aligned in Figure 3.4.

Table 3. 3 Percentage identities between mosquito GST protein sequences.

	Percent identity							
	GSTE2	**GSTE4**	**AgGSTE2**	**AgGSTE4**	**GSTT1**	**GSTT2**	**AgGSTT1**	**AgGSTT2**
GSTE2	-	54.5	71.5	55.4	21.2	15.3	20.7	17.1
GSTE4		-	52.9	71.0	22.3	18.8	19.2	18.8
AgGSTE2			-	53.4	21.7	17.6	23.1	20.4
AgGSTE4				-	18.7	16.0	20.9	20.0
GSTT1					-	52.4	66.8	46.7
GSTT2						-	54.2	51.1
AgGSTT1							-	50.9
AgGSTT2								-

Percentage similarity was determined using the DNASTAR software. The grey highlights indicate the percentage similarity between putative *Ae. aegypti* and *An. gambiae* (Ag) orthologs.

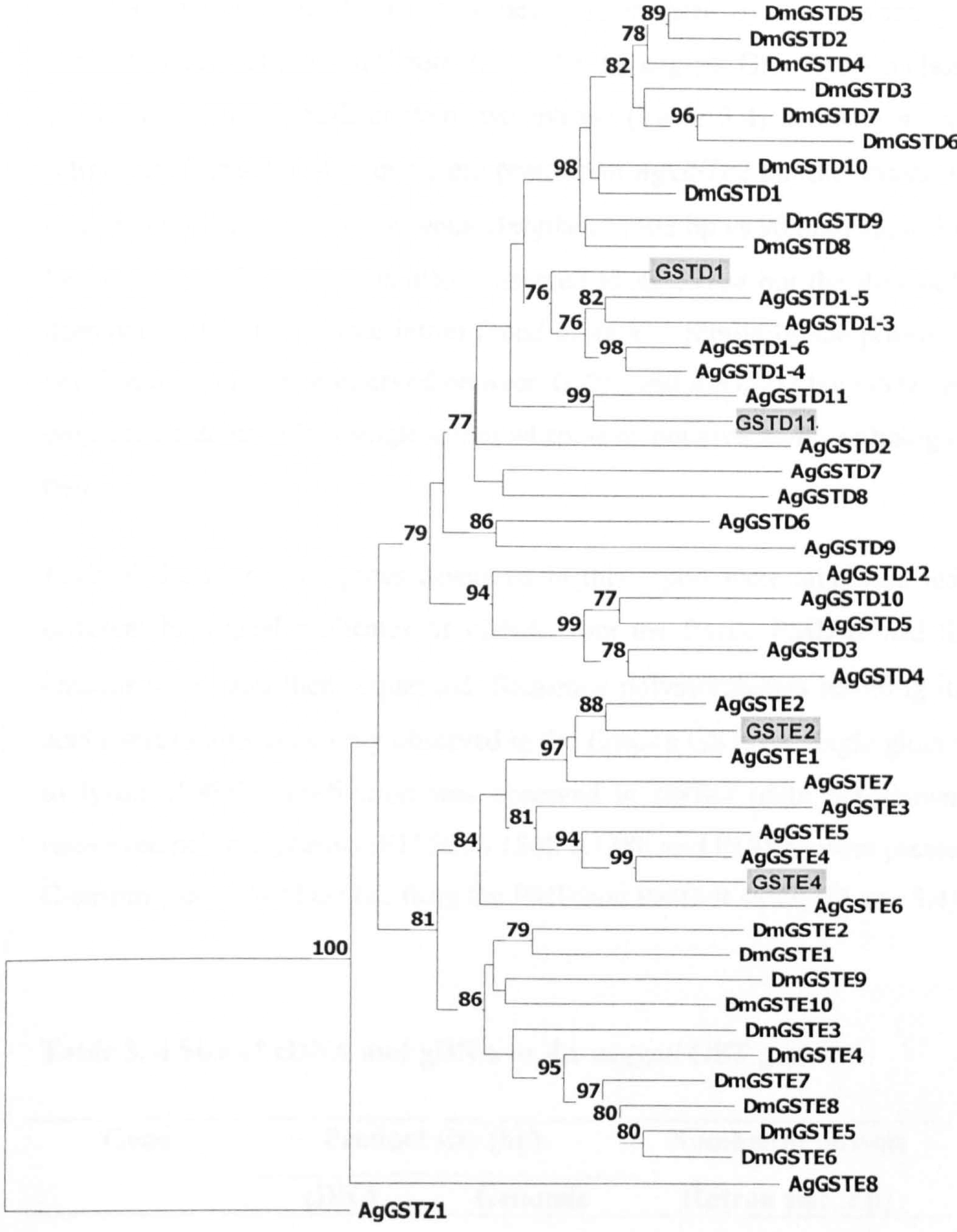


Figure 3. 3 Phylogenetic relationships of Epsilon and Delta GST classes in insects

Amino acid sequences were aligned using ClustalW and a distance neighbour-joining tree was generated using TREECON. Nodes with distance bootstrap values (1000 replicates) of >70% are shown. GSTs highlighted in grey are from *Ae. aegypti*. Ag = *An. gambiae* and Dm = *D. melanogaster*.

The coding sequence of all four genes is interrupted by two introns (positions marked as vertical lines in Figure 3.4). The *Ae. aegypti* GST Epsilon class genes, *GSTe2* and *GSTe4*, both contain two introns (Table 3.4) at identical positions. Introns are located at the equivalent position in *AgGSTe2* but the second intron is considerably longer in *Aedes* versus *Anopheles* (503 bp vs 90 bp) (Table 3.4). The location of the first intron is also conserved in *AgGSTe4* but the *Anopheles* gene does not contain the second intron found in *Aedes*. Similarly, the positions of the two Theta introns are conserved between *GSTt1* and *AgGSTt1* but *GSTt2* from *An. gambiae* contains only a single intron whereas its putative *Aedes* ortholog contains two.

Each of the four GST genes described in this report were amplified using five different biological replicates of cDNA from the PMD, PMD-R and the New Orleans strain and then sequenced. Sequence polymorphisms resulting in amino acid substitutions were only observed in the Epsilon GSTs. A single glutamic acid to lysine (E89K) substitution was observed in *GSTe4* (data not shown). Four conserved polymorphisms (F115C, V150I, A178E and E198A) were present in the C-terminal domain of *GSTe2* from the PMD and PMD-R strain (Figure 3.4).

Table 3. 4 Size of cDNA and gDNA in *Ae. aegypti* GST genes.

Gene	Product size (bp)		Number of introns
	cDNA	Genomic	(Intron size; bp)
GSTe2	666	1230	2 (61 and 503)
GSTe4	672	812	2 (75 and 65)
GSTt1	687	802	2 (53 and 62)
GSTt2	681	1150	2 (400 and 69)

```
        -----------------------------------------------------------
GSTe2   MT---KLILYTLHVSPPCRAVELCAKALGLELEQKTVNLLTKEHLTPEFMK-|MNPQHTVP 56
GSTe4   MG---KVQLYTAKLSPPGRAVELTAKAIGLDLDVHPINLIAGDHLKPEFVK-|MNPQHTIP 56
GSTt1   MS---KLRYFYDLMSQPSRMLYIFLESTKIPYERCLVNLGKG|EHLT-DKFKAINRFQKVP 56
GSTt2   MANGRNIRFYYDLISQPCRALYIFLEQNKIHYQKCPIALRKW|EHTTPEYLQNVNRFGKVP 60

        -----------------------------------------------------------
GSTe2   -VLDDNGTIVCESHAIMIYLVSKYGKDDSLYSKELVKQAKLNAALHFESG----VLFARS 111
GSTe4   LIVDEDGTIVYDSHAIIIYLVSKYAKDDSLYPEDIATRAKINAALHFDSG----VLFARL 112
GSTt1   CIVDKNDLHLAESVAIVRYLAREYPFSDHWYPKDSQKRARIDEYLEWQQHNTRAVCATYF 116
GSTt2   AIVDGKNFKLAESIAILRYLAREFTVPDHWYPRDSRRRARVDEYLEWQHSNTRLHCAGYV 120

        ---C-------------------------------------I-------------
GSTe2   RFVF|EPILFAGG---SEIPADRAEYVQKAYQLLEDTLVD---DYVVGNSLTIADFSCVSS 165
GSTe4   RFYL|EPILYYGS---PDTPQDKIDYACKAYQLLNDTLVD---EYIVGNRMTLADLSCIAS 166
GSTt1   QYVWLRPKLMGTKVNPERAEEYKQKMEDCLDFIESDYLGGGNPFLVGNEISVADLFAACE 176
GSTt2   RYVWRGP-LRGETMDPRVAKRLKAEMVGCLDFIETNVLQRDVHFIAGDEISIADLVAACE 179

        ------------E-----------------A-----------------------
GSTe2   VSSIMGVIPMDKAKFPKIYGWLDRLKALPYYEEANGSGAEQVAQFVLSQKEKN-AQKA   222
GSTe4   IASYHAIFPIDAAKYPKLAAWVQRLEKLPYYKGTNQEGAEELAAVYRDRLAQNRAGKK   224
GSTt1   IEQP|KMAGFDPCVGRPKMTAWMARVREAT--NPHYDEAHKLVYRIAPDSVPKPKL---   229
GSTt2   IEQP|KLAGYDARVGRPKLTAWMQRVKETT--QPDYDEAHKVLNKFAPTAT--------   227
```

Figure 3. 4 The deduced amino acid sequences of *Ae. aegypti* Epsilon and Theta GSTs.

Sequences shown are from the New Orleans strain. Four amino acid substitutions found in *GSTe2* from PMD and PMD-R strain are shown in line 1 of the alignment. The amino acid sequences were aligned using ClustalW. Letters in bold indicate 100% conservation between the 4 sequences. Dashes are used to denote gaps introduced for maximum alignment. Vertical lines indicate the intron positions. GenBank accession numbers for *GSTe2*, *GSTe4*, *GSTt1* and *GSTt2* are AY819710, AY819709, AY819711 and AY819712, respectively.

3.3.2 BAC library screening using PCR

Screening of the *Ae. aegypti* BAC library with primers specific to *GSTe4* identified a single positive clone (ND17A9). PCR screening of the BAC DNA isolated from this clone with primers designed for partial *GSTe2* gave a positive signal (Figure 3.5A), whose identity was confirmed by DNA sequencing (data not shown). The average insert size of the BAC clones in the NDL library is 122kb (Jimenez *et al.*, 2004) and this result indicates that the two Epsilon GSTs are in close proximity in the genome. Clusters of Epsilon GST genes are found in *D. melanogaster* and *An. gambiae* (Ding *et al.*, 2003; Sawicki *et al.*, 2003). An attempt to amplify the intergenic region between *GSTe2* and *GSTe4* to obtain other putative member of this GST gene cluster was unsuccessful. This may indicate that genes in this region are separated by several kilo base pairs. Screening the BAC library with the Theta GST primers for both *GSTt1* and *GSTt2*, led to the identification of a single positive clone, ND2C20 (Figure 3.5B), again suggesting that the *Aedes* Theta GSTs are close together. However, PCR amplification of the intergenic region between *GSTt1* and *GSTt2* in this clone was not attempted.

3.4 DISCUSSION

Nine GSTs were identified from the *Ae. aegypti* EST database. Phylogenetic analysis identified clear orthologs for the majority of these genes in *An. gambiae*. This finding suggested that GST gene family in *Ae. aegypti* and *An. gambiae* diverged prior the split of these species (approximately 100 million years ago) (Krzywinski *et al.*, 2001).However when the mosquitoes are compared with the fruitfly, it is apparent that the insect-specific GST classes (Epsilon and Delta) have evolved separately in these two insect lineages which diverged approximately 250 million years ago (Severson *et al.*, 2004).

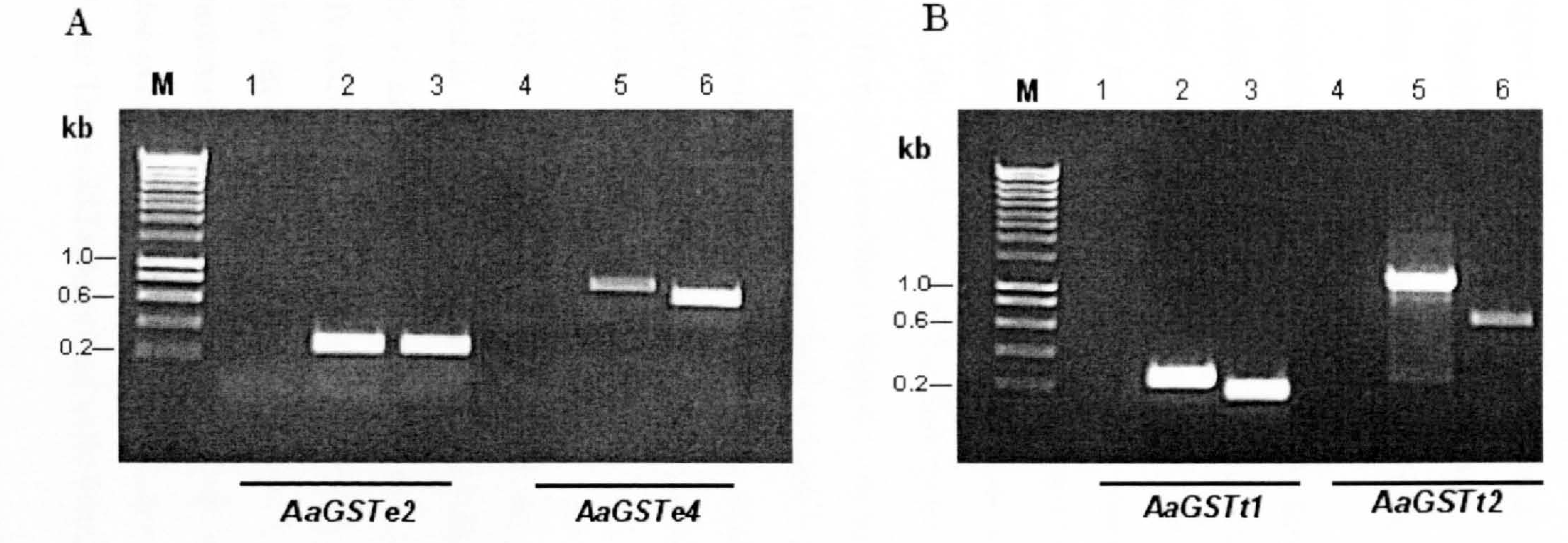


Figure 3. 5 BAC library screening for *Ae. aegypi* GSTs using PCR.

(A) PCR screening for Epsilon GSTs; *GSTe2* and *GSTe4* in ND17A9 clone, M indicates to DNA marker; lane 1 and 4 are negative controls; lane 2 and 5 represent to ND17A9 plasmid from BAC DNA; lane 3 and 6 are PMD-R cDNA from *Ae. aegypti*. **(B)** PCR screening for Theta GSTs; *GSTt1* and *GSTt2* in ND2C20 clone, lane 1 and 4 are negative controls; lane 2 and 5 represent to ND2C20 plasmid from BAC DNA; lane 3 and 6 are PMD-R cDNA from adult *Ae. aegypti*.

The coding sequences and the intron positions of the GSTs are very conserved between *Ae. aegypti* and *An. gambiae,* but the intron sizes are considerably large in *Ae aegypti.* The genome size of *Ae. aegypti* is predicted to be approximately 3 times bigger than *Anopheles* (Severson *et al.*, 2004)and therefore it is not surprising to find larger introns in this species.

Polymorphisms in the GST sequences between the three strains of *Ae. aegypti* were observed. Although only a small number of clones were sequenced, and therefore these results are tentative, four amino acid polymorphisms were observed within *GSTe2* between the susceptible and resistant strain. It is not known if these substitutions affect the catalytic activity of GSTe2. However, since three of these four substitutions are conserved between GSTe2 from the insecticide resistant *An. gambiae* and the susceptible *Ae. aegypti* strain, and the GSTe2 protein from *An. gambiae* is known to metabolise DDT (Ortelli *et al.*, 2003), these substitutions are thought unlikely to have a dramatic effect on substrate specificity. One mutation (alanine at position 178 to glutamic acid) found in the resistant strain is unique to the PMD strain and so it could possibly be important in determining the insecticide resistance phenotype.

BAC DNA screening indicated that the Epsilon and Theta class GSTs are clustered in the *Aedes* genome. Eight Epsilon GSTs, are clustered in *An. gambiae* (Ortelli *et al.*, 2003). It is expected that further sequencing of this region will identify additional *Ae. aegypti* Epsilon GSTs. In contrast, the Theta GSTs in *An. gambiae* are not clustered. Both *AgGSTt1* and *AgGSTt2* are located on the X chromosome but on polytene band, 4B and 1A, respectively (details on *An. gambiae* genome database). The preliminary data from this study suggests that the two *Aedes* Theta GSTs described in this chapter are tandemly arranged.

The results in this chapter describe the identification and cloning of eight novel *Aedes* GSTs. At the time of this study, sequencing of the *Aedes aegypti* genome

was at a preliminary stage. However during the writing of this thesis, the whole genome shotgun sequence was released in an unassembled form. As expected, additional sequences with high identity to GSTs were found in this dataset. This is discussed further in the final chapter.

Chapter 4

QUANTIFICATION OF GST EXPRESSION

4.1 INTRODUCTION

Five of the eight epsilon class GSTs in *An. gambiae* are expressed at significantly higher levels in a DDT resistant strain relative to the susceptible strain (Ding *et al.*, 2003) and one of these, *AgGSTe2*, has been shown to confer increased DDT metabolism in the resistant strain (Ortelli *et al.*, 2003). In the previous chapter, the cloning of the ortholog of this gene from *Ae aegypti* was described. In this chapter, the expression of multiple *Ae. aegypti* GSTs in strains differing in their susceptibility to insecticides was determined using quantitative PCR. Four GST genes were used in this study including the two Epsilon GSTs, *GSTe2* and *GSTe4*. Unclassified GSTu3 (*GST-2*) was also included in this study as it has been associated with DDT resistance in *Ae. aegypti* from South America (Grant & Hammock, 1992). Finally, a GST from the Theta class (*GSTt1*), a class not previously correlated with insecticide resistance was included as a control.

The incorporation of SYBR GREEN I into double stranded PCR products was determined by the acquisition of fluorescence, and this was used to quantify the copy number of the gene. The ribosomal protein gene, *SP7* (accession number AY380336) was used as an internal control to normalise for variation in concentration of the templates. To compare the expression in different samples, the ratio between the copy number of the gene of interest and the copy number of the internal gene was calculated. The expression levels of the two Epsilon genes, *GSTt1* and *GSTu3* were established for different life stages in three strains of *Ae. aegypti*.

4.2 MATERIALS AND METHODS

4.2.1 Mosquito Strains

The mosquitoes used in this study were from the susceptible New Orleans, PMD and DDT/permethrin resistant PMD-R strains described in Chapter 2.

4.2.2 Sequencing of GST Genes

The full-length of *GSTe2, GSTe4, GSTu3* and *GSTt1* were amplified as described in section 3.2.5 using cDNA from the susceptible New Orleans, PMD and DDT/permethrin resistant PMD-R strains. The PCR products were ligated into the pGEM-T easy vector and then transformed into *E. coli* JM109 competent cells (Promega). DNA sequencing was performed to verify the sequence integrity.

4.2.3 Quantitative PCR on cDNA

4.2.3.1 cDNA synthesis

Total RNA was extracted from 3 replicate sets (10 mosquitoes per replicate) of 4th instar larvae, pupae, one-day old adult males or females from each of the three strains as described in section 3.2.3. Total RNA was reverse transcribed to cDNA as described in section 3.2.4.

4.2.3.2 Plasmid construction

The *GSTe2, GSTe4, GSTu3* and *GSTt1* standard plasmids were constructed by the insertion of the full length cDNA from the PMD-R strain into pGEM-T easy vector as described in section 4.2.2. An alternative *GSTe2* plasmid was established from the New Orleans susceptible strain as the primer binding sites encompassed a polymorphic nucleotide. The partial ribosomal S7 gene, used as an internal control plasmid, was amplified using primers aaS7F and aaS7R (Table 4.1) and ligated into the pGEM-T easy vector. Plasmid concentrations were determined using a ND-1000 spectrophotometer (NanoDrop Technologies).

Table 4. 1 Primer sequences for quantitative PCR.

Gene	Primer name	Primer sequences (5' to 3')	cDNA size (bp)	Annealing/Detection Temp (°C)
GSTe2	GSTe2F2	TATGTGCAAAAGGCTTACCAACTG	270	60/84
	GSTe2SusF2	TATGTGCAAAAGGCATACCAACTG		
	GSTe2R	TGCCTTTTGAGCATTCTTCTCC		
GSTe4	GSTe4F2	CAAGATTGACTACGCGTGCAAG	283	60/84
	GSTe4R	CGAACAATGCAATTACTTCTT		
GSTu3	GSTu3F2	ATCCTGGAAGCCCTAGGAAC	258	60/83
	GSTu3R	TTAGAAAGGTTCCTCCAGCTTG		
GSTt1	GSTt1F2	GAGATTTCCGTTGCGGATCTG	195	60/84
	GSTt1R	CAACTTCGGCTTCGGAACG		
S7	aaS7F	GCAAGCACGTCGTGTTCATCGGC	296	60/87
	aaS7R	GAACGTAACGTCACGTCCGGTCAG		

GSTe2SusF2 is the forward primer for amplifying *GSTe2* from the New Orleans strain.

4.2.3.3 Quantitative PCR and standard curve preparation

The *GSTe2*, *GSTe4*, *GSTu3*, *GSTt1* and S7 standard plasmids were diluted to produce a range of concentrations from 1fg/µl to 1ng/µl. The specific primer pairs for each gene were designed to give products less than 300bp. The primers used in quantitative PCR reaction are shown in Table 4.1. Alternative primers were used for amplifying *GSTe2* from the susceptible strain as the primer binding sites encompassed a polymorphic nucleotide. The plasmid and cDNA templates were amplified using QuantiTect SYBR Green PCR Kit (Qiagen). Quantitative PCR was conducted by amplifying 1 µl of cDNA with 0.5 µM of each primer and 1X SYBR Green PCR Master Mix in a total volume of 10 µl. The amplification conditions were 95°C for 15 min. followed by 35 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s with fluorescence read at 82°C. Plasmid DNA standards and negative controls were included in the same plate, for each experiment. Three biological replicates from each strain and life stage were used as templates. Each sample was analysed in duplicate in each experiment and the results were averaged from three independent experiments. The incorporation of SYBR Green I into double stranded PCR products was quantified using the DNA Engine Opticon (MJ Research). The relationship between fluorescence and the cycle number was plotted and the crossing line was produced to obtain the standard concentration related to the cycle number as shown in Figure 4.1. To achieve the straight line of the standard curve, the cycle number was plotted with the logarithmic value of the standard concentrations as demonstrated in Figure 4.2.

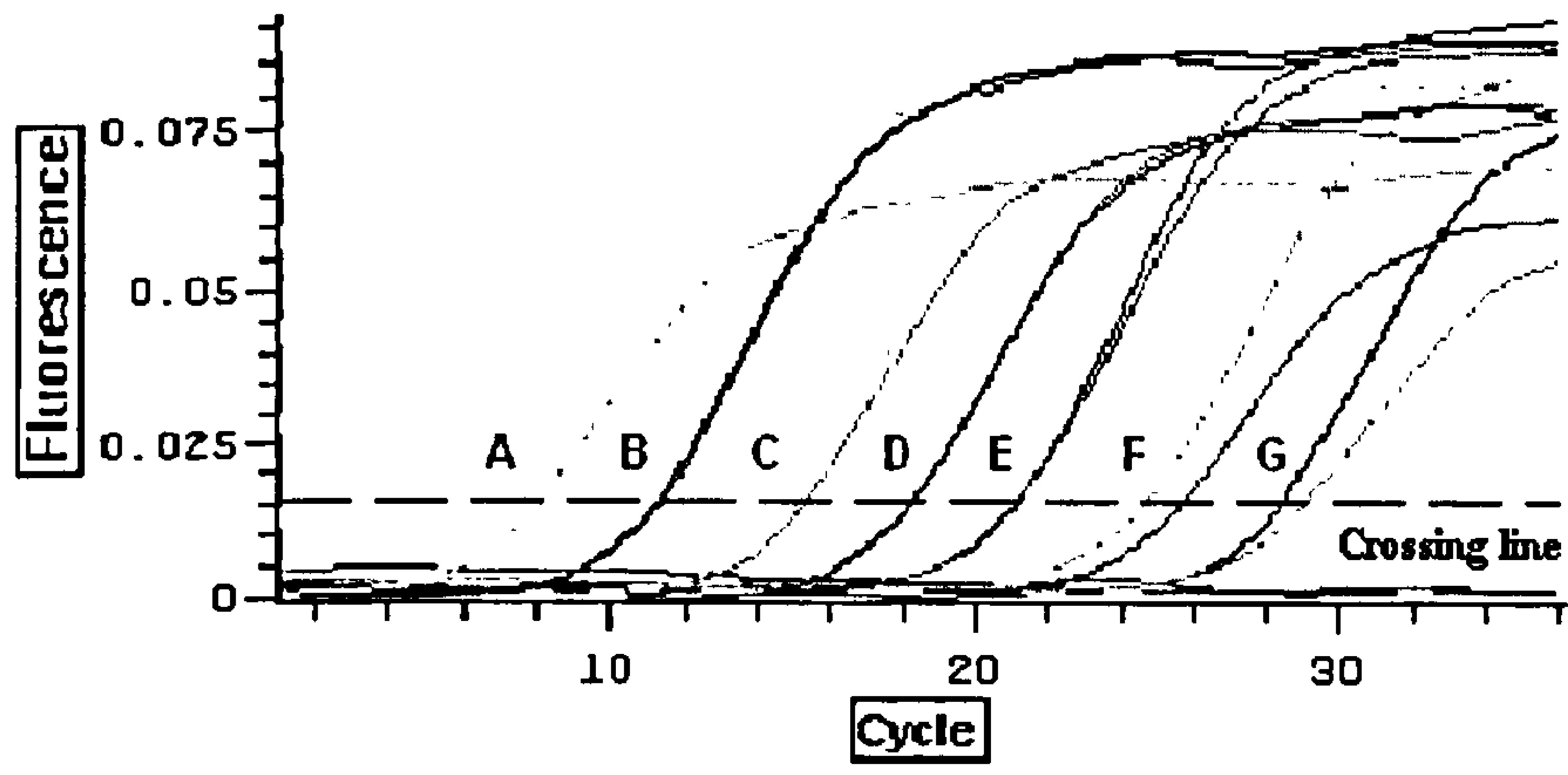


Figure 4. 1 Standard curves for quantitative PCR.

The X-axis represents the cycle number and the Y-axis indicates the value of the fluorescence acquisition. The crossing line was created to extrapolate the standard plasmid concentrations in different cycles. A to G indicate the 10 fold dilution of standard concentrations ranging 1ng/μl to 1fg/μl.

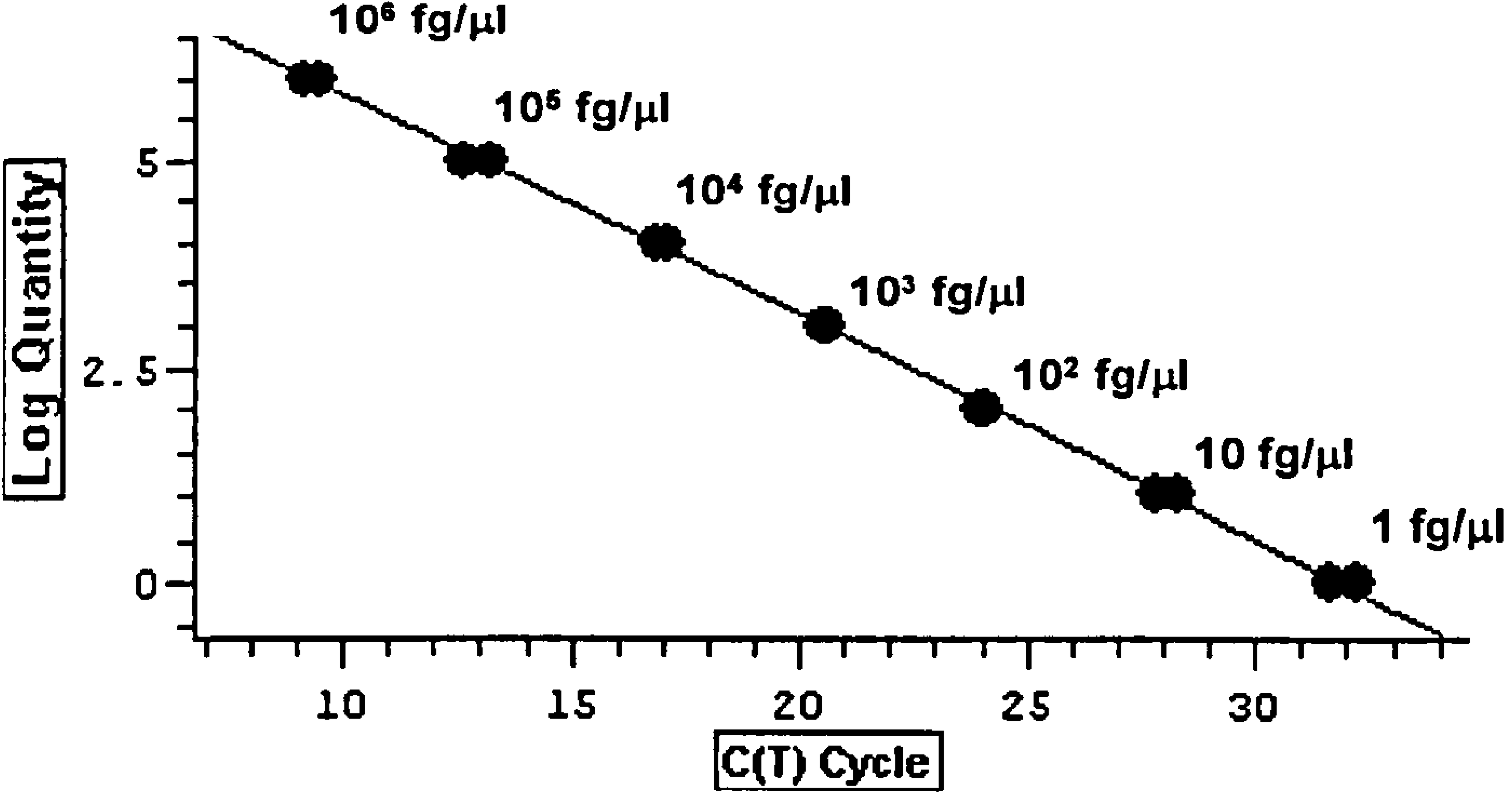


Figure 4. 2 Standard curve for quantitative PCR.

Log concentration of standard plasmid plotted against the number of cycles.

4.2.3.4 Copy number calculation

The number of copies of mRNA for each gene of interest was calculated by measuring the incorporation of the fluorescent dye into the double stranded PCR product and comparing this value to a standard curve produced from the PCR amplification of the same gene fragment from plasmids of known concentrations. The mRNA copy number of each transcript was calculated as shown in the equation as below. To normalise for variation in total cDNA concentration in different batches, the copy number of a ribosomal gene, *S7* was similarly determined. The normalised mRNA copy number was calculated by dividing the copy number of target transcript with copy number of *S7* transcript.

4.3 RESULTS

4.3.1 Quantitative Analysis of *Ae. aegypti* GST Expression

Elevated expression of Epsilon GSTs has been implicated in conferring resistance to DDT in *An. gambiae*. To determine whether the *Ae. aegypti* GSTs are also elevated in DDT resistant mosquitoes, the mRNA copy number of the *Ae. aegypti* GST genes in different life stages of the three strains was determined. The relative copy number of each gene was calculated by normalising with the ribosomal S7 gene. Expression of all four genes was detected in all life stages assayed (Figure 4.3). The expression of *GSTe2* was significantly higher in the DDT-resistant PMD-R strain in all life stages except adult male ($p = 0.19$) ($p < 0.001$ in pupae and adult female, and $p < 0.05$ in larvae) when compared to the susceptible strain, and in all life stages ($p < 0.001$ in pupae and adult female, $p < 0.01$ in adult males and $p < 0.05$ in larvae) when compared to the parental PMD strain (Table 4.2). In contrast no significant differences in *GSTe2* expression was detected between the parental PMD strain and the New Orleans strain in any life stage (Table 4.2).

Copy number / fg = Moles/fg of plasmid X Avogadro's constant

<u>Example for copies number calculation:</u>

Moles/fg of plasmid = Molecular weight of GSTe2 + Molecular weight of pGEM T easy vector

Full length of GSTe2 = 660 bp

A+T = 334 X 697 = 232798 g/mole

C+G = 332 X 712 = 236384 g/mole

= 469182 g/mole

T easy vector = 2124135 g/mole

= 2593317 g/mole

∴ Moles/fg of plasmid $= 10^{-15} / 2593317$

$= 3.8561 \times 10^{-22}$

Copy number /fg $= 3.8561 \times 10^{-22} \times 6.023 \times 10^{23}$

= 232.25 copies/fg

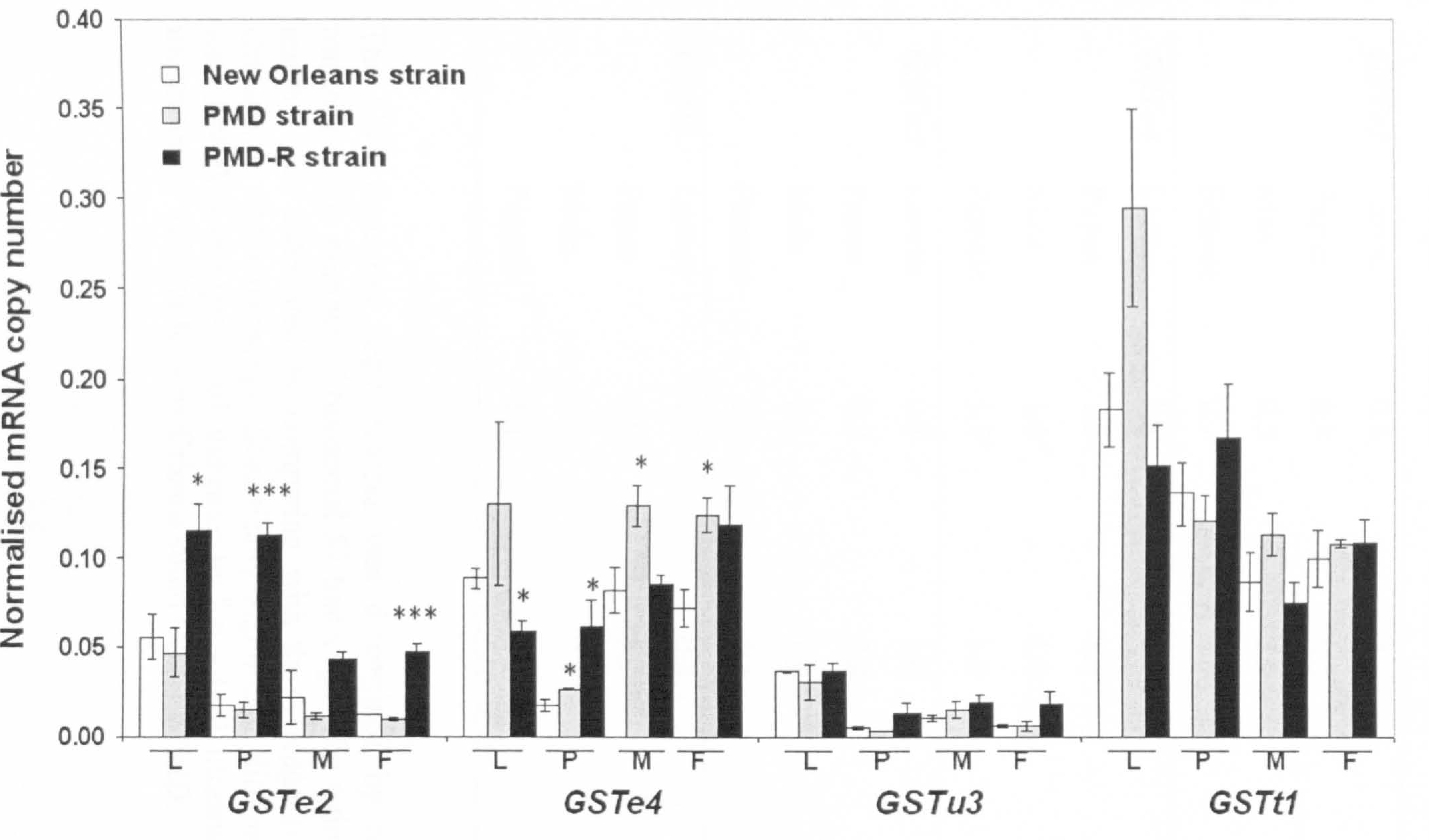


Figure 4. 3 Quantification of GST mRNA expression level in three strains of *Ae. aegypti*.

Complementary DNA from three different biological replicates (ten mosquitoes each) was used as templates. Four life-stages were analysed; larvae (L), pupae (P), adult male (M), and adult female (F). Each sample was analysed in duplicate in each experiment and the results were averaged from three independent experiments. The mRNA copy numbers were determined by comparison with known concentrations of standard plasmids and normalised against the copy number of the ribosomal S7 transcript. Error bars indicate standard error of the mean. Statistically significant differences were evaluated with ANOVA test ($p< 0.001$ indicated by *** and $p < 0.05$ as * relative to New Orleans strain.

Table 4. 2 Quantitative PCR results of *Ae. aegypti* GSTs.

Gene	Life Stages	RATIO OF COPY NUMBER		
		PMD/NO	PMD-R/NO	PMD-R/PMD
GSTe2	Larvae	0.8	2.1[c]	2.4[c]
	Pupae	0.8	6.5[a]	7.7[a]
	Male	0.5	2.0	3.8[b]
	Female	0.7[c]	3.9[a]	5.3[a]
GSTe4	Larvae	1.5	0.6[c]	0.4
	Pupae	1.5[c]	3.6[c]	2.3
	Male	1.6[c]	1.0	0.6[c]
	Female	1.7[c]	1.6	1.0
GSTu3	Larvae	0.8	1.0	1.2
	Pupae	0.7	2.8	3.7
	Male	1.4	1.9	1.3
	Female	1.0	3.2	3.2
GSTt1	Larvae	1.6	0.8	0.5
	Pupae	0.9	1.2	1.4
	Male	1.3	0.8	0.6
	Female	1.1	1.1	1.0

The GST transcript copy number was determined by normalising with the transcript copy number of ribosomal *S7* transcript. The ratio of the average copy number was calculated by comparing with the average copy number of New Orleans or PMD transcript. Statistically significant differences were evaluated with ANOVA test ($p < 0.001$ indicates by [a], $p < 0.01$ illustrates as [b] and $p < 0.05$ illustrates as [c] relative to New Orleans strain (NO) or PMD.

For *GSTe4*, *GSTu3* and *GSTt1*, gene expression was not correlated with insecticide resistance status. However, the expression of *GSTe4* fluctuated in parental PMD and PMD-R strain in different stages relative to the susceptible strain. Expression of *GSTe4* was significantly higher in pupae and adults of parental PMD strain, but not in PMD-R strain ($p < 0.05$). In PMD-R strain, differences in *GSTe4* expression were observed in immature stages when compared with the susceptible strain (lower expression in larva stage, but higher expression in pupae).

4.4 CONCLUSIONS

The expression of four *Ae. aegypti* genes encoding GSTs, *GSTe2*, *GSTe4*, *GSTu3* and *GSTt1* were measured at mRNA levels. *GSTe2* was significantly over-expressed in all life-stages, except in adult male mosquitoes in PMD-R compared to parental PMD and the susceptible New Orleans strain. The elevated expression of *GSTe2* in PMD-R, over the parental PMD strain suggests that this is a consequence of DDT and permethrin selection. Interestingly, *GSTu3* is up-regulated in a DDT resistant strain from South America (Grant & Hammock, 1992; Grant & Mutsumura, 1989) but there is no significant increase in the expression of this gene in the Thai DDT resistant strain, and thus the results suggest that different GST based resistance mechanisms have evolved in the two geographically distant populations of *Ae. aegypti.*

GSTe2 expression was slightly lower in parental PMD than the susceptible New Orleans strain in all life-stages and hence *GSTe2* expression did not, correlate with total GST activity level in adult and immature stages as determined in CHAPTER 2. The correlation between GST activities against CDNB and the mRNA levels are shown in Figure 4.4. There is no correlation between GST activity and *GSTe2* mRNA levels ($R^2 = 0.17$) or the sum of the mRNA level from all four GST genes, *GSTe2*, *GSTe4*, *GSTu3* and *GSTt1* ($R^2 = 0.41$). In adult mosquitoes, CDNB activity was approximately 2 fold higher in the parental PMD relative to New Orleans susceptible strain (Table 2.2), but selection with DDT did not result in any further

increase in total GST activity. However this pattern is not reflected in *GSTe2* expression levels; GSTe2 transcript levels do not differ between New Orleans susceptible strain and parental PMD, but selection of the parental PMD strain results in an approximately 4 fold increase in GSTe2 expression. Clearly, this is only a preliminary study as only subset of GSTs were studied, but this initial results suggest that the biochemical assay with CDNB may not be an accurate measure of GST expression levels. This subject is investigated further in the following chapter. Expression of the remaining genes, *GSTe4*, *GSTu3* and *GSTt1*, was not associated with DDT or permethrin resistance.

The mechanism responsible for the up regulation of *GSTe2* in the PMD-R strain of *Ae. aegypti* is still unknown. Over expression of *GSTu3* in *Ae. aegypti* from South America is regulated by an unidentified *trans*-acting transcriptional factor (Grant & Hammock, 1992). The overexpression of Epsilon GSTs in *An. gambiae* is due to increases in transcript copy number rather than gene amplification (Ding *et al.*, 2003). Specifically, over expression of *AgGSTe2* in *An. gambiae* is at least partially caused by the deletion of two adenosines in the *GSTe2* promoter of the DDT resistant strain (Ding *et al.*, 2005). Further studies are needed in *Ae. aegypti* to determine the factors responsible for the elevated GSTe2 expression in the resistant strain.

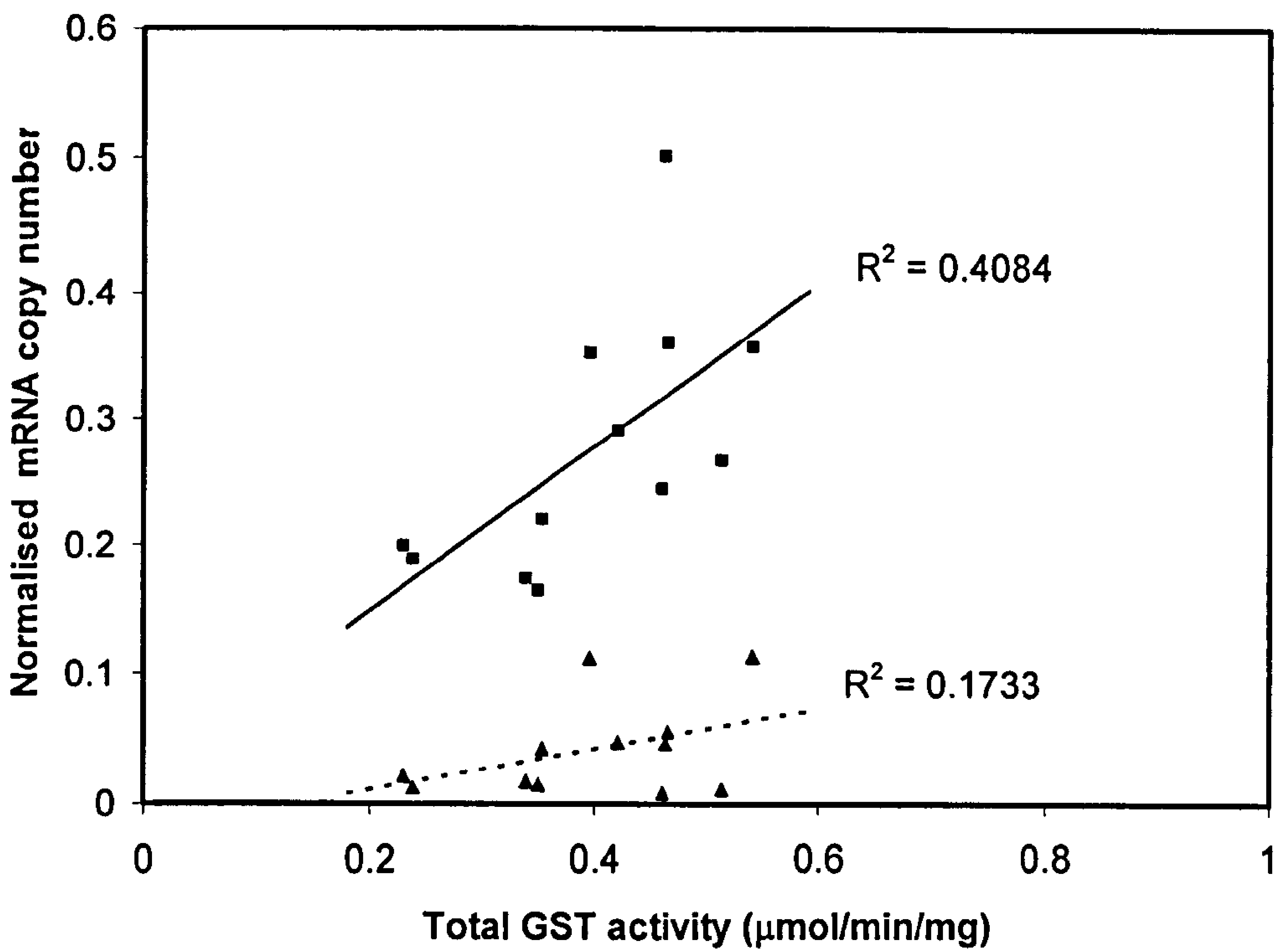


Figure 4. 4 Relationship between total GST activity against CDNB and the mRNA copy number of GSTs from *Ae. aegypti.*

The triangles and the dashed line are the values from the normalised GSTe2 mRNA copy number. Squares and the solid line indicate the values from the sum of the normalised mRNA copy number from four GST genes, *GSTe2*, *GSTe4*, *GSTu3* and *GSTt1*.

Chapter 5

IN VITRO EXPRESSION OF GSTE2 AND CHARACTERISATION OF THE RECOMBINANT PROTEIN

5.1 INTRODUCTION

In the previous chapter, it was shown that *GSTe2* is significantly over expressed in a DDT resistant strain of *Ae. aegypti*. The ortholog of this gene in *An. gambiae* is efficient at metabolising DDT (Ortelli et al, 2003). The aim of this chapter was to express and characterise the recombinant GSTe2 protein in *Ae. aegypti* and determine its role in insecticide resistance in this species.

Recombinant *Ae. aegypti* GSTe2 was expressed using the pET-3a bacterial expression vector. The recombinant protein was purified and characterised using a range of substrates including DDT. Some GSTs are able to bind heme (Harwaldt *et al.*, 2002; van Rossum *et al.*, 2004; Vander Jagt *et al.*, 1985). It has been hypothesised that GSTs may play a protective role in the insect midgut by reducing heme toxicity after a blood meal. Therefore the affinity of recombinant GSTe2 for heme was determined.

5.2 MATERIALS AND METHODS

5.2.1 Mosquito Strains

The *Ae. aegypti* strain used for the GSTe2 expression was the DDT/permethrin resistant PMD-R strain described in CHAPTER 2.

5.2.2 Protein Expression

In vitro GSTe2 expression was performed using the pET bacterial expression system (Novagen). The *GSTe2*-containing plasmid was transformed into *E. coli* expression host cells, BL21 (DE3) pLysS. Expression of the foreign gene is induced by T7 RNA polymerase production, in the presence of isopropyl β-D-thiogalactoside (IPTG).

5.2.2.1 Plasmid construction

The full coding region of *GSTe2* was amplified from cDNA from the PMD-R strain of *Ae. aegypti* using the forward primer aaGSTe2BamF (5'-CGGAATTCGGATCCAT-GACGAAGCTCATTTTGTACACG-3') and the reverse primer aaGSTe2BamR (5'CGGAATTCGGATCCTTATGCC-TTTTGAGCATTCTTCTCC-3'). These primers contain the initiation and stop codon, respectively, flanked by *BamHI* restriction sites (shown underlined). PCR amplification was performed using ProofStart DNA Polymerase Kit (Qiagen) in 1 x PCR buffer (containing Tris-HCl, KCl, $(NH_4)_2SO_4$, 1.5 mM $MgSO_4$, bovine serum albumin, Triton X-100; pH 8.7) 0.3 mM dNTPs, 1 μM of each primers and 2.5 units of ProofStart DNA polymerase in a final volume of 50 μl. Conditions for the PCR were 95°C for 5 min. 35 cycles of 95°C for 30s, 55°C for 30s and 72°C for 1 min. and finally 72°C for 1 min. The PCR product was digested with *Bam*HI and purified using MinElute PCR purification kit (Qiagen). The full-length of *GSTe2* was ligated into the *BamHI* site of the pET3a vector (Novagen) at 16°C overnight. After transforming into the non-expression host, *E. coli* JM109, colonies were screened by PCR using the T7 promoter primer (5'-TAATACGACTCACTATAGGG-3') and aaGSTe2BamR to verify that the product had been inserted in the correct orientation. Positive colonies were isolated and grown overnight in 2 ml LB-medium containing ampicillin (50 μg/ml) at 37°C with shaking at 250 rpm. The plasmids were purified and sequenced with the T7

promoter primer and T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3'), which flank the pET3a multiple cloning site, to verify the sequence integrity.

5.2.2.2 Transformation into E. coli BL21 (DE3) pLysS competent cells

The expression host *E. coli* BL21 (DE3) pLysS was made chemically competent according to Sambrook *et al.* (Sambrook *et al.*, 1989). The cells were grown on LB-agar containing chloramphenicol (34 μg/ml) overnight at 37°C. A single colony was used to inoculate 50 ml LB-medium and cultured for 3 hrs at 37°C with shaking at 250 rpm. The cell suspension was split into two tubes and incubated on ice for 10 min. After centrifugation at 4000g at 4°C for 10 min, the pellets were resuspended in 5 ml of pre-chilled 0.1 M calcium chloride ($CaCl_2$). The centrifugation was repeated and the pellets were resuspended in 1 ml of ice-cold $CaCl_2$. The competent cells were stored at 4°C overnight to increase the transformation efficiency.

The *GSTe2* plasmid was transformed into the *E. coli* BL21 (DE3) pLysS completent cells as followes; 1 μl of plasmid was added to 200 μl of pre-chilled competent cells and then incubated on ice for 20 min. The plasmids were introduced into competent cells by heat-pulse in a 42°C water bath for 45 seconds. After incubating the tube on ice for 2 minutes, 1.8 ml of pre-heated SOC medium was added and the cells were incubated at 37°C for 45 minutes with shaking at 250 rpm. One hundred microlitres of transformed cells were grown on LB-agar plate containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml).

5.2.2.3 Expression of the GSTe2 gene

A single colony containing the recombinant plasmid was grown in 2 ml of LB-medium containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) at 30°C overnight. This culture was used to inoculate 100 ml LB-medium and grown for a further 3 hours. Expression was induced by addition of 0.4 mM isopropyl β-D-thiogalactoside (IPTG) and after an additional incubation at 30°C for 3 hours;

bacterial cells were collected by centrifugation at 5000g, 4°C for 10 min. Pelleted cells were resuspended in lysis buffer containing 4.29 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, 0.137 mM NaCl and 0.1% Tween, pH 7.3. After snap freezing in liquid nitrogen, the cells were stored at –20°C or used for purification.

5.2.2.4 Protein purification

The cell lysate was thawed and incubated with 2 Units/ml of DNase RQI at room temperature for 20 minute to digest bacterial genomic DNA. After centrifugation to remove the cell debris, the soluble protein was collected for purification. The recombinant protein contains a T7•Tag sequence (11 amino acids) at the N-terminal and was purified using the T7•Tag Affinity Purification Kit (Novagen) according to the manufacturer's protocol. Briefly, the cell lysate was applied to T7•Tag antibody agarose column (1 ml bed volume), equilibrated with 1X T7•Tag Bind/Wash buffer (4.29 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, 0.137 M NaCl, 1% v/v Tween-20, pH7.3). After washing the column with 10 column volumes (10 ml) of 1X T7•Tag Bind/Wash buffer, the bound proteins were eluted with 5 ml 1X T7•Tag Elute buffer (0.1 M citric acid pH 3.5). The eluted proteins, 1 ml/fraction were collected in 150 μl of 1X T7•Tag Neutralization buffer (2 M Tris base pH 10.4). The enzyme purity and subunit size were resolved by SDS-PAGE. Protein concentration was determined using the Bio-Rad Protein Reagent (Bio-Rad) with bovine serum albumin for the standard protein as described in section 2.2.6. Recombinant protein fractions were pooled and loaded onto PD-10 gel filtration columns to change the buffer to 50 mM sodium phosphate buffer, pH 6.5. The recombinant protein was then concentrated using Microcon YM-30 columns (Amicon) and collected in the presence of 40% (v/v) glycerol and 15 mM DTT and stored at –20°C.

5.2.2.5 SDS-PAGE analysis

Polyacrylamide gel electrophoresis was performed using a Mini-Protein II electrophoresis unit (Bio-Rad). A 15 % resolving gel and 5% stacking gel were prepared according to Laemmli system (Laemmli, 1970). Proteins were solubilised in 2 X SDS sample buffer (0.125 M Tris-HCl, 4% w/v SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% w/v bromophenol blue, pH 6.8) and boiled for 3 minutes before loading onto the polyacrylamide gel. Electrophoresis was performed in electrode buffer containing 0.025 M Tris, 0.192 M glycine, 0.1% w/v SDS, pH 8.3 and carried out at 120 constant volts. SDS-PAGE molecular weight low range (Bio-Rad) and SeeBlue prestained standard protein (Invitrogen) was applied as standard markers. To visualise the proteins, gels were stained with Coomassie Blue staining solution (0.025% w/v Coomassie Brilliant Blue R250, 40% v/v methanol, 7% v/v acetic acid) and then destained with 40% v/v methanol, 7% v/v acetic acid.

5.2.3 Western Blot Analysis

Batches of ten 4th instar larvae were homogenised in 0.1 M phosphate buffer pH 6.5 containing 10 mM DTT, and their protein content determined as described in section 2.2.6. Fifty micrograms of protein from each homogenate were resolved on individual lanes of a 4-20% gradient Tris-HCl Ready Gel (Bio-Rad). Purified recombinant GSTE2-2 from *Ae. aegypti* (10, 20, 50 and 100 ng per lane) and 10 μg of homogenates from the DDT-resistant ZAN/U strain of *An. gambiae* were also run as controls. The proteins were transferred to an ECL hybond nitrocellulose membrane (Amersham Biosciences) using a Mini-Trans Blot Cell Assembly (Bio-Rad) in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, 0.1% v/v SDS, pH 8.3) with cooling system for 1 h at 70 volts. Immunoblotting was performed using ECL Advance Western Blotting Detection Kit (Amersham Biosciences). The membrane was blocked overnight at 4°C with 3% (w/v) ECL Advance Blocking Agent in TBS-T (20 mM Tris-HCl, 0.136 M NaCl, 0.1% v/v Tween-20, pH 7.6). After washing with TBS-T, the membrane

was probed with a 1:5000 dilution of polyclonal antibody raised against the purified recombinant GSTE2-2 protein from *An. gambiae* (Ortelli *et al.*, 2003) for 1 h on an orbital shaker. The membrane was rinsed briefly with wash buffer (TBS-T) and washed for 20 minutes followed by 5 x 10 minutes with fresh changes of wash buffer at room temperature. The bound antiserum was detected by incubation with a 1:50000 dilution of Peroxidase-labelled Anti-Rabbit Antiserum (Amersham) for 1 h. The washing steps were carried out as described above. Bound antibody was detected using ECL Advance Blotting Detection Kit (Amersham Bioscience). The blot was incubated with the detection solution, prepared by mixing equal volume of solution A and B (1 ml/ cm^2), for 5 min. After draining the detection solution, the membrane was wrapped with SaranWrap and exposed to Hyperfilm™ ECL (Amersharm Bioscience, UK Ltd.) for 15 seconds. The bound proteins were visualised by autoradiography.

5.2.4 Characterisation of GSTE2-2

5.2.4.1 Substrate specificity

GST activity against 1-choro-2, 4-dinitrobenzene (CDNB) was determined as described previously in section 2.2.3. GST activity toward 1, 2-dichloro-4-nitrobenzene (DCNB) was measured according to the method of Habig *et al.* (Habig *et al.*, 1974). Briefly, the formation of GSH-DCNB was measured at 340 nm for 2 min. using VERSAMax microplate reader (Molecular Devices). The reaction mixture contains 10 µl of sample in 200 µl of substrate mixture (1mM DCNB and 10 mM GSH in 0.1M phosphate buffer pH 6.5). The molar extinction coefficient 8.5 mM^{-1} cm^{-1} was used for GST activity calculation. One unit of GST activity is defined as 1 µmol of GSH conjugation per minute. DDT-dehydrochlorinase activity was determined by measuring conversion of DDT to DDE as described previously in section 2.2.5.

Glutathione peroxidase activity was determined at 340 nm by coupling the reduction of cumene hydroperoxide (CHP) by GSH to the oxidation of NADPH by oxidized GSSG with glutathione reductase as described previously (Simmons *et al.*, 1989). The 200 μl reaction consisted of 10 μl of enzyme and 190 μl of substrate mixture (1mM GSH, 1 mM EDTA, 0.2 mM NADPH and 1 unit/ml of GSH reductase in 50 mM potassium phosphate buffer pH 7.0). The mixture was incubated at 25°C for 5 min. and then the reaction was initiated by addition of 1.5 mM of cumene hydroperoxide. The peroxidase activity was monitored by the consumption of NADPH at 25°C for 4 min. A molar extinction coefficient of 6.22 mM^{-1} cm^{-1} was used for activity calculation. The reaction was subtracted for non-enzymatic oxidation of NADPH by the peroxidase substrate.

Kinetic studies were performed by varying the concentration of CDNB (0.025-2 mM) or GSH (0.5-40 mM) at fixed concentrations of 15 mM GSH and 2 mM CDNB, respectively. The kinetic parameters (V_{max} and K_m) were analysed by non-linear regression analysis using GraphPad Prism 4 software. The turnover number, k_{cat} was calculated using the molecular mass of GSTe2 (24737.70 daltons per subunit).

5.2.4.2 Hematin binding assay

Hematin binding to GST was monitored by measuring the inhibition of GST activity. A 1μM hematin solution was prepared by dissolving 0.0633 g hematin in 1 ml 0.5 M NaOH and then bringing the volume to 50 ml with 50 mM potassium phosphate buffer pH 7.0. The inhibition of GST activity by hematin was determined by incubating the enzyme with 1 mM GSH and 1 mM CDNB in the presence or absence of hematin. The reaction rate was measured at 340 nM for 2 minutes. The IC_{50} value was determined by plotting sigmoidal dose response of fractional GST activity against log concentration of hematin using GraphPad Prism 4 software.

The binding of hematin to GST was also determined by following the quenching of the intrinsic protein fluorescence in the presence of hematin as described previously (Vander Jagt *et al.*, 1985). A 1 μM solution of recombinant GST was incubated in 0.02 M potassium phosphate buffer containing 0.1 M NaCl, pH 6.5 in the presence of varying hematin concentration, at 25°C for 3 minutes. The changes of intrinsic fluorescence of tryptophan in the primary structure of GST was monitored using Luminescence spectrometer LS50B (Perkin Elmer Life and Analytical Sciences) with the excitation and emission wavelengths of 280 and 363 nm, respectively. The K_D value was calculated by double reciprocal plots of the intrinsic fluorescence of GST with the concentration of free hematin.

5.3 RESULTS

5.3.1 Cloning of GSTe2

The coding region of *Ae. aegypti GSTe2* was amplified using ProofStart DNA polymerase. This enzyme contains a proofreading activity (3'→ 5' exonuclease activity) to verify and remove any errors from the mis-incorporation of dNTPs. The deduced amino acid sequences of GSTe2 from *Ae. aegypti* was aligned with its ortholog in *An. gambiae* (Figure 5.1).

5.3.2 Protein Expression and Purification

As expression of *GSTe2* was elevated in the DDT resistant PMD-R strain, the ability of the enzyme to catalyse the detoxification of DDT was established. The homodimer GSTe2 from the PMD-R strain was expressed *in vitro* using the pET3a *E. coli* expression system. In initial attempts at expressing the recombinant protein the culture was grown at 37°C but this resulted in the majority of the recombinant GSTE2-2 being sequestered in insoluble inclusion bodies (Figure 5.2A). Only by reducing the incubation temperature to 30°C was sufficient soluble protein produced to enable purification (Figure 5.2B).

```
AaGSTE2        MTKLILYTLHVSPPCRAVELCAKALGLELEQKTVNLLTKEHLTPEFMKMNPQHTVPVLDD 60
AgGSTE2        MSNLVLYTLHLSPPCRAVELTAKALGLELEQKTINLLTGDHLKPEFVKLNPQHTIPVLDD 60

AaGSTE2        NGTIVCESHAIMIYLVSKYGKDDSLYSKELVKQAKLNAALHFESGVLFARLRFVFEPILF 120
AgGSTE2        NGTIITESHAIMIYLVTKYGKDDSLYPKDPVKQARVNSALHFESGVLFARMRFNFERILF 120

AaGSTE2        AGGSEIPADRAEYVQKAYQLLEDTLVDDYIVGNSLTIADFSCVSSVSSIMGVIPMDKEKF 180
AgGSTE2        FGKSDIPEDRVEYVQKSYELLEDTLVDDFVAGPTMTIADFSCISTISSIMGVVPLEQSKH 180

AaGSTE2        PKIYGWLDRLKALPYYEAANGSGAEQVAQFVLSQKEKNAQKA 222
AgGSTE2        PRIYAWIDRLKQLPYYEEANGGGTDLGKFVLAKKEENA-KA 221
```

Figure 5. 1 Alignment of deduced amino acid sequences of GSTe2 from *Ae. aegypti* (Aa) and *An. gambiae* (Ag).

The amino acid sequences were aligned using ClustalW. Highlighted residues are conserved between the two species. Dashes are used to denote gaps introduced for maximum alignment.

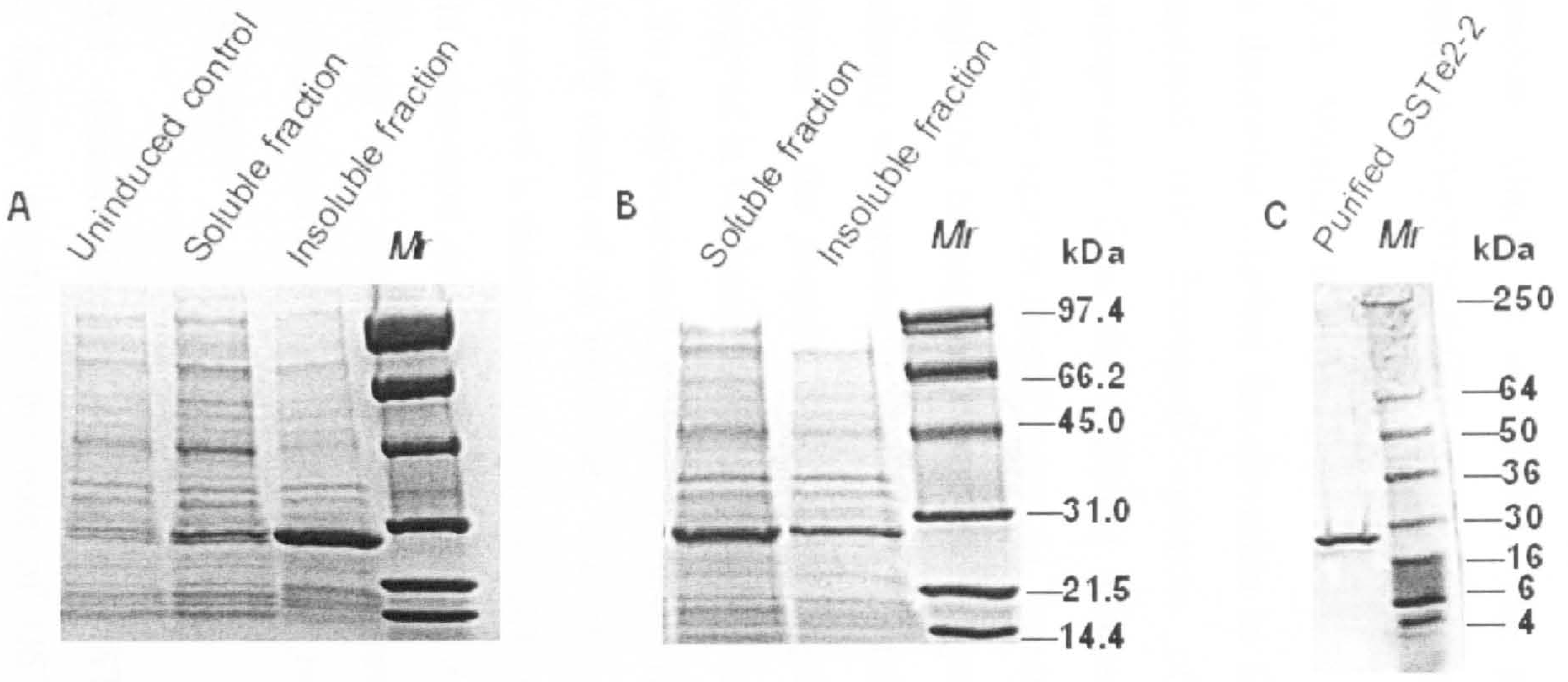


Figure 5. 2 SDS-PAGE profiles of GSTE2-2 expression and purification.

Proteins were separated by electrophoresis on 4-20% Tris-HCl Ready Gels (Bio-Rad). Protein bands were visualized by staining with Coomassie Blue. Lanes marked Mr contained molecular mass standard proteins. **(A)** Growth and induction at 37°C. **(B)** Growth and induction at 30°C. **(C)** GSTE2-2 after purification using T7•Tag antibody agarose column.

The recombinant GSTE2-2 did not bind to an S-hexylglutathione affinity column (data not shown), but was successfully purified by affinity chromatography using monoclonal antibodies against the T7 tag that is fused to the N-terminal end of the recombinant protein. This was typical of the recombinant GSTE2-2 from *An. gambiae* (Ortelli *et al.*, 2003). The purity of recombinant GSTE2-2 was determined by SDS-PAGE as shown in Figure 5.2C.

5.3.3 Western Blot Analysis

To determine whether the elevation in *GSTe2* expression in the PMD-R strain translated into increased amounts of protein, western blots containing homogenates from the different strains were probed with antiserum raised previously against recombinant *An. gambiae* GSTE2-2 (Ortelli *et al.*, 2003). A single band of approximately 25kDa (predicted size of GSTE2-2 is 24737.70 Daltons) was present in the PMD-R strain, but not observed in either the New Orleans or the parental PMD strain (Figure 5.3). A band of very similar size is observed in the lanes containing the recombinant GSTE2-2 from *Ae. aegypti* and in the positive control, which contained crude homogenate from the DDT resistant ZAN/U strain of *An. gambiae.* The intensity of the band in the PMD-R strain of *Ae. aegypti* is fainter than in the ZAN/U strain of *An. gambiae* despite loading 5 fold more total *Ae. aegypti* protein than *An. gambiae* protein. However, since the antibody was raised against the *An. gambiae* GST, no conclusions about the relative amount of GSTE2-2 in the two strains can be drawn from these experiments.

5.3.4 Genetic Control of Elevated GSTE2-2 Expression

To further investigate the role of GSTE2-2 in conferring DDT resistance, homogenates from the F1 progeny from crosses between the New Orleans susceptible and PMD-R resistant strains described in Chapter 2 were probed with the anti-AgGSTE2-2 antibody. Ten 4th instar larvae from the F1 progeny were

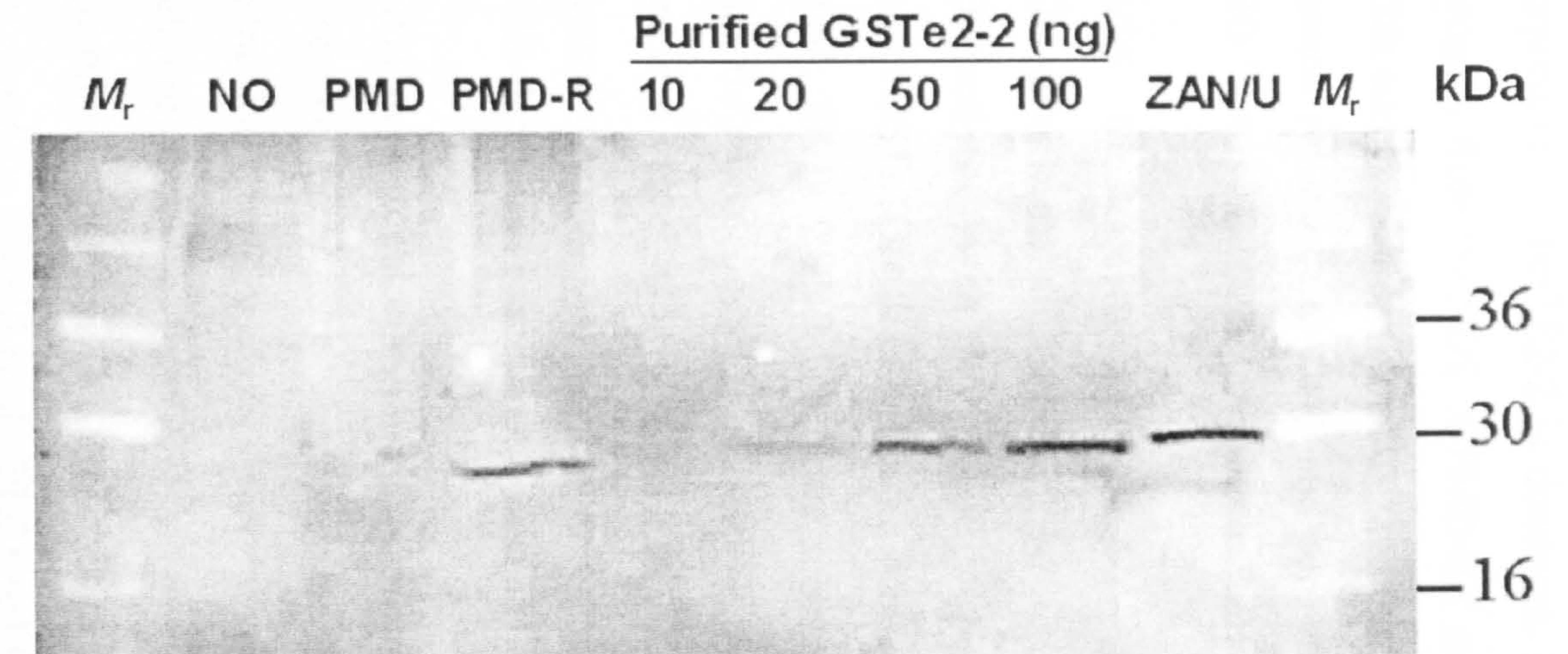


Figure 5. 3 Immunological cross reactivity with polyclonal antibody raised against recombinant GSTE2-2 from *An. gambiae*.

Protein (50μg) from 4th instar larvae from New Orleans (NO), PMD and PMD-R strains and purified recombinant GSTe2 (10, 20, 50 and 100 ng) were resolved on 4-20% Tris-HCl Ready Gel (Bio-Rad). Protein (10 μg) from *An. gambiae* (ZAN/U) was used as positive control. Proteins were transferred to a nitrocellulose membrane and probed with GSTE2-2 antibody, 1:5000 (Ortelli *et al.*, 2003) for 1h. Peroxidase labeled anti-rabbit antibody (1:50000; Amersham Pharmacia Biotech)) was used as a second antibody. Proteins were visualized by enhancing the chemiluminescence using ECL Advance Blotting Detection Kit (Amersham Biosciences). Lane *Mr* indicates the molecular masses of standard protein.

homogenised and 50 μg of protein were used for western blot analysis as described in section 5.2.3. Expression of GSTE2-2 was elevated in the F1 progeny of both reciprocal crosses, over the susceptible strain (Figure 5.4) suggesting that the over expression of GSTE2-2 is dominant. More protein was detectable in the in F1 progeny from the NO♀ X PMD-R♂ cross than from the PMD-R♀ X NO♂ cross. This agrees with the bioassay data that the F1 progeny NO♀ X PMD-R♂ showed greater resistance to DDT and permethrin than progeny from the PMD-R♀ X NO♂ cross (Table 2.1 and Figure 2.2).

5.3.5 Biochemistry of GSTE2-2

The specific activities of GSTE2-2 with various substrates are shown in Table 5.1. The activities with CDNB and DCNB are within the range reported for other insect GSTs (Ortelli *et al.*, 2003; Ranson *et al.*, 1997; Sawicki *et al.*, 2003; Vontas *et al.*, 2002). CDNB is a better substrate for recombinant *An. gambiae* GSTE2-2 than for recombinant GSTE2-2 from *Ae. aegypti* as evidenced by the higher *V*max and *k*cat values and the lower Km^{CDNB} of the *An. gambiae* enzyme. Only the *Ae. aegypti* enzyme displayed glutathione peroxidase activity as measured by the GSH-dependent reduction of cumene hydroperoxide.

DDT dehydrochlorinase activity of the recombinant GSTE2-2 was quantified as described previously. Approximately 98% recovery of DDT or DDE after extraction and analysis was obtained. The specific activity of DDT dehydrochlorinase for GSTE2-2 was 4.16 nmol of DDE formation per μg recombinant protein. This activity was 1.5 fold higher than that for GSTE2-2 from *An. gambiae* (Ortelli *et al.*, 2003) and is the highest DDTase activity reported for any insect GST. Control assays with no GSH or denatured recombinant enzyme had no detectable DDE production. This verified that the conversion of DDT to DDE in the experimental reactions was catalysed by the recombinant GSTE2-2.

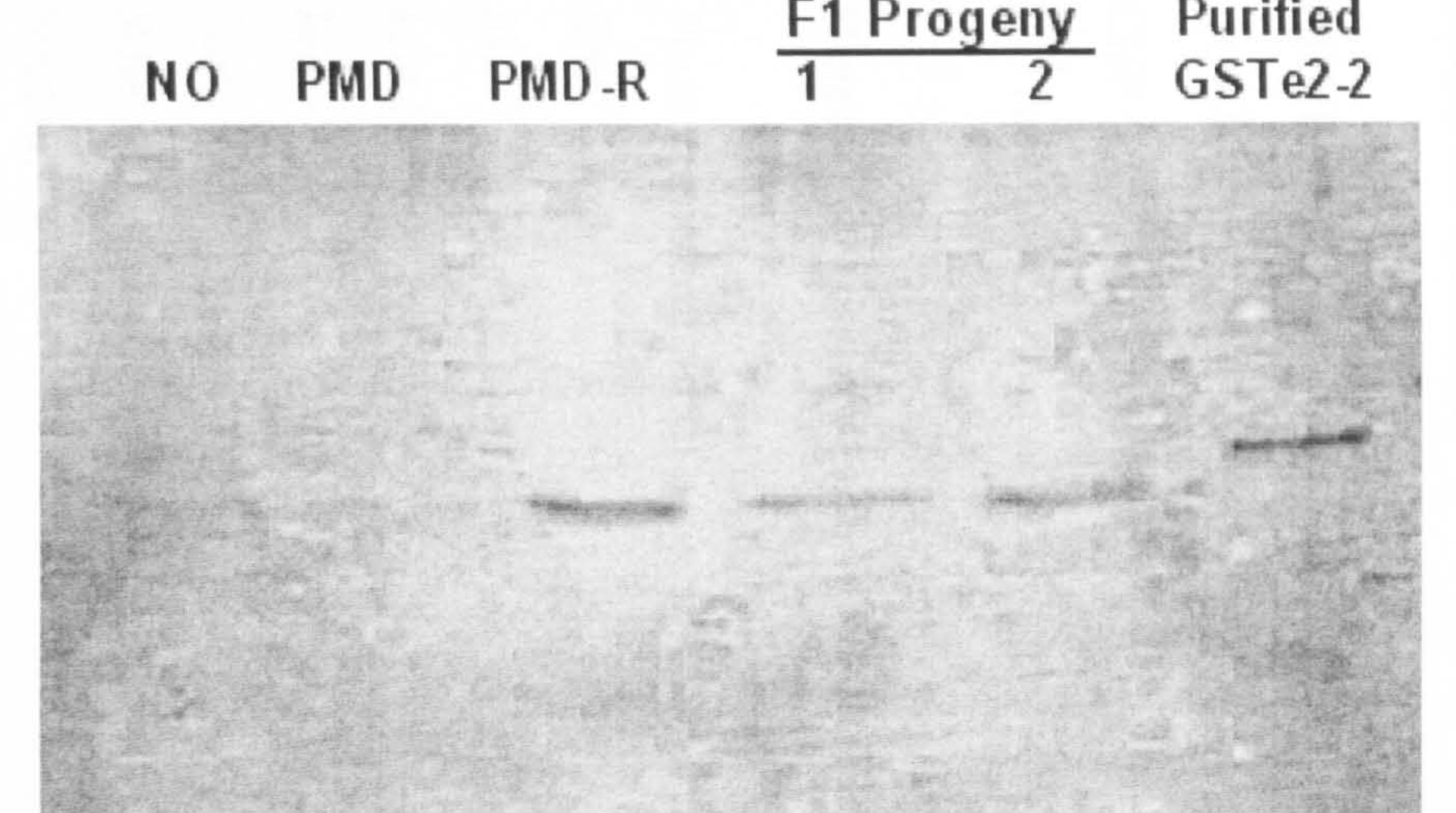


Figure 5. 4 Western blot probed of parental strains and F1 progeny from genetic crosses probed with anti-GSTE2-2 serum from *An. gambiae.*

Protein (50μg) from 4th instar larvae mosquitoes from New Orleans (NO), PMD, PMD-R strains, F1 progeny1; PMD-R♀ X NO♂, F1 progeny 2; NO♀ X PMD-R♂ and purified recombinant GSTe2 (100 ng) were resolved on 15% SDS-PAGE. Western blot was performed as described previously in section 5.2.3.

Table 5. 1 Substrate specificities and kinetic parameters for recombinant GSTE2-2 from *Ae. aegypti* and *An. gambiae*.

	Specific activity of GSTE2-2	
	Ae. aegypti	*An. gambiae*
Substrate specificity		
CDNB (μmol/min/mg)	6.77 ± 0.54	12.5 ± 0.58
DCNB (μmol/min/mg)	2.89 ± 0.46	5.87 ± 0.24
CHP (μmol/min/mg)	0.11 ± 0.01	ND
DDTase activity (nmol DDE/μg)	4.16 ± 0.28	2.77
Kinetic parameters		
*V*max (μmol/min/mg)	5.00 ± 0.44	13.10 ± 0.40
Km^{GSH} (mM)	7.57 ± 1.17	6.72 ± 1.70
Km^{CDNB} (mM)	0.18 ± 0.04	0.07 ± 0.01
*k*cat (s-1)	4.12	10.84 ± 0.33
$kcat / Km^{GSH}$ (s-1 . mM-1)	0.54	1.61
$kcat / Km^{CDNB}$ (s-1 . mM-1)	22.89	157.1
S-hexyl glutathione agarose binding	Unbound	Unbound

Three independent assays were performed. Results show mean ± SD. Kinetic studies were determined by varying the concentration of GSH (0.5-60mM) and the concentration of CDNB (0.025-2.0 mM) at fixed concentrations of CDNB of 2mM and GSH of 15mM, respectively. Substrate specificity and kinetic parameters of AgGSTE2-2 from *An. gambiae* are given for comparison (Ortelli *et al.*, 2003). ND indicates not detectable.

5.3.6 Hematin Binding to GSTE2-2

The effect of hematin on GSTE2-2 activity was determined by competitive inhibition assays. Hematin was dissolved in 10 mM NaOH solution, giving a final concentration of NaOH in the inhibition assays of less than 2.5 mM, which did not affect the GST activity (data not shown). GST activity with CDNB was determined in the presence of varying concentrations of hematin (0.005-50 μM). The percentage inhibition was determined by dividing GST activities in the presence of hematin with GST activity without hematin and plotted against the logarithms of hematin concentration to produce the sigmoidal dose response curve shown in Figure 5.5. The hematin concentration giving 50 % inhibition of GSTE2-2 activity (IC_{50}) was 3.71 ± 1.40 μM.

The binding of hematin to recombinant GSTE2-2 was also analysed by monitoring the quenching of intrinsic protein fluorescence. The dissociation constant (K_D) value was determined by the double-reciprocal plot of the changes of intrinsic fluorescence and the concentration of hematin. K_D is the ratio of the concentration of dissociated to bound compound and indicates the affinity of ligand to the protein (The lower the K_D the higher affinity of the compound). The K_D value for hematin in binding to GSTE2-2 is 67.9 ± 2.0 μM (Figure 5.6).

5.4 DISCUSSION

Western blot analysis confirmed that antibodies raised against recombinant AgGSTE2-2 were able to detect *Ae. aegypti* GSTE2. Immunological cross reactivity has been used as a mean of classifying GSTs (Fournier *et al.*, 1992) and this result therefore gives further support to the relationship between these two enzymes. The *Anopheles* antiserum was used to confirm that *Aedes* GSTE2-2 is up regulated in the PMD-R strain relative to the susceptible strain at the protein level. As was found with its ortholog in *Anopheles* the recombinant GSTE2-2 from *Aedes* is efficient at metabolising DDT to the non-toxic DDE.

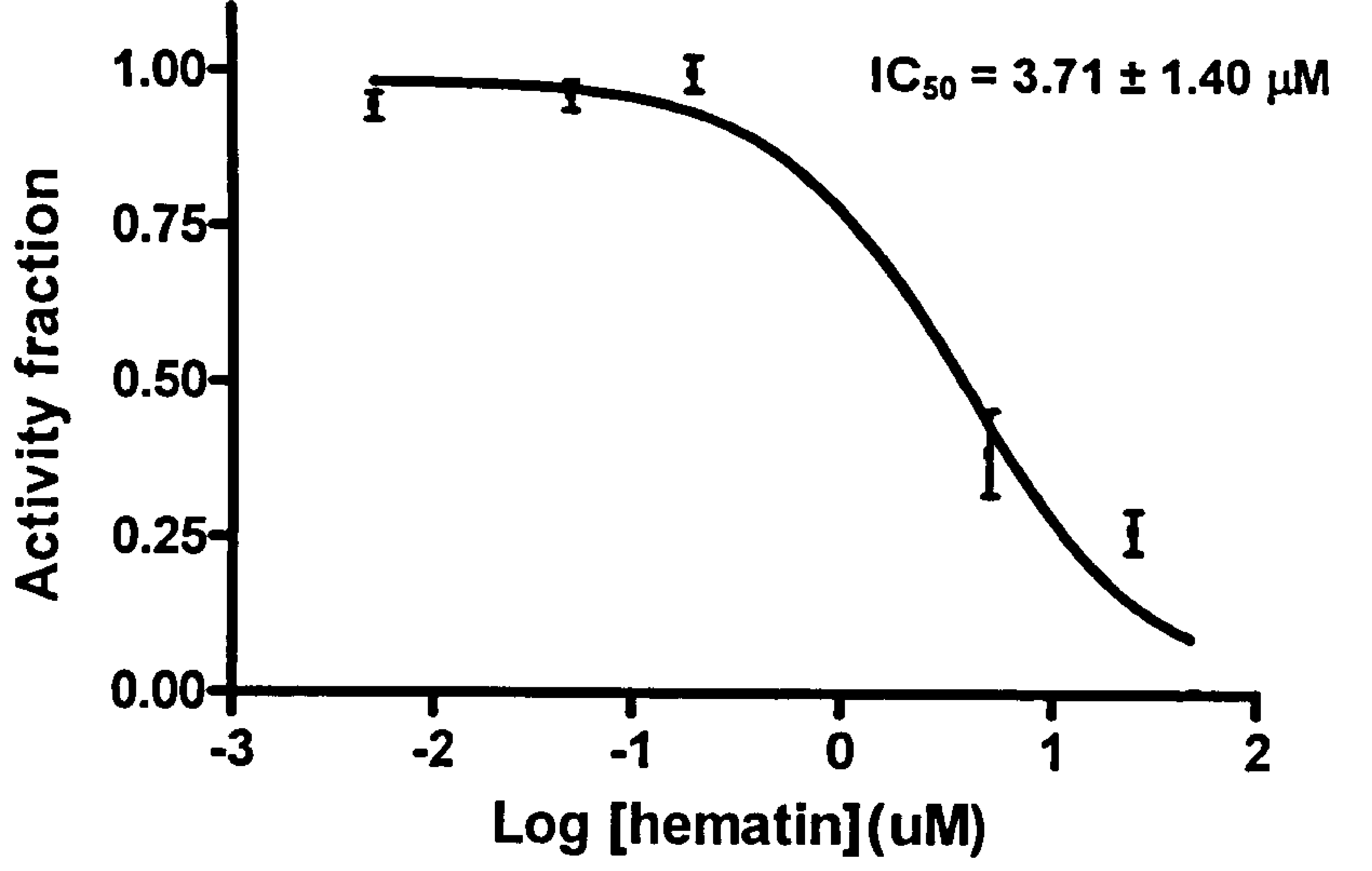


Figure 5. 5 Inhibition of GSTE2-2 activity by hematin.

The inhibition study was performed in 1mM GSH and 1 mM CDNB in 50 mM potassium phosphate buffer pH 7.0 in the absence and presence hematin (from 0.005 μM to 50 μM). IC50 value is the concentration of hematin resulting in 50% inhibition of GST activity, determined by sigmoidal dose response best fit-value using GraphPad Prism 4 software. Three independent experiments were performed.

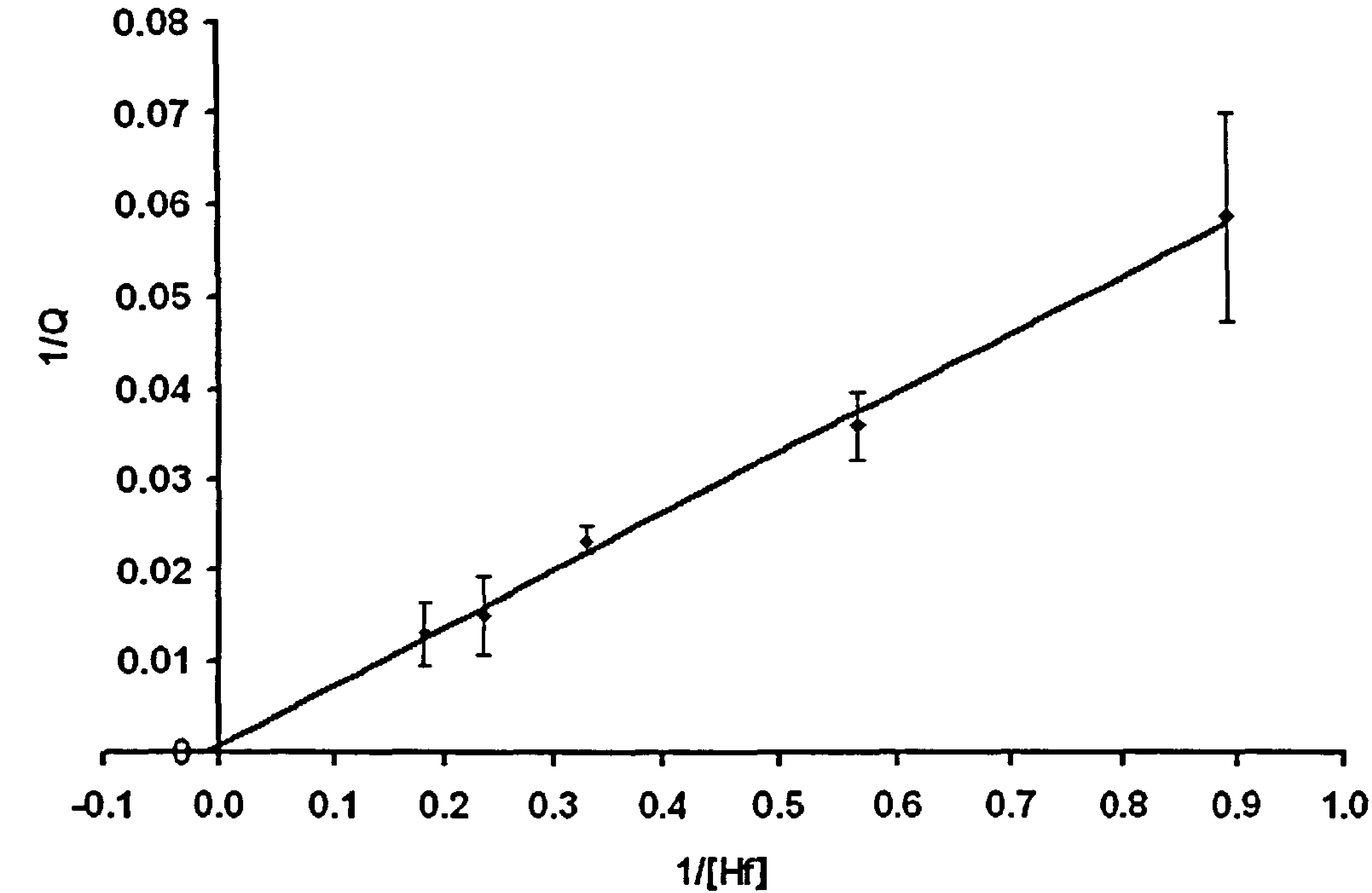


Figure 5. 6 Double reciprocal plot of the intrinsic fluorescence intensity (1/Q) of GSTE2-2 against the concentration of free hematin ($1/H_f$). K_D= 67.9± 2.0 μM.

Taken together these results suggest that increased expression of GSTe2 in *Ae. aegypti* contributes to the elevated level of DDT resistance seen in the PMD-R strain.

To investigate the genetic factors responsible for the over expression of GSTE2, the amount of enzyme in the progeny of the reciprocal F1 crosses was determined. Elevated level of GSTE2 were detected in the F1 progeny compared to the New Orleans susceptible and the parental PMD strain, regardless of the direction of the cross indicating that the over expression is a dominant or semi-dominant trait. Slightly more GSTe2 was detectable in the NO♀ X PMD-R♂ cross than the PMD-R♀ X NO♂. The NO♀ X PMD-R♂ progeny also showed higher level of resistance to DDT than the progeny of the reciprocal cross (Chapter 2) adding additional support to the role of GSTe2 in conferring DDT resistance.

The genes conferring insecticide resistance has been mapped in *An. gambiae*. Several loci responsible for DDT resistance (Ranson *et al.*, 2000) and permethrin resistance (Ranson *et al.*, 2004) were identified, but none of these were linked to the X chromosome. In addition, the location of GSTe2 gene responsible for DDT resistance in *An. gambiae* is located on chromosome 3R suggesting that the resistance in *Anopheles* is not the influence of the sex chromosome. In *Aedes* sex is not determined by X and Y sex chromosome as in *Anopheles*, but by sex determining locus, which is located in chromosome 1 (Clements, 1992). It is not known if the locus conferring GSTe2 expression is linked to this sex determining locus. In *Aedes aegypti*, several genes responsible for DDT resistance are linked to Chromosome 2 and 3 (Munstermann & Graig, 1979; Severson *et al.*, 1997). The location of GSTe2 gene encoding the enzyme responsible for insecticide resistance in PMD-R strain is still unknown. The differences in the level of resistance in the F1 progeny from reciprocal crosses in this study need further investigation. Results of the genetic resistance and the expression of GSTE2-2 in F1 crosses demonstrated that this resistance phenotype is more complex than just the over

expression of this gene. Only the up-regulation of GSTe2 has been found in this study. However, other unidentified resistance mechanisms may be acting in the PMD-R strain.

GSTE2-2 protein levels in the parental strain and F1 crosses did not correlate with total GST activity supporting the suggestion in Chapter 4 that CDNB was not the optimal substrate for GSTE2-2. Low GST activity toward CDNB has been reported in several recombinant GST enzymes. The average of GST activity against CDNB of Epsilon GSTs is generally lower than that for Delta GSTs (Jirajaroenrat *et al.*, 2001; Ortelli *et al.*, 2003; Ranson *et al.*, 1997; Ranson *et al.*, 2001; Sawicki *et al.*, 2003; Tang & Tu, 1994). One Epsilon GST, DmGSTE1-1 from *D. melanogaster*, showed the CDNB activity below the sensitivity of the assay (Sawicki *et al.*, 2003).

Aedes GSTE2-2 has peroxidase activity as measured with CHP comparable with that of DmGSTS1-1 in *D. melanogaster* (0.17±0.02 μmol/min/mg), an enzyme involved in the conjugation of lipid peroxidation end products (Singh *et al.*, 2001). It is possible that the peroxidase activity of GSTE2-2 may be conferring some resistance to the secondary effects of insecticide exposure in *Ae. aegypti*. Exposure to pyrethroids causes oxidative stress. GSTs with peroxidase activity can protect cells against oxidative stress. This has been shown to be important resistance mechanism in pyrethroid resistant strain of planthoppers (Vontas *et al.*, 2002). Interestingly, the *Anopheles* ortholog lacks peroxidase activity. This may explain the lack of cross-resistance to DDT and pyrethroids in the ZAN/U strain (Ortelli *et al.*, 2003).

Hematin has an affinity for GSTE2-2, indicated by both the inhibition of GST activity by hematin (IC_{50} = 3.71 ± 1.40 μM) and the interruption of intrinsic fluorescence of GSTE2-2 by hematin (K_D = 67.9± 2.0 μM). Although, the affinity of GSTE2-2 to hematin is an order of magnitude less than that of blood sucking

parasites (van Rossum *et al.*, 2004), the binding of heme may still be an important property of GSTE2-2. Haematophagous insects encounter an oxidative stress after blood meal due to heme released by the degradation of haemoglobin in the midgut. Free heme triggers the production of reactive oxygen species (ROS), which can damage the host cells (Sadrzadeh *et al.*, 1984; Tappel, 1955). Heme detoxification can be accomplished by multiple mechanisms; for instance, the polymerization of heme to hemozoin (an insoluble dark blown pigment) (Oliveira *et al.*, 2000), the binding of heme with peritrophic matrix (PM) (Pascoa *et al.*, 2002) or the binding of heme to proteins leading to the nontoxic form (dans Petretski *et al.*, 1995). The role of GSTs in heme binding in mosquito was not further investigated but it is interesting to note that in *An. gambiae* several GSTs, including *GSTe2* are up-regulated 3h after blood feeding (Marinotti *et al.*, 2005).

Chapter 6

IDENTIFICATION, EXPRESSION AND CHARATERISATION OF UNCLASSIFIED GSTS

6.1 INTRODUCTION

The name of 'unclassified' was utilised by Ding *et al.* (Ding *et al.*, 2003) for *An. gambiae* GSTs for which phylogenetic analysis indicates that they do not belong to any of the six classes and for which function is unknown. There are three unclassified GSTs in *An. gambiae*, designated *GSTu1*, *GSTu2* and *GSTu3*. Orthologs of unclassified GST are not found in *Drosophila*. Therefore, it has been speculated that these GSTs may have some specific role in mosquitoes, perhaps related to blood feeding. GSTu3 has been previously studied in *Ae. agypti* and named GST-2 (Grant & Hammock, 1992). Up-regulation of this gene was related to DDT resistance in a strain of *Ae. aegypti* from South America at both the mRNA and protein level by using Northern and Western blot analysis. In contrast, expression of GSTu3 is not correlated with DDT resistance in the Thai PMD-R strain (Chapter 4). In this chapter, GSTu3 was expressed and characterised and the ability of this enzyme to catalyse the detoxification of DDT was determined.

6.2 MATERIALS AND METHODS

6.2.1 Identification of Unclassified GSTs

Putative orthologs of the *An. gambiae* unclassified GST genes were identified in *Ae. aegypti* by searching the TIGR EST database (http://www.tigr.org) as described previously (section 3.2.1).

6.2.2 Expression of Unclassified GSTs

6.2.2.1 cDNA cloning and construction of expression vector

Total RNA was extracted from one-day old adults from the PMD-R strain and cDNA synthesis was performed as described in section 3.2.3. The full-length cDNA of the three unclassified GSTs were amplified using PCR primer pairs specific for *GSTu1*, *GSTu2* and *GSTu3* genes (Table 6.1). The upstream primers were designed to start with the initiation codon (ATG) and downstream primers spanned the stop codon. PCR amplification was performed using ProofStart PCR Kit as described in section 5.2.2.1. After purification, PCR products were A-tailed in the presence of 0.2 mM dATP and 5 units of *Taq* DNA Polymerase at 70°C for 30 minutes. A-tailed PCR products were ligated into pGEM-T easy vector and transformed into JM109 competent cells as described in section 3.2.6.2. After PCR screening, the plasmids were purified and used as template for PCR amplification using *Taq* DNA Polymerase. These PCR products were then cloned into the pET SUMO vector (Invitrogen). The ligation reaction was carried out in the presence of 1X Ligation Buffer, 50 ng of pET SUMO vector and 4 Weiss units of T4 DNA Ligase and incubated overnight at 15°C.

Table 6. 1 Primers used to produce the full-length *Ae. aegypti* unclassified GSTs.

Forward primers were designed to start with ATG for the initiation codon. Reverse primers contained a stop codon (underlined).

Gene	Primer name	Primer sequences (5'- 3')
GSTu1	GSTu1F	ATGAAAATCTATGCCGTATCG
	GSTu1R1	TCATTTCTTAACTTTTTTGATGGGATG
GSTu2	GSTu2F	ATGCCCATGAGTTTGTATTACAG
	GSTu2R	TACATTCCCTCGGTCACG
GSTu3	GSTu3F	ATGGCTCCAATTGTGCTGTATC
	GSTu3R1	TTAGAAAGGTTCCTCCAGCTTG

The ligation products were transformed into *E. coli* JM109 competent cells as described in section 5.2.2.1. After growing overnight on LB-agar plates containing 50μg/ml kanamycin, the colonies were screened by PCR using specific forward primers (as shown in Table 6.1) and T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3') to determine the correct orientation. Positive colonies were isolated and grown overnight in 2 ml LB-medium containing 50 μg/ml kanamycin at 37°C with shaking at 250 rpm. The plasmids were purified and sequenced with SUMO forward primer (5'-AGATTCTTGTACGACGGTATTAG-3') and T7 reverse primer (5'-TAGTTATTGCTCAGCGGTGG-3'), which flank the cloning site of the pET SUMO vector.

6.2.2.2 In vitro expression of the GST genes

The BL21 (DE3) pLysS competent cells were prepared and the positive plasmids from section 6.2.2.1 were transformed into BL21 (DE3) pLysS competent cells as described in section 5.2.2.2. One hundred microlitres of transformed cells were grown on an LB-agar plate containing kanamycin (50 μg/ml) and chloramphenicol (34 μg/ml). A single colony was grown overnight in 2 ml LB-medium with antibiotics as above and then stored in 8 % (v/v) of glycerol at –80°C until used.

The glycerol stock was used to inoculate 2 ml of LB-medium containing kanamycin (50 μg/ml) and chloramphenicol (34 μg/ml) and incubated at 37°C overnight. This culture was used to inoculate 100 ml LB-medium and grown for a further 3 hours. Expression was induced by addition of 1 mM IPTG and after an additional incubation at 37°C for 3 h, bacterial cells were harvested by centrifugation at 5000g, 4°C for 10 min. Pelleted cells were resuspended in 5 ml of 1X Binding Buffer containing 0.5 M NaCl, 20 mM Tris-HCl, 20 mM imidazole, pH 7.9. After snap freezing in liquid nitrogen, the cells were stored at –80°C or used for purification.

6.2.3 Protein Purification of Recombinant GSTs

The cell lysate was thawed at 37°C and incubated with 2 Units/ml of DNase RQI at room temperature for 20 minutes to digest bacterial genomic DNA. After centrifugation to remove the cell debris, the soluble protein was collected for purification. The recombinant protein contained a 6X His tag and SUMO fusion protein (13 kDa) at the N-terminal and this enabled purification using the His•Bind resin (Novagen). The cell lysate was applied to Nickle (Ni^{2+})-charged His•Bind resin column (1ml bed volume), pre-equilibrated with 5 column volumes of 1X Binding buffer. After washing with 10 column volumes of 1X Binding buffer, the column was washed with 10 column volumes of 1 X Wash buffer (0.5 M NaCl, 20 mM Tris-HCl, 60 mM imidazole, pH 7.9) followed by 10 column volumes of Wash buffer containing 80 mM imidazole. The bound proteins were eluted with 6 column volumes of 1X Elute buffer containing 0.5 M NaCl, 20 mM Tris-HCl, 1 M imidazole, pH 7.9. The eluted proteins were collected in 1.5 ml/fractions and monitored for protein purity by SDS-PAGE. The fractions containing recombinant protein were pooled and loaded onto PD-10 column to desalt and remove imidazole. Recombinant protein was eluted with 50 mM Tris-HCL pH 8.0 and then concentrated using Microcon YM-30 columns. Concentrated protein was collected for SUMO protease treatment.

6.2.3.1 SUMO protease treatment and removal

The recombinant fusion protein was cleaved by SUMO protease to generate the native protein. Ten units of SUMO protease (Invitrogen) were added to 20 μg of fusion protein in 1X SUMO protease buffer (50 mM Tris-HCl, pH 8.0, 2% Igepal (NP), 10 mM DTT) and incubated overnight at 4°C. The proteins were resolved by SDS-PAGE to verify the efficacy of the cleavage reaction.

The SUMO protein and SUMO protease were purified from the recombinant GST using Ni^{2+}-charged His•Bind resin column. Both the SUMO protein and SUMO protease contain polyhistidines tag at the N-terminal and will therefore bind to the column. The cleavage reaction was diluted to 10 times in 1X Bind buffer to reduce the DTT concentration and applied to the column. The flow-through fractions were collected and loaded onto PD-10 column to desalt and remove imidazole. The proteins were eluted with 50 mM phosphate buffer pH 6.5 and then concentrated using Microcon YM-30 columns. Recombinant proteins were collected in the presence of 40% (v/v) glycerol and 15 mM DTT and stored at –20°C.

6.2.4 MALDI-TOF Mass Spectrometry Analysis

Matrix Assisted Laser Desorption/Time of Flight (MALDI-TOF) mass spectrometry analysis was performed. Briefly, tryptic digestion of purified recombinant protein was carried out using sequencing grade trypsin (Promega). The single band of recombinant GSTU3-3 from SDS-PAGE was cut into small pieces and then washed 2 x15 minutes with 200 µl of dH_2O. The gel slice was dehydrated with 100 µl of 50 % (v/v) acetonitrile for 10 minutes. After removing the acetonitrile, the gel was incubated with 100 µl of 50 mM ammonium bicarbonate for 10 minutes and then replaced with 100 µl 50% (v/v) acetonitrile. After removing all acetonitrile, digestion was carried out by adding 20 µl of 10 ng/µl of trypsin and incubated overnight at 37°C. All supernatant was transferred to a fresh tube and keep for mass spectrometric analysis, which performed by Dr Yang Wu. One microlitre of trypsin-digested sample was mixed with 1 µl of matrix (α-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile, 0.1% v/v trifluoroacetic acid) on the metal MALDI-TOF target plate. After drying, the sample was then subjected to MALDI-TOF mass spectrometry analysis using Kratos Analytical mass spectrometer (Shimadzu Group Company). Internal mass calibration was performed using trypsin autodigestion products. A peptide mass map was generated by Kratos PC Axima CFRplus V2.4.1 software (Shimadzu

Group Company). Database searching was performed using monoisotopic peptide masses obtained from MALDI-TOF mass spectrophotometry. Mascot search program (http://www.matrixscience.com) was used to identify the peptide sequences in the NCBI database.

6.2.5 Characterisation of Unclassified GSTs

6.2.5.1 Biochemical studies

Substrate specificity for CDNB and DCNB were determined as explained in section 2.2.3 and 5.2.4.1. Se-independent peroxidase and DDT dehydrochlorinase assay were carried out as described in section 5.2.4.1.

Hematin binding assays were performed to investigate the interference of hematin against GST activity. The inhibition study was carried out using the CDNB assay as described in section 5.2.4.2 in the presence of various concentration of hematin. The affinity binding of hematin to GST was measured by following the quenching of the intrinsic protein fluorescence in the presence of hematin as described in section 5.2.4.2.

6.2.6 Immunobloting

Recombinant GSTU3-3 and GSTE2-2 from *Ae. aegypti* were diluted in 0.1 M phosphate buffer pH 6.5 to obtain protein concentrations ranging from 250 ng to 3 ng/μl The proteins were manually spotted onto an ECL hybond nitrocellulose membrane.. The membrane was blocked overnight at 4°C with 3% (w/v) ECL Advance Blocking Agent in TBS-T (20 mM Tris-HCl, 0.136 M NaCl, 0.1% v/v Tween-20, pH7.6). After washing with TBS-T, the membrane was probed with a 1:5000 dilution of polyclonal antibody raised against the purified recombinant GSTE2-2 protein from *An. gambiae* (Ortelli *et al.*, 2003) or 1:2500 dilution polyclonal antibody raised against the recombinant GSTD1-6 protein from *An. gambiae* (Ranson *et al.*, 1997) for 1 h on an orbital shaker. The membrane was

washed and then the bound proteins were probed with a 1:50000 dilution of Peroxidase-labelled Anti-Rabbit Antiserum as described in section 5.2.3.

6.3 RESULTS

6.3.1 Identification of Unclassified GSTs

Three unclassified GSTs were identified, which are clear orthologs of *GSTu1*, *GSTu2* and *GSTu3* from *An. gambiae* (Figure 6.1). The deduced amino acids of all three genes are aligned in Figure 6.2. *GSTu2* and *GSTu3* consist of 218 amino acids, whereas *GSTu1* contains 231 amino acids. The percentage similarities between the *Aedes* and *Anopheles* orthologs range from 58 to 75.9 at amino acid level (Table 6.2).

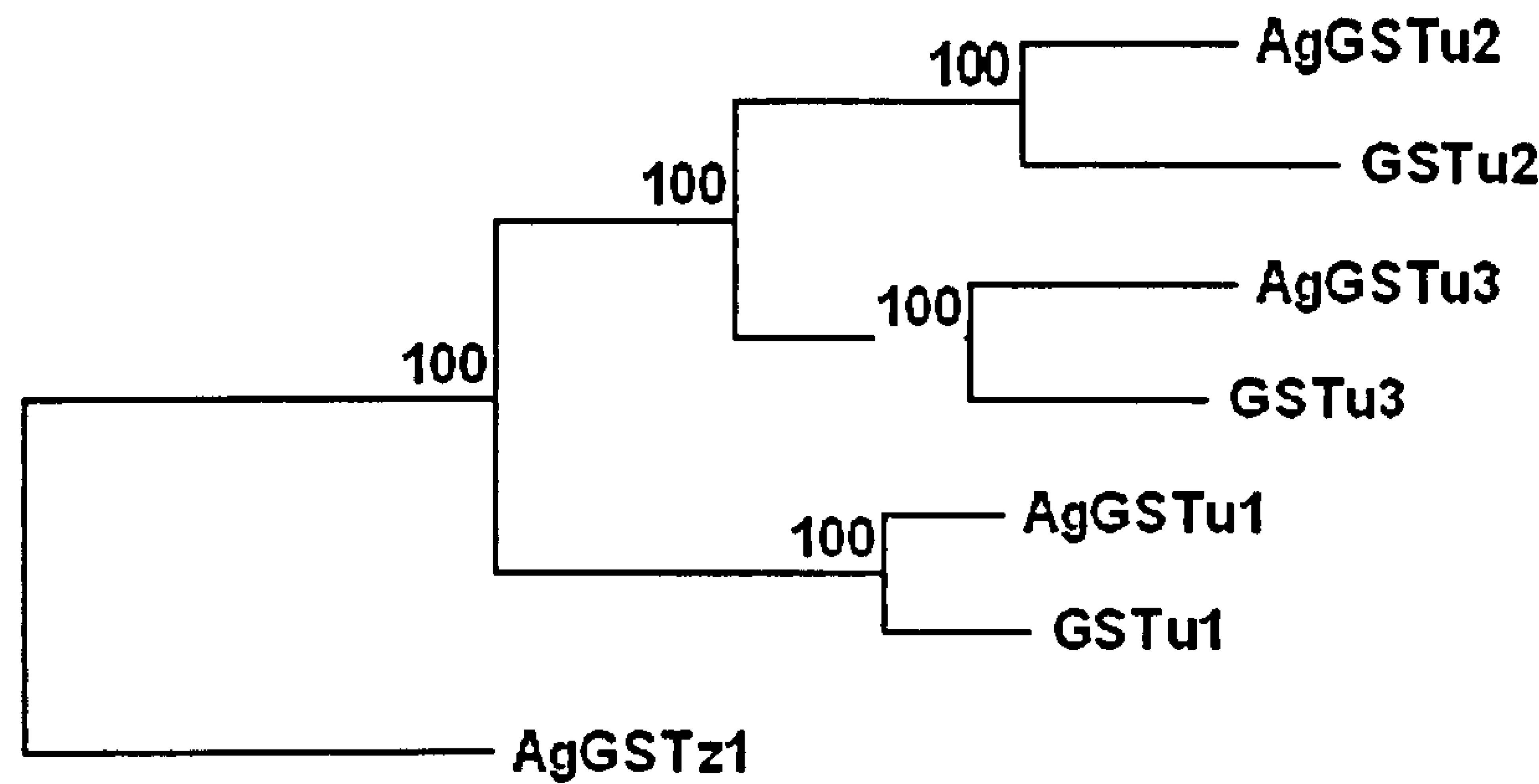


Figure 6. 1 Phylogenetic relationship of unclassified GST orthologous proteins.

The distance neighbour-joining method was performed as described in section 3.2.3.

```
GSTu2    -MPMSLYYSKMSPPARSVLLLIQELGLTGIQLKEVDVQGGGTRTEEFLKMNPEHTIPTLD 59
GSTu3    MAPIVLYHFPMSPPSRSALLVARNLGLD-VEVKILNLMAGEHMQEEFVKINPQHTVPTVV 59
GSTu1    ---MKIYAVSDGPPSLAVRMALKALDIA-HEHVPVDYGKGEHMTEDYAKMNPQKEIPVLD 56

GSTu2    DNGFYLWESRAILTYLVDAYRPGHDLYPNIPREKAQINRVLHHELSAFHPKTLGQMGAIY 119
GSTu3    DDDYVLWESKAIATYLVEQHQPDSTLYPADPKQRGIINQRLYFDSTVLFARAYAAVAPLM 119
GSTu1    DDGFFLSESNAILQYLCDKYAPDSPLYPKDPKERALVNHRLCFNLSFLYPQISAYVMAPI 116

GSTu2    RRETSVVTDEMKAKINEAYTNLELFLV--RNDWFAGENVTVADLCLLPTISTMVHVGFDL 177
GSTu3    RQGATSIPQDKKDAILEALGTLNGYLD--GQDWVAGENTTVADLCLLATVSSLEKLGVDL 177
GSTu1    FFDYERTPMGLK-KLHIALAAFETYMSRLGSKFAAGDHLTIADFPLVTSVMCLEGINFNI 175

GSTu2    SKHPRLAAWYENCK---------------VLKGYEEDQAVSQQIGQLFKELVTEGM 218
GSTu3    SDLPNITAWLERCK---------------SLPGFEENEEGASMFGNGLKSKLEEPF 218
GSTu1    DQYPLVKAWYANFKQQYPELWAISAVGMAEITEFEKNPPDLSGMEHPIHPIKKVKK 231
```

Figure 6. 2 Multiple alignment of deduced amino acid sequences of unclassified GSTs from *Ae. aegypti.*

Sequences shown are from PMD-R strain. The amino acid sequences were aligned using ClustalW. Gray shade represents to the conserved amino acid in all three genes. Dashes are used to denote gaps introduced for maximum alignment.

Table 6. 2 Sequence identities between *Aedes* and *Anopheles* GSTs (similarities of *Aedes* unclassified GSTs to *Anopheles* orthologs indicate in grey highlight).

	Percent identity					
	GSTu1	GSTu2	GSTu3	AgGSTu1	AgGSTu2	AgGSTu3
GSTu1	-	26.5	27.9	75.9	29.1	26.0
GSTu2		-	37.4	25.1	58.0	32.4
GSTu3			-	26.5	39.7	62.1
AgGSTu1				-	29.1	26.9
AgGSTu2						38.8
AgGSTu3						-

6.3.2 Cloning and Expression of Unclassified GSTs

The unclassified GST genes, *GSTu1*, *GSTu2* and *GSTu3* from PMD-R strain were cloned into the pET SUMO vector. The coding regions of the GSTs were amplified from PMD-R cDNA using a high fidelity DNA polymerase. In the first attempt, the PCR products, after A-tailing, were ligated directly into the SUMO vector but no positive colonies were obtained. Therefore an intermediate cloning step into the pGEM-T easy vector was performed. These plasmids were used as the template for the amplification of GST genes to obtain the A-overhang PCR products, and then ligated into the SUMO vector (Map of pET SUMO as shown in appendices). Positive colonies, containing the GST constructs were isolated and the recombinant proteins were expressed under IPTG induction. The majority of the expressed proteins were retained in insoluble inclusion bodies (Figure 6.3A, B and Figure 6.4) but purification of the small amount of soluble recombinant GSTs from the cell lysate was attempted.

6.3.3 Purification of Recombinant Unclassified GSTs

Cell lysates from the recombinant GST proteins were purified using His•Bind resin. The purification was carried out under native condition; cell lysate containing 5 mM imidazole was applied to the column and after washing, the bound protein was eluted with buffer containing 1 M imidazole, which contains structure similar to histidine and competes with histidine to bind with Ni^{2+} ions in the resin. The recombinant GSTU1 and GSTU2 proteins did not bind to the column (Figure 6.3A and B). GST activity levels were performed to confirm the presence of GSTU1 and GSTU2 in the cell lysate; 4.67 and 3.31mOD/min/10μl, respectively, which indicated that the expression level of these proteins were very low. Only 6x histidine-tagged recombinant GSTU3-3 bound to the column and was successfully purified from the majority of the *E. coli* proteins. However, under these elution conditions a number of impurities co-eluted with the recombinant GSTU3-3 (data not shown). To reduce the non-specific binding, binding buffer

containing 20 mM imidazole was used and higher stringency washing buffer, containing 80 mM imidazole was used.

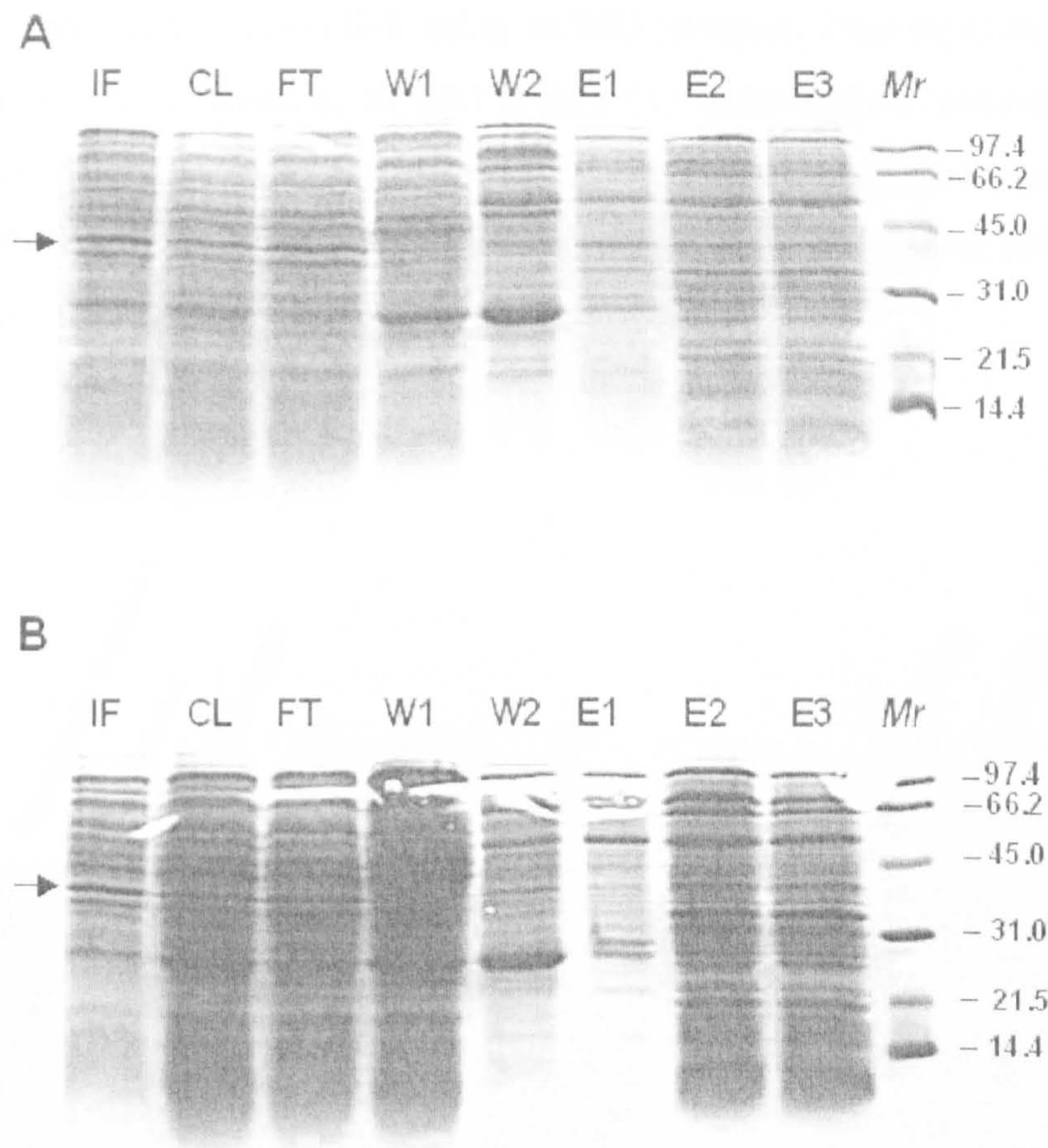


Figure 6. 3 SDS-PAGE profiles of GSTU1-1 (A) and GSTU2-2 (B) expression and purification.

Cell lysate was loaded onto His Bind resin. After flow-through collection, the resin was washed with 1X Bind buffer containing 5 mM imidazole and the followed by 1X Wash buffer containing 60 mM imidazole. The bound proteins were eluted with 1 M imidazole in 1X Elution buffer. Proteins were subjected to 15% separating polyacrylamide gel. Protein bands were visualized by staining with Coomassie Blue. Lanes contained Molecular mass standard proteins (*Mr*). IF: insoluble fraction; CL: cell lysate; FT: flow-through; W1: 5 mM imidazole wash; W2: 60 mM imidazole wash; E1-E3: eluate fraction number 1-3 (1ml/fraction). Arrow indicates to the expected size of the fusion target protein (approximately 31kDa).

The purification profile is shown in Figure 6.4. A protein of approximately 31 kDa formed the vast majority of the protein in the eluted fraction. The purified SUMO-fused recombinant GSTU3-3 was cleaved to remove SUMO protein and obtain the native recombinant GSTU3-3 using SUMO protease. Two separate bands were found, which consisted of SUMO protein (13 kDa) and recombinant GSTU3-3 (approximately 25 kDa) as shown in Figure 6.4B. Recombinant GSTU3-3 was separated from the SUMO protein and protease using a His•Bind column (Figure 6. 4B).

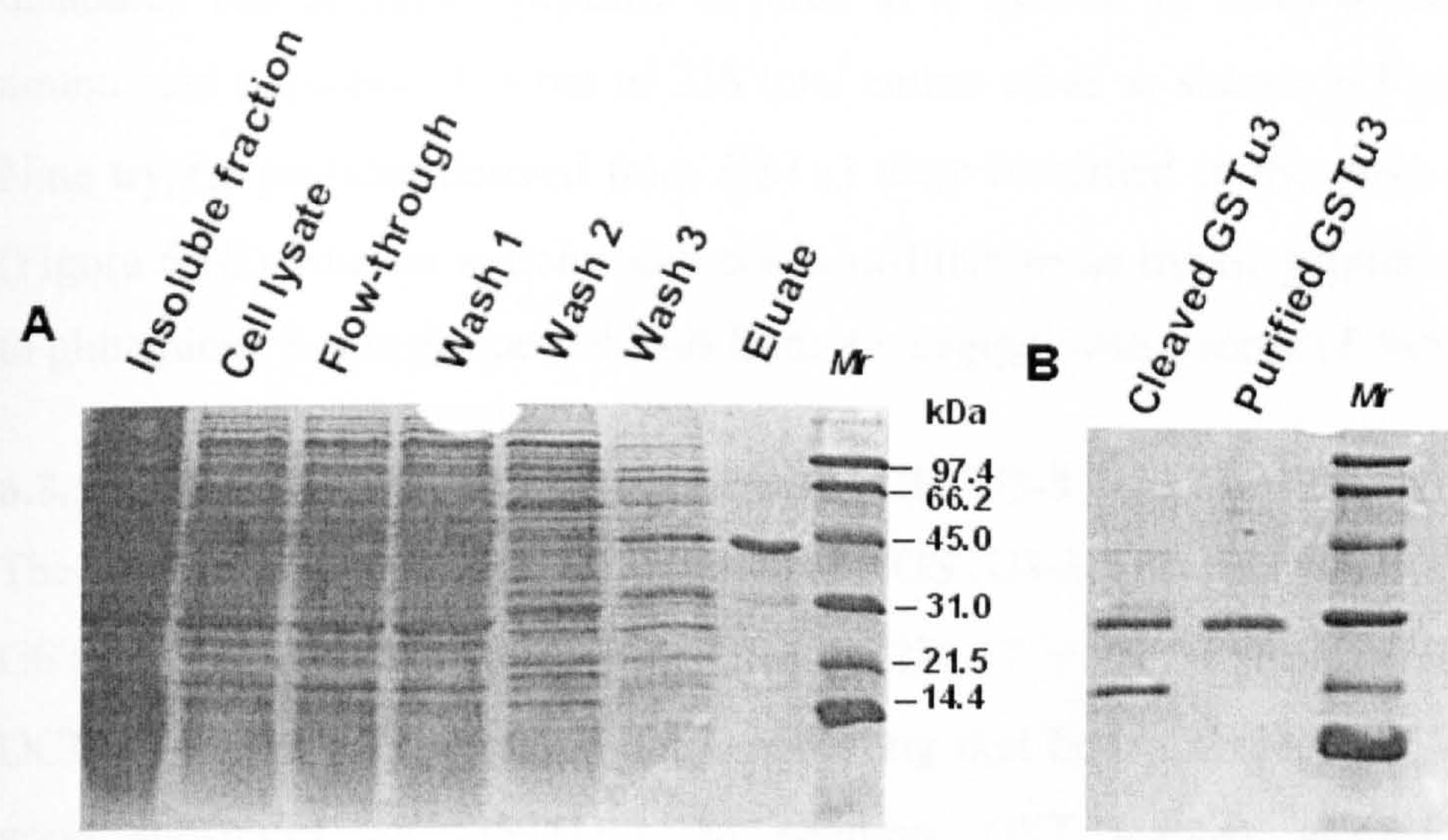


Figure 6. 4 SDS-PAGE profile of GSTU3-3 expression and purification.

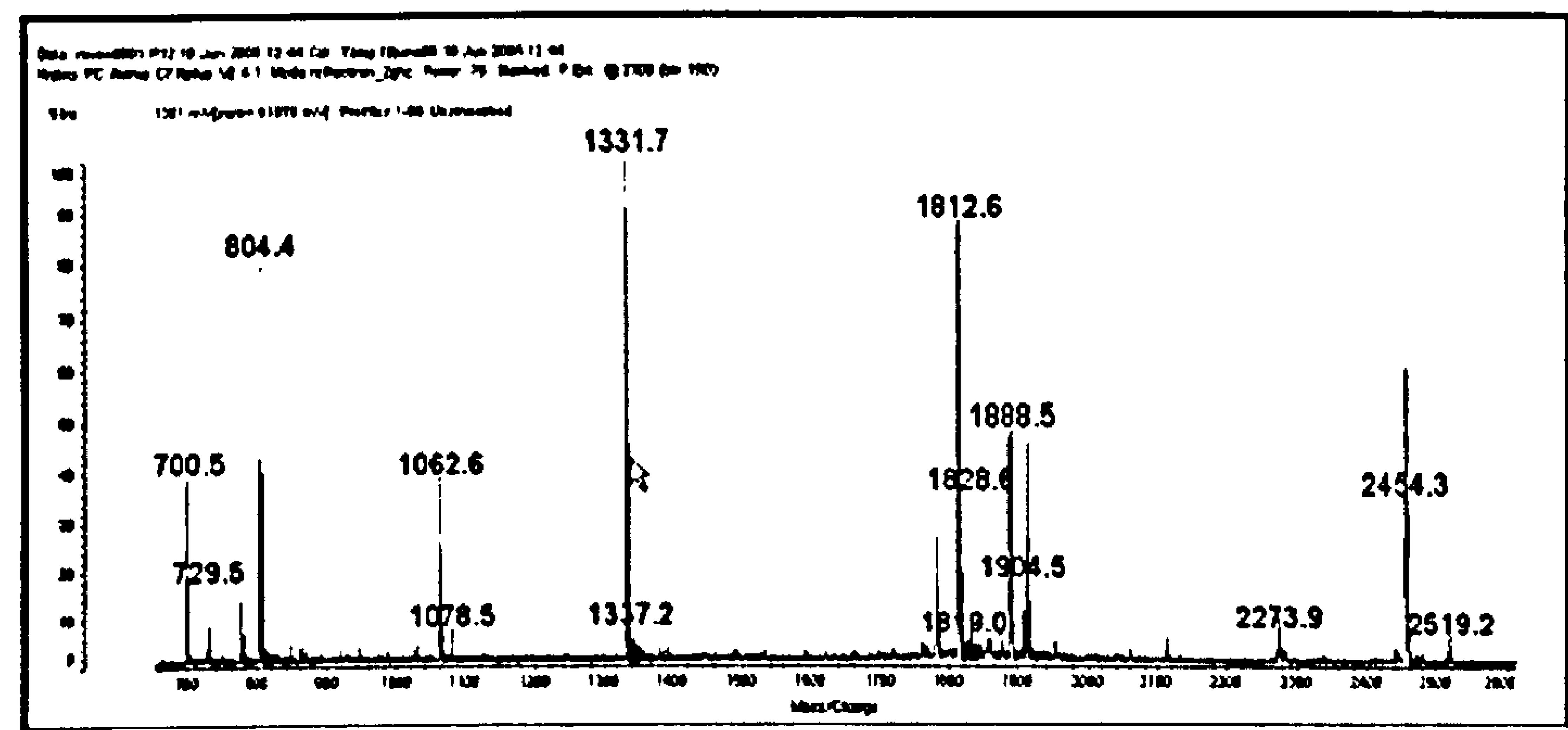
Proteins were subjected to 15% separating polyacrylamide gel. Protein bands were visualized by staining with Coomassie Blue. Lanes contained Molecular mass standard proteins are labeled *Mr*. (A) Purification profile of SUMO-fused recombinant GSTU3-3 using a His•Bind column. Wash 1, 2 and 3 contained 20, 60 and 80 mM imidazole, respectively. Protein was eluted in buffer contained 1 M imidazole. (B) SUMO protease-cleaved GSTU3-3 and purified GSTU3-3 in the flow-though after the second step purification using His•Bind column.

6.3.4 MALDI-TOF Mass Analysis

MALDI-TOF mass spectrometry analysis can be used to identify peptides and proteins. The sample is first co-crystallised with matrix compound (α-cyano-4-hydroxycinnamic acid). The application of laser radiation to this mixture, leads to the vaporisation of the matrix-carried sample and subsequently the ionisation of both positive and negative ions in the sample. The ions contain a different mass, and the mass/charge ratio determines the arrival time at the time-of-flight mass (TOF) analyser detector. The mass spectrum of trypsin-digested recombinant GSTU3-3 is shown in Figure 6.5A. All masses are reported as monoisotopic mass values. Peptides were identified with the Mascot search program against the NCBI database. The identified peptides covered 47% (shown in bold) of the GSTu3 amino acid sequence, 103 out of 218 total amino acids as shown in Figure 6.5B. Nine tryptic peptides derived from GSTu3 were identified in the mass spectrum (Figure 6.5C). Mascot search result confirmed that these tryptic peptides matched to glutathione S-transferase (GST-2) from *Ae. aegypti* with a score of 99%.

6.3.5 Characterisation of Recombinant GSTU3-3

The substrate specificities of recombinant GSTU3-3 are shown in Table 6.3. GSTU3-3 has high activity with CDNB (295.47 ± 41.08 μmol/min/mg) and DCNB (5.83 ± 0.72 μmol/min/mg), indicating that both CDNB and DCNB are good substrates for GSTU3-3. In addition, GST activity against cumene hydroperoxide is detectable, but at a very low level. The DDTase activity of recombinant GSTU3-3 was measured as the conversion of DDT to DDE. However, the activity of GSTU3-3 toward DDT is undetectable.



B

```
  1 MAPIVLYHFP MSPPSRSALL VARNLGLDVE VKILNLMAGE HMQEEFVKIN
 51 PQHTVPTVVD DDYVLWESKA IATYLVEQHQ PDSTLYPADP KQRGIINQRL
101 YFDSTVLFAR AYAAVAPLMR QGATSIPQDK KDAILEALGT LNGYLDGQDW
151 VAGENTTVAD LCLLATVSSL EKLGVDLSDL PNITAWLERC KSLPGFEENE
201 EGASMFGNGL KSKLEEPF
```

C

Residue number in GSTu3	Mass	GSTu3 peptide sequence
17 - 23	729.37	SALLVAR
24 - 32	986.49	NLGLDVEVK
33 - 48	1888.52	ILNLMAGEHMQEEFVK
94 - 99	700.44	GIINQR
100 - 110	1331.66	LYFDSTVLFAR
111 - 120	1062.61	AYAAVAPLMR
173 - 189	1911.64	LGVDLSDLPNITAWLER
192 - 211	2113.31	SLPGFEENEEGASMFGNGLK
212 - 218	849.42	SKLEEPF

Figure 6. 5 MALDI-TOF mass spectrometry analysis.

(A) MALDI-TOF mass spectrum of tryptic peptides of purified GSTU3-3. The x axis indicates the mass charge ratio and the y axis represents to the percent intensity for each peptide. (B) The putative amino acid sequence of recombinant GSTU3-3, Matched tryptic peptides are shown in bold. (C) The peptide residues identified in enzymatic digestions of the recombinant GSTU3-3 by MALDI-TOF mass spectrometry, which corresponds to the mass charge ratio indicates in number as shown in each peak in panel A, the peptide sequences.

Table 6. 3 Substrate specificities for recombinant GSTU3-3 from *Ae. aegypti.*
Three independent assays were performed. Results show mean ± SD. ND indicates not detectable.

Substrate	Specific activity of GSTU3-3
CDNB (μmol/min/mg)	295.47 ± 41.08
DCNB (μmol/min/mg)	5.83 ± 0.72
CHP (μmol/min/mg)	0.093 ± 0.054
DDTase activity (nmol DDE/μg)	ND

The inhibition of recombinant GSTU3-3 activity by hematin was determined by varying the concentration of hematin in the fixed concentration of GSH and CDNB at 1 mM. The IC_{50} value of 37.5 ± 0.1 μM for GSTU3-3 (Figure 6.6) is approximately 10 fold higher than that for GSTE2-2 (as shown in Figure 5.6). The dissociation constant (K_D) for hematin is 2.58 ± 0.46 μM (Figure 6.7), indicating the binding of GSTU3-3 to hematin is higher than GSTE2-2 (K_D = 67.9 ± 2.0 μM). Although, the inhibition type of heme on recombinant GSTE2-2 and GSTU3-3 was not determined further. Studied in mammalian GSTs indicated that heme inhibit GST activity by non competitive manner, suggesting the heme-binding site on GST is separated from active site (Vander Jagt *et al.*, 1985).

6.3.6 Immunobloting

Polyclonal antiserum raised against the *An. gambiae* Delta and Epsilon GSTs, GSTD1-6 and GSTE2-2, were used to investigate their cross-reactivity with recombinant GSTE2-2 and GSTU3-3. Polyclonal antibody against AgGSTE2-2 bound to *Aedes* GSTE2-2 but not GSTU3-3 (Figure 6.8A). The results with the polyclonal antibody against AgGSTD1-6 show the cross-reactivity with recombinant GSTE2-2 and GSTU3-3 as clear differences in intensity of spots between different concentrations (Figure 6.8B).

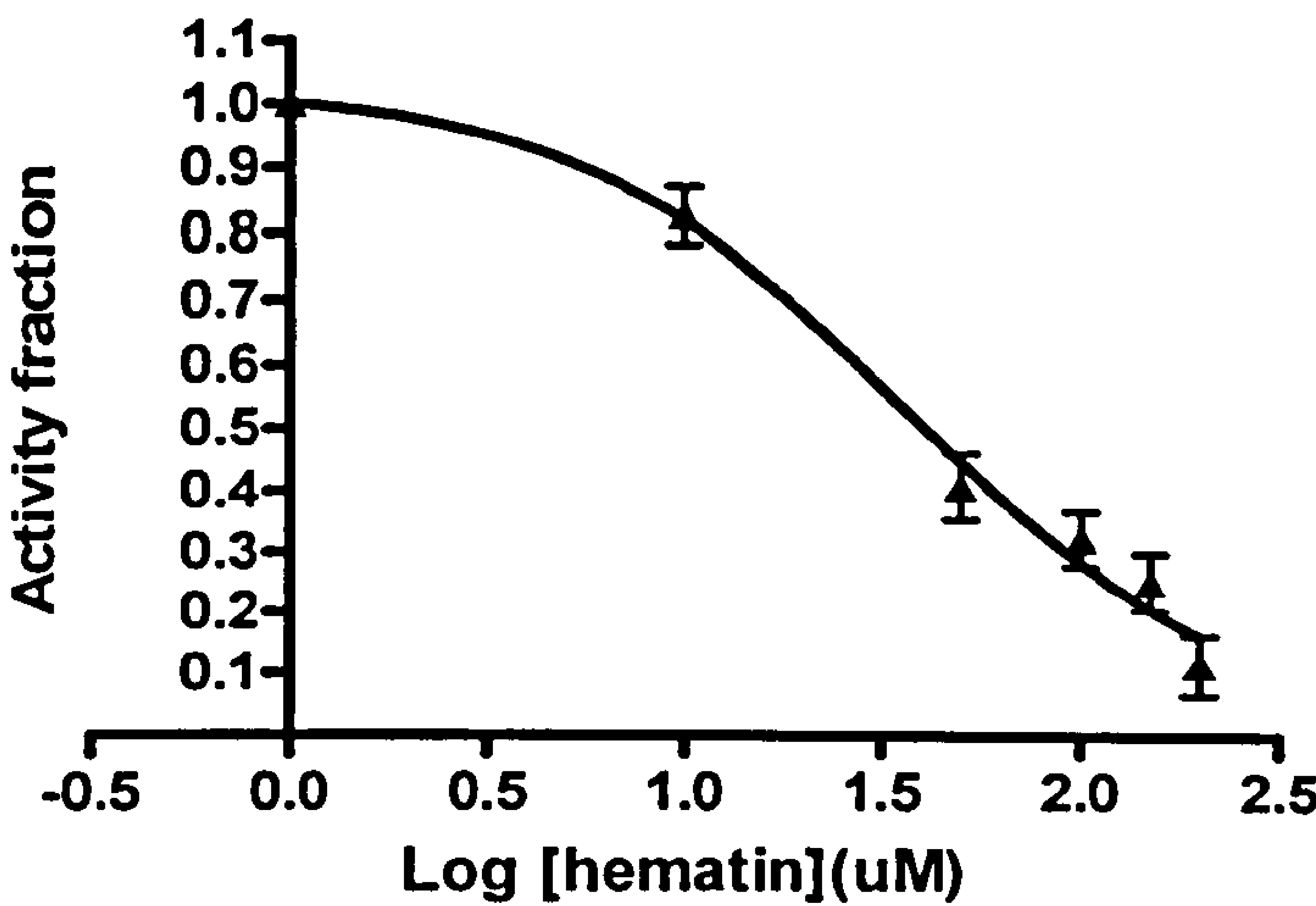


Figure 6. 6 Inhibition of GSTU3-3 by hematin with CDNB as a model substrate.

Data represents the mean ± SD of three replicate experiments. IC_{50} is the concentration of hematin giving 50% inhibition of enzyme activity. The IC_{50} value is 37.5 ± 0.1 μM.

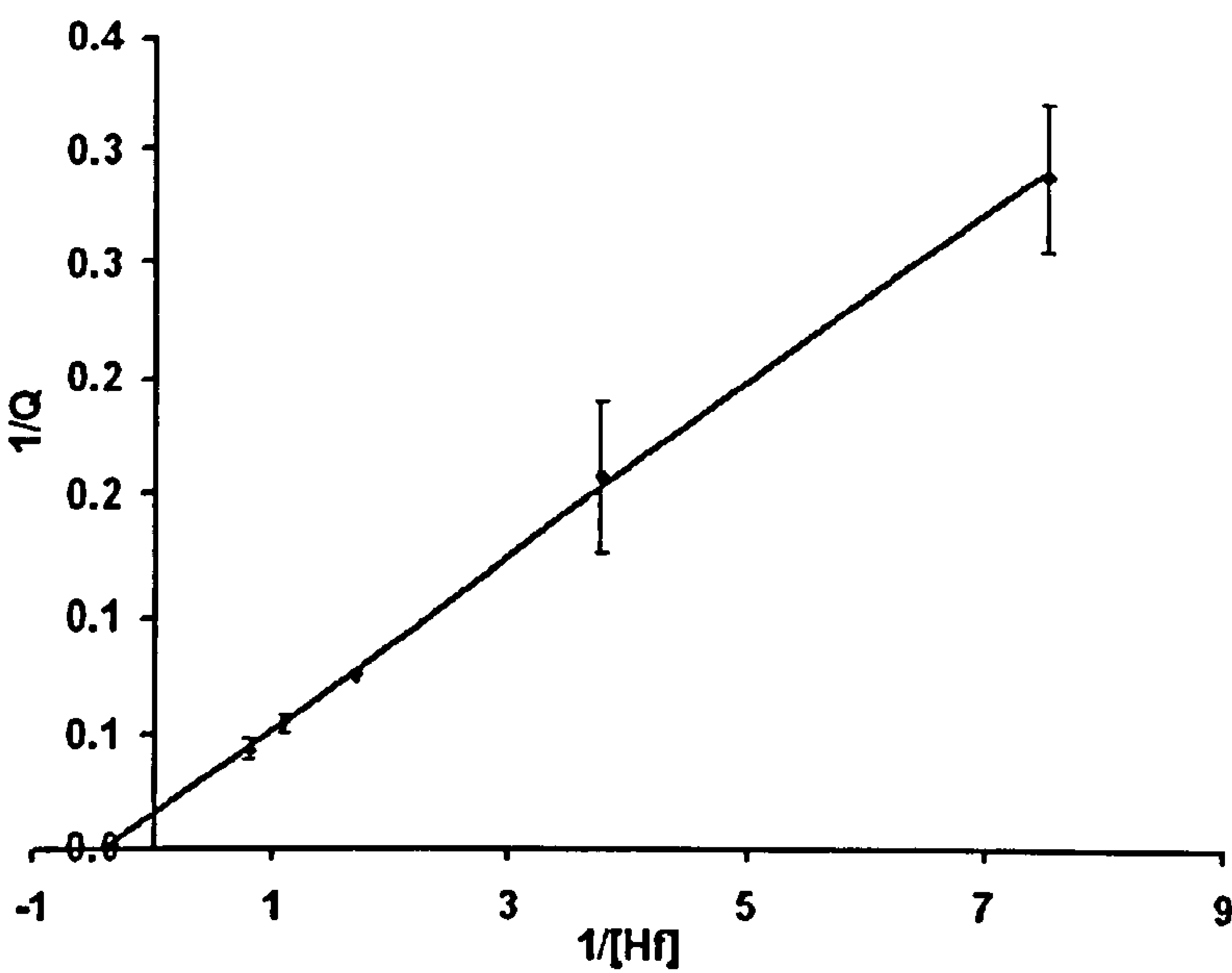


Figure 6. 7 Double reciprocal plot of the intrinsic fluorescence intensity (1/Q) of GSTU3-3 against the concentration of free hematin ($1/H_f$). K_D = 2.58 ± 0.46 μM.

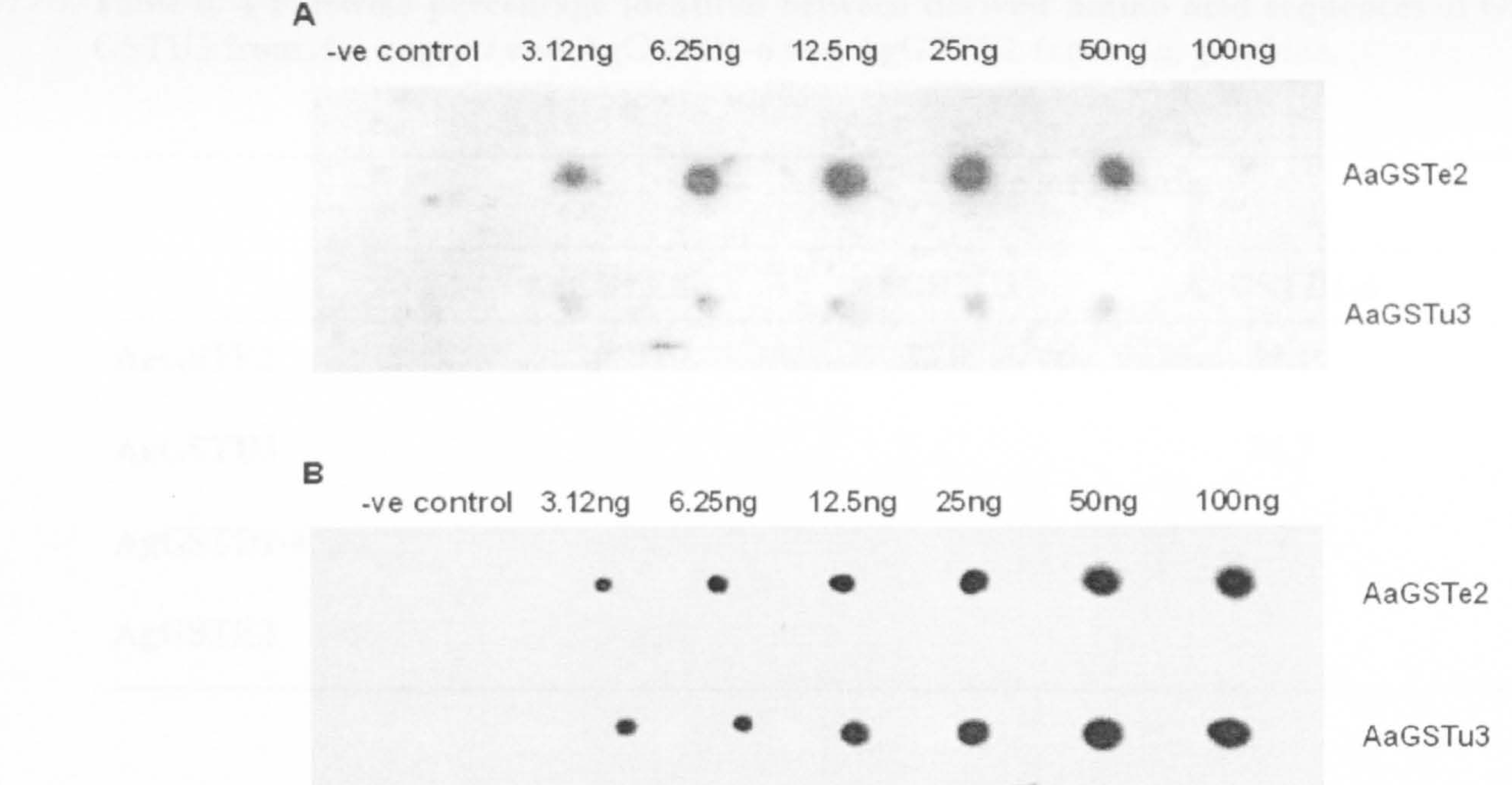


Figure 6. 8 Immunoblot analysis.

One microlitres purified recombinant AaGSTE2-2 and AaGSTU3-3 (3.12ng-100ng) were spot on nitrocellulose membrane. Proteins were probed with antibody raised against AgGSTE2-2 (1:5000 dilution) **(A)** and AgGSTd1-6 (1:1000 dilution) **(B)**. Peroxidase labeled anti-rabbit antibody (1:50000; Amersham Pharmacia Biotech)) was used as a second antibody. Proteins were visualised by enhancing the chemiluminescence using ECL Advance Blotting Detection Kit (Amersham Biosciences). Negative control was performed using 1 μl 0.1 M phosphate buffer indicates in –ve control.

Table 6. 4 Pairwise percentage identities between derived amino acid sequences of GSTE2 and GSTU3 from *Ae. aegypti* and AgGSTD1-6 and AgGSTE2 from *An. gambiae*.

	Percent identity			
	AaGSTE2	**AaGSTU3**	**AgGSTD1-6**	**AgGSTE2**
AaGSTE2	-	33.0	35.1	71.2
AaGSTU3		-	36.7	34.9
AgGSTD1-6			-	36.9
AgGSTE2				-

Aedes aegypti and *An. gambiae* GSTE2 share more than 70% of identical residues, In contrast approximately 35% identities were observed in GSTE2 and GSTU3 relative to AgGSTD1-6 (Table 6.4).

6.4 DISCUSSION

The purification of GSTU1 and GSTU2 was not successful due to either the lower expression of these proteins or the lack of the affinity binding to Ni^{2+} ions in the His Bind resin. These might be due to the inaccessibility or the degradation of 6xHis tag or indicate that the binding conditions were incorrect. An attempt to purify these proteins under denaturing conditions to expose the tag was made, but the same result was found. pET SUMO vector has been used to increase the expression and the solubility of the recombinant proteins (Invitrogen), but this system was not success for GSTU1 and GSTU2. However the purification conditions were not optimised further for these proteins. Only unclassified GSTU3-3 was successfully purified and its identity confirmed by MALDI-TOF mass analysis.

In a previous study, three different GST isozymes were purified and characterized in *Ae. aegypti*, one of these isozymes, GST-2 (GSTU3) was over expressed at mRNA and protein level in a DDT resistant strain (Grant & Hammock, 1992; Grant & Matsamura, 1988; Grant & Mutsumura, 1989). However, this gene is not over expressed in PMD-R, Thai mosquito strain (Chapter 4). DDTase activity was not detected in the recombinant GSTU3-3 enzyme. Collectively, the results of this study suggest GSTU3 is not responsible for DDT resistance in PMD-R strain from Thailand. One explanation for this result is that alternative DDT resistance mechanisms have involved in different strain. However, in the study by Grant *et al.* (Grant & Hammock, 1992) the mRNA expression of *GSTu3* was measured by

Northern blot analysis using a 123 bp N-terminal cDNA probe from a highly conserved region in GSTs. This probe may have cross hybridised with other GST genes, which were responsible for DDT resistance. Another reason is that GST-2 may represent more than one GST isoenzyme. Grant et al. (Grant & Hammock, 1992) demonstrated that GST-2 protein also up regulated in resistant strain. This protein was measured using the primary antibody raised against GST-2 isozyme, which may cross react with other GST proteins involved GST-2.

Recombinant GSTU3-3 showed higher GST activity toward the model substrate, CDNB than recombinant GSTE2-2. Both recombinant enzymes also bind to hematin. GSTU3-3 was higher affinity for heme than GSTE2-2, but bound heme less efficiently than that for GSTE2-2. This may indicate that the binding site of heme is not the same as the active site of GSTE2-2, but causes interference in the activity of this protein. These results suggest that in addition to the enzymatic function these GSTs bind with non substrate compounds such as hematin. The binding of hematin by GST has been proposed to be the adaptation of the nematode *Haemonchus contours* to blood feeding (van Rossum *et al.*, 2004).

AgGSTE2-2 antiserum is able to bind to recombinant AaGSTE2-2, but this antibody does not cross-react with AaGSTU3-3 under the conditions of this assay suggesting that GSTu3 does not belong to Epsilon class. In contrast, AgGSTD1-6 antiserum bound to recombinant AaGSTE2-2 and AaGSTU3-3. This result may suggest that these two recombinant proteins have a common epitope that is recognised by the antibody raised against AgGSTD1-6. Previously, insect GSTs were classified into 2 board classes based on the sequence identity and the immunological cross-reactivity (Sheehan *et al.*, 2001). The sequences that share more than 40% identity were assigned to the same classes. Class I GSTs known as Delta class has been found mainly in insects (Ranson *et al.*, 1998; Tang & Tu, 1994), whereas Omega, Zeta, Sigma and Theta classes were identified subsequently to be a member of class II GSTs (Chelvanayagam *et al.*, 2001).

Although, AgGSTD1-6 shows approximately 35% identity to AaGSTE2-2 and AaGSTU3-3 while, immunological cross-reactivity was observed in both recombinant proteins suggested that these GSTs might be previously included in Delta class (class I GSTs).

Chapter 7

IDENTIFICATION OF TRANSCRIPTS PRODUCED BY ALTERNATIVE SPLICING OF *Aedes aegypti* GST GENES

7.1 INTRODUCTION

Alternative splicing is the process of intron splicing from pre-mRNAs by joining together different combinations of exons to produce different mature mRNA transcripts. The different transcripts lead to the synthesis of variant proteins, which may have different properties or functions (Stamm, S.et al., 2005, Graveley, B., 2001 and Black, D. 2000). In general, splice sites start with 5'- GU and end with the sequence AG-3' (Figure 7.1), however this GU/AG rule is not always followed (Wu & Krainer, 1999). Alternative splicing can be divided into five splicing types: exon skipping/ inclusion, alternative 5' splice sites, alternative 3' splice sites, mutually exclusive cassette exons and retained intron (Figure 7.2) (Graveley, 2001).

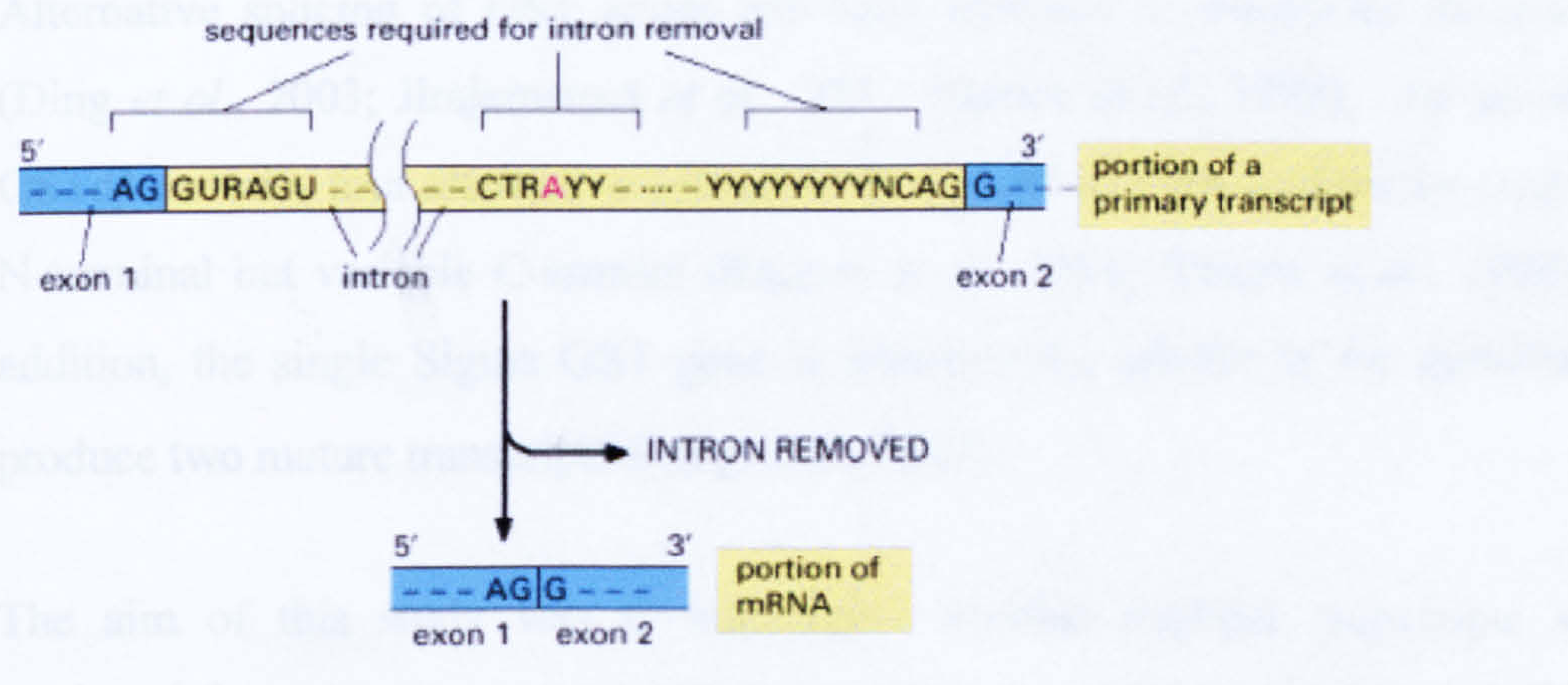


Figure 7. 1 Splicing signal sequences Figure from molecular biology of the cell (Alberts *et al.*, 2002).

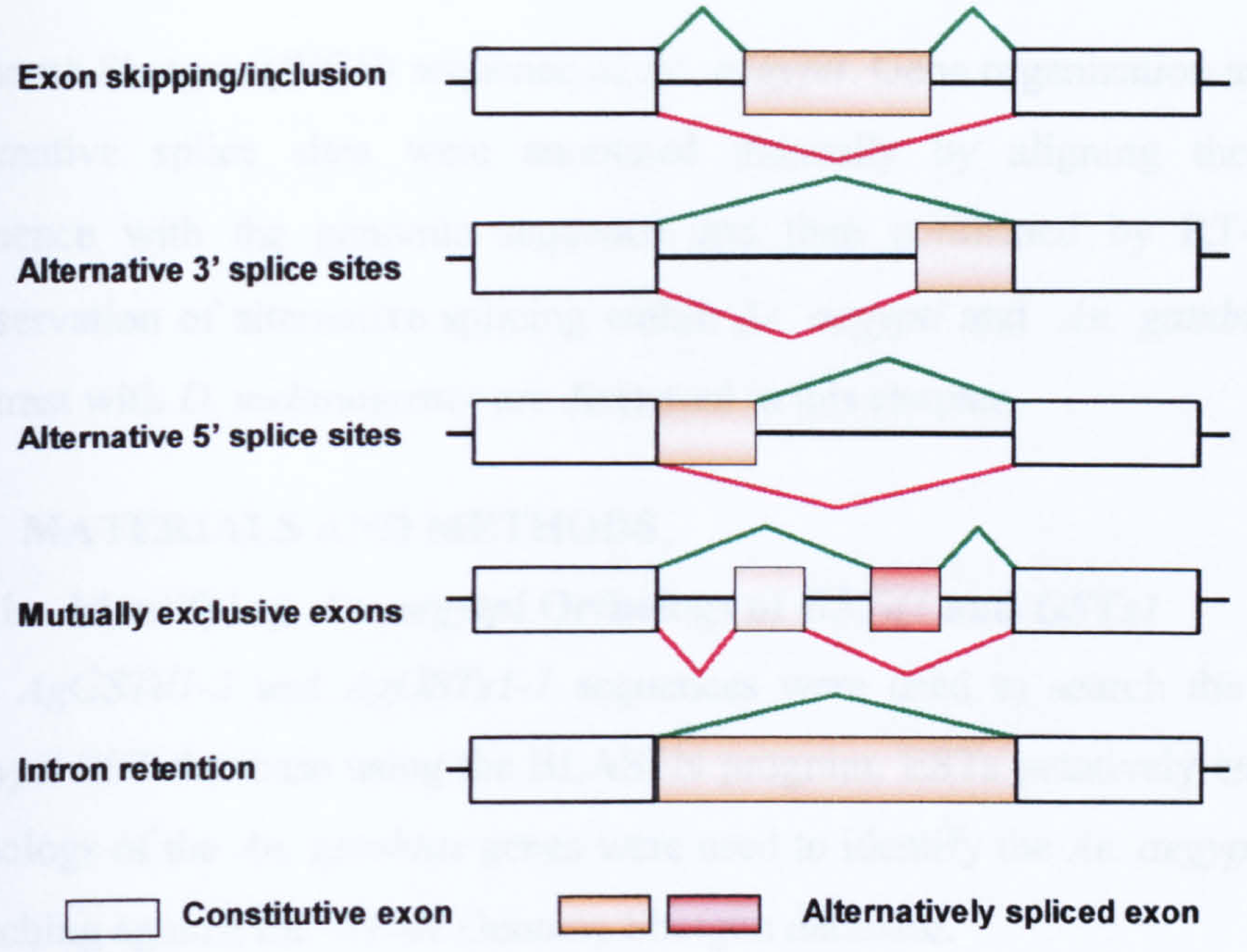


Figure 7. 2 Types of alternative splicing.

The angle lines between the boxes represent the possible alternative splicing pattern. Constitutive exons are shown as white boxes. Alternatively spliced exons are represented as boxes with different shading. Figure is from http://med.stanford.edu/sgtc/research/alternative splicing.html.

Alternative splicing of GST genes has been reported in *Anopheles* mosquitoes (Ding *et al.*, 2003; Jirajaroenrat *et al.*, 2001; Ranson *et al.*, 1998). *An. gambiae* GSTd1 encodes four alternative spliced isoforms. All of them contain the common N-terminal but variable C-termini (Ranson *et al.*, 1998; Ranson *et al.*, 1997). In addition, the single Sigma GST gene is alternatively spliced in *An. gambiae* to produce two mature transcripts (Ding *et al.*, 2003).

The aim of this study was to investigate whether multiple transcripts were produced from the *Ae. aegypti* orthologs of the *An. gambiae GSTd1* and *GSTs1* genes. EST sequences derived from these genes were identified in the *Ae. aegypti* TIGR database. These EST sequences were searched again against the Whole

Genome Shotgun (WGS) sequence of *Ae. aegypti*. Gene organization and putative alternative splice sites were annotated manually by aligning the transcript sequence with the genomic sequence and then confirmed by RT-PCR. The conservation of alternative splicing within *Ae. aegypti* and *An. gambiae* and the contrast with *D. melanogaster* are discussed in this chapter.

7.2 MATERIALS AND METHODS

7.2.1 Identifying *Ae. aegytpi* Orthologs of *GSTd1* and *GSTs1*

The *AgGSTd1-2* and *AgGSTs1-1* sequences were used to search the TIGR *Ae. aegypti* EST database using the BLASTN program. ESTs putatively encoding the orthologs of the *An. gambiae* genes were used to identify the *Ae. aegypti* genes by searching against the Whole Genome Shotgun database.

7.2.2 Prediction of Alternative Splice Variants

The putative structure of the *Ae. aegypti GSTs1* and *GSTd1* genes was determined by searching the nr database at GenBank to identify genomic regions with high identity to GST sequences. The protein coding regions were manually annotated on matched WGS sequences using the MapDraw programme (DNAstar software). Deduced amino acid sequences and exon/intron boundaries were identified and verified by comparison with EST sequences, where available. Three putative splice variants were identified for *GSTd1* (*GSTd1-1, GST d1-2* and *GSTd1-3*) and two for *GSTs1* (*GSTs1-1* and *GSTs1-2*)

7.2.3 Confirmation of Splice Variant of GSTd1 and GSTs1

Primers were designed to amplify each of the putative alternative transcripts from *GSTd1* and *GSTs1* (Table 7.1). PCR reactions were performed as described in section 3.2.5. The PCR products were cloned into pGEM-T easy vector (section 3.2.6). DNA sequencing was performed as explained in section 3.2.7.

Table 7. 1 Primers used to amplify *Ae. aegypti* alternatively spliced GSTs.

Gene	Primer name	Primer sequence (5'-3')
GSTd1	GSTd1F	ATGGATTTCTACTACCTGCCAG
	GSTd1-1R	TCACTTCTCGAAGTACTTG
	GSTd1-2R	CTACTGCGCAGGGGCTTTAAC
	GSTd1-3R	TTACATTCCGGACAGGAAC
GSTs1	GSTs1F	ATGCCGGATTACAAGGTCTAC
	GSTs1exon4R	TTAGATCTCAGTTTGTGGTCG
	GSTs1exon5R	ACTCAGACGGAAATCGTTAATC

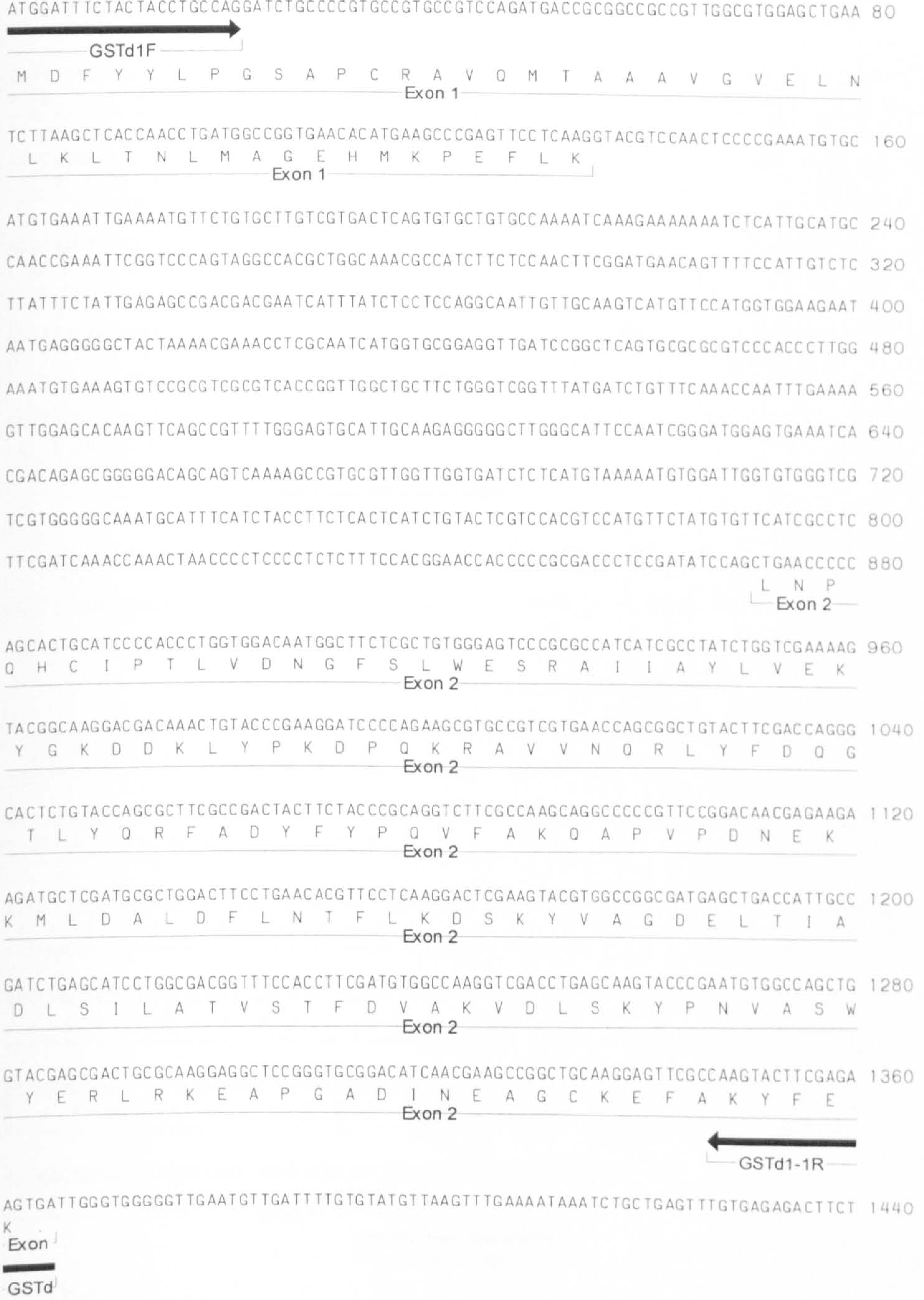
7.3 RESULTS

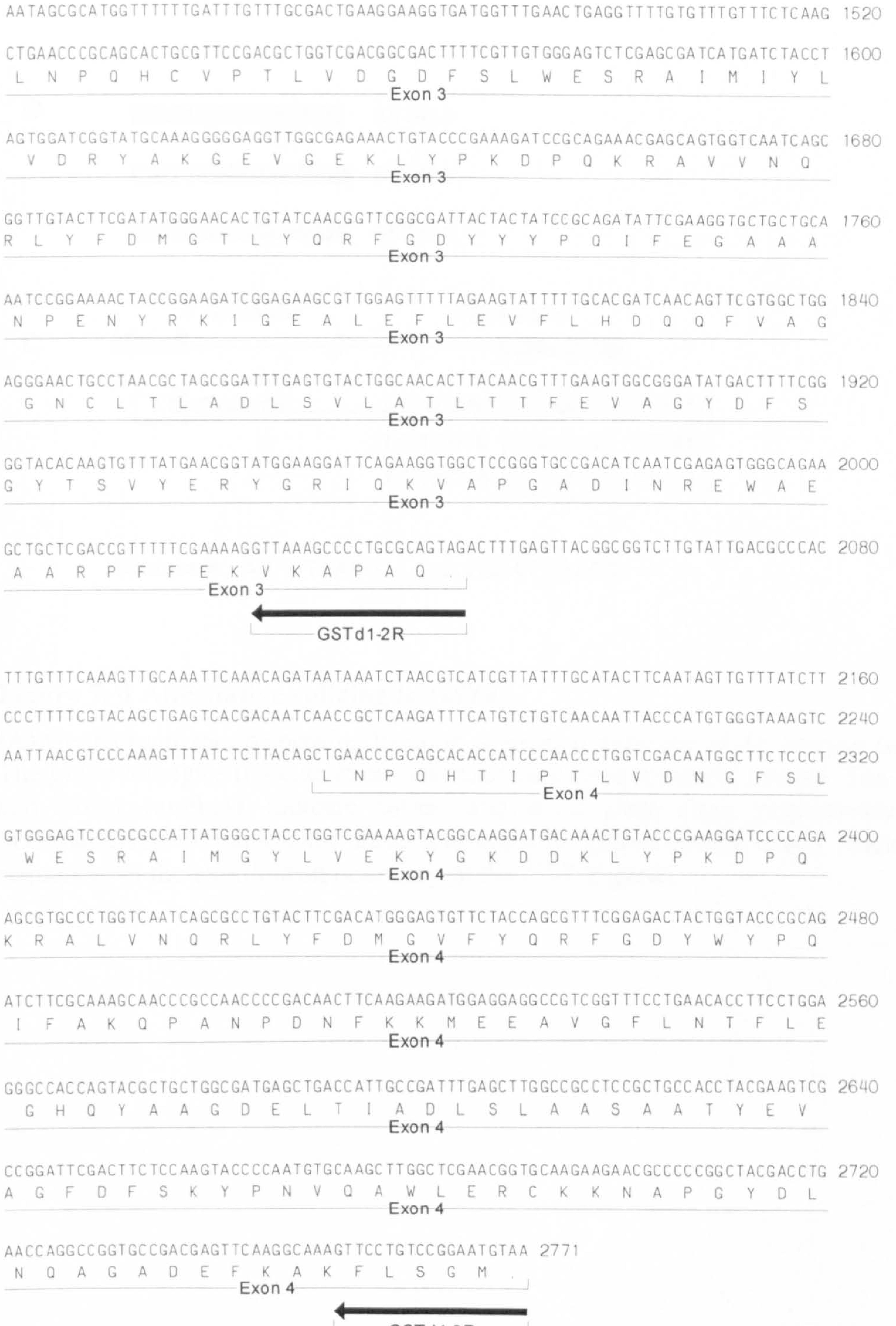
7.3.1 Alternative Splicing in *GSTd1*

Two transcripts putatively from *Ae. aegypti GSTd1* were retrieved from the EST database and were used to identify genomic fragments encoding the *GSTd1* gene. Two WGS scaffolds (AAGE01035830 and AAGE01064758), were retrieved and aligned to determine the structure of the *GSTd1* gene. The positions of the four exons, designated exon 1 to 4, and three introns are shown in Figure 7.3 and 7.4A. Exon 1 encodes the N-terminal of a GST gene, whereas exons 2, 3 and 4 are 3'-alternatively spliced exons encoding the C termini of three distinct GSTs, *GSTd1-1*, *GSTd1-2* and *GSTd1-3* (Figure 7.4B). All exon/intron boundaries show splice signals consistent with the GT-AG rule as presented in Figure 7.4C. To confirm that this predicted organization of the *GSTd1* gene was correct and that, all three transcripts are expressed, the putative transcripts were amplified from adult cDNA and sequenced. The deduced amino acid sequences are shown in Figure 7.5. The 45 amino acids from coding exon 1 are identical in all transcripts at the N-terminal end, while the remaining sequences were variable matching either exon 2, 3 or 4. The deduced amino acid sequence of *GSTd1-1*, *GSTd1-2* and *GSTd1-3* consisted of 209, 211 and 219 amino acids, respectively.

Figure 7. 3 Nucleotide sequence and predicted exon locations in *Ae. aegypti GSTd1* gene.

The primer sequences used to confirm the putative splicing pattern are marked.




1520
1600
Exon 3
1680
Exon 3
1760
Exon 3
1840
Exon 3
1920
Exon 3
2000
Exon 3
2080
Exon 3
GSTd1-2R
2160
2240
2320
Exon 4
2400
Exon 4
2480
Exon 4
2560
Exon 4
2640
Exon 4
2720
Exon 4
2771
Exon 4
GSTd1-3R

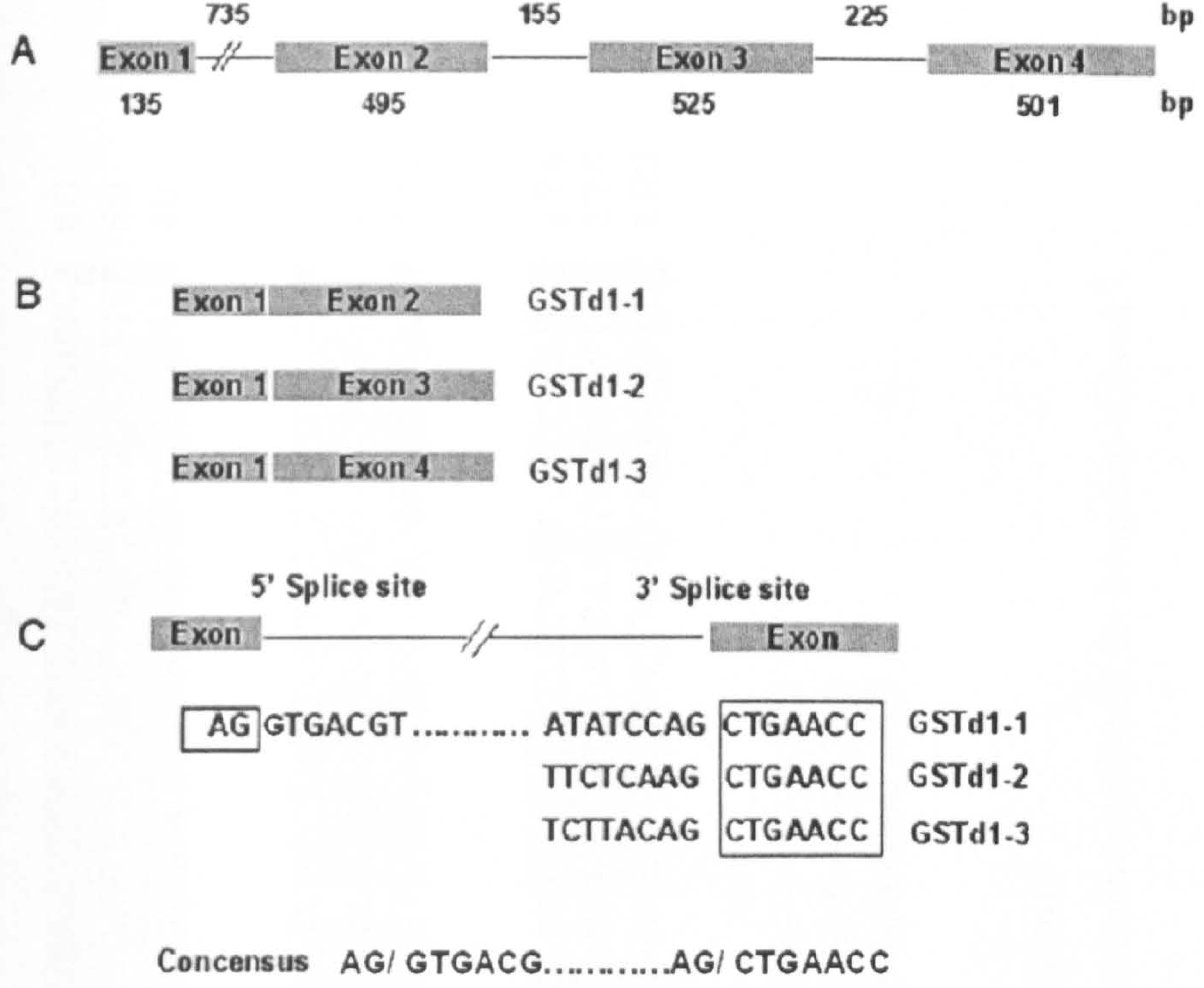


Figure 7. 4 Alternative splicing in *GSTd1*.

(A) Gene organization showing location of exon and introns of *Ae. aegypti GSTd1*. The gray rectangles indicate exons and the black lines represent introns. The upper and lower numbers indicate intron and exon sizes (bp), respectively. **(B)** Schematic representation the three alternatively spliced products **(C)** Nucleotide sequences at the exon/intron boundary in the *GSTd1* gene.

```
GSTd1-1    MDFYYLPGSAPCRAVQMTAAAVGVELNLKLTNLMAGEHMKPEFLKLNPQHCIPTLVDNGF 60
GSTd1-3    MDFYYLPGSAPCRAVQMTAAAVGVELNLKLTNLMAGEHMKPEFLKLNPQHTIPTLVDNGF 60
GSTd1-2    MDFYYLPGSAPCRAVQMTAAAVGVELNLKLTNLMAGEHMKPEFLKLNPQHCVPTLVDGDF 60

GSTd1-1    SLWESRAIIAYLVEKYGK---DDKLYPKDPQKRAVVNQRLYFDQGTLYQRFADYFYPQVF 117
GSTd1-3    SLWESRAIMGYLVEKYGK---DDKLYPKDPQKRALVNQRLYFDMGVFYQRFGDYWYPQIF 117
GSTd1-2    SLWESRAIMIYLVDRYAKGEVGEKLYPKDPQKRAVVNQRLYFDMGTLYQRFGDYYYPQIF 120

GSTd1-1    AKQAPVPDNEKKMLDALDFLNTFLKDSKYVAG-DELTIADLSILATVSTFDVAKVDLSKY 176
GSTd1-3    AKQPANPENFKKMEEAVGFLNTFLEGHQYAAG-DELTIADLSLAASAATYEVAGFDFSKY 176
GSTd1-2    EGAAANPENYRKIGEALEFLEVFLHDQQFVAGGNCLTLADLSVLATLTTFEVAGYDFSGY 180

GSTd1-1    PNVASWYERLRKEAPGADINEAGCKEFAKYFEK------ 209
GSTd1-3    PNVQAWLERCKKNAPGYDLNQAGADEFKAKFLSGM---- 211
GSTd1-2    TSVYERYGRIQKVAPGADINREWAEAARPFFEKVKAPAQ 219
```

Figure 7. 5 Alignment of the three GST proteins from the *GSTd1* gene.

Gray highlights indicate conserved sequences in the three proteins. The arrow represents the boundary of exon 1. Dashes are used to denote gaps introduced for maximum alignment.

Amino acid sequence alignment of alternatively spliced *GSTd1* isoforms in *Ae. aegypti* and *An. gambiae* indicated that the N-terminal exons are highly conserved (Figure 7.6). *Aedes aegypti GSTd1* demonstrates a similar pattern of alternative splicing to that, which has been previously reported in *Anopheles GSTd1* (Pongjaroenkit *et al.*, 2001; Ranson *et al.*, 1998). However, four transcripts are produced in *Anopheles* whereas only three are found in *Ae. aegypti*. The percent identity of spliced *GSTd1* in *Ae. aegypti* and *An. gambiae* range from 55-85% at the amino acid level (Table 7.2). The alternatively spliced *GSTd1* from *Aedes* and *Anopheles* are probable orthologs and the duplication of the C-terminal exons presumably occurred before the divergence of these two lineages (Figure 7.12).

7.3.2 Alternative Splicing in *GSTs1*

Two putative Sigma GSTs were identified in the EST database and used to retrieve the corresponding genomic locus/loci. Both were contained within four WGS scaffolds without overlapping (AAGE01051524, AAGE01631930, AAGE01044281 and AAGE01133014). The gene structure was determined using DNAStar software and BLASTX. The *Aedes aegypti* Sigma GST gene spans 7678 bp and contains six exons (Figure 7.7). Two alternative transcripts are produced by alternative splicing of this gene, *GSTs1-1* and *GSTs1-2*. The intron and exon structure of these transcripts is shown in Figure 7.8A. *GSTs1-1* is composed of four exons (exon1-4), while only three exons (exon1, exon 5 and 6) comprise *GSTs1-2* (Figure 7.8B). *GSTs1-1* and *GSTs1-2* were amplified using GSTs1F forward primer and GSTs1exon4R and GSTs1exon5R primers (Table 7.1) indicating that both cDNAs are expressed in adult mosquitoes (Figure 7.9). The amplified cDNAs correspond with the full-length of *GSTs1-1* and the partial *GSTs1-2* (612 and 303 bp) as demonstrated in Figure 7.8.

```
                                                                  ↓
GSTd1-2      MDFYYLPGSAPCRA-V-QMTAAAVGVELNLKLTNLMAGEHMKPEFLKLNPQHCVPTLVDG 58
AgGSTd1-5    MDFYYLPGSAPVPCRP-RLDGGPVGVELNLKLTDLMKGEHMKPEFLKLNPQHCVATLVDS 59
GSTd1-3      MDFYYLPGSA--PCRAVQMTAAAVGVELNLKLTNLMAGEHMKPEFLKLNPQHTIPTLVDN 58
AgGSTd1-6    MDFYYLPGSAPVPCRP-RLDGGPVGVELNLKLTDLMKGEHMKPEFLKLNPQHCIPTLVDN 59
AgGSTd1-4    MDFYYLPGSAPVPCRP-RLDGGPVGVELNLKLTDLMKGEHMKPEFLKINPQHCIPTLVDN 59
GSTd1-1      MDFYYLPGSA--PCRAVQMTAAAVGVELNLKLTNLMAGEHMKPEFLKLNPQHCIPTLVDN 58
AgGSTd1-3    MDFYYLPGSAPVPCRP-RLDGGPVGVELNLKLTDLMKGEHMKPEFLKLNPQHCIPTLVDE 59

GSTd1-2      -DFSLWESRAIMIYLVDRYAKGE--VGEKLYPKDPQKRAVVNQRLYFDMGTLYQRFGDYY 115
AgGSTd1-5    -GFALWESRAIMCYLVEKYGKPC--NNDSLYPTDPQKRAIVNQRLYFDMGTLYQRFGDYY 116
GSTd1-3      -GFSLWESRAIMGYLVEKYGK-----DDKLYPKDPQKRALVNQRLYFDMGVFYQRFGDYW 112
AgGSTd1-6    -GFALWESRAIQIYLAEKYGK-----DDKLYPKDPQKRAVVNQRLYFDMGTLYQRFADYH 113
AgGSTd1-4    -GFALWESRAICTYLAEKYGK-----DDKLYPKDPQKRAVVNQRMYFDMGTLYQRFANYY 113
GSTd1-1      -GFSLWESRAIIAYLVEKYGK-----DDKLYPKDPQKRAVVNQRLYFDQGTLYQRFADYF 112
AgGSTd1-3    DGFVLWESRAIQIYLVEKYCAHDPALAERLYPGDPRRRAVVHQRLFFDVAILYQRFAEYY 119

GSTd1-2      YPQIFEGAAAN-PENYRKIGEALEFLEVFLHDQQFVAGGNCLTLADLSVLATLTTFEVAG 174
AgGSTd1-5    YPQIFEGAPAN-EANFAKIGEALAFLDTFLEGERFVAGGNGYSLADISLYATLTTFEVAG 175
GSTd1-3      YPQIFAKQPAN-PENFKKMEEAVGFLNTFLEGHQYAAG-DELTIADLSLAASAATYEVAG 170
AgGSTd1-6    YPQIFAKQPAN-PENEKKMKDAVGFLNTFLEGQEYAAG-NDLTIADLSLAATIATYEVGG 171
AgGSTd1-4    YPQIFAKQPAN-PENEQKMKDAVGFLNTFLDGHKYVAG-DSLTIADLSILATISTYDVAG 171
GSTd1-1      YPQVFAKQAPV-PDNEKKMLDALDFLNTFLKDSKYVAG-DELTIADLSILATVSTFDVAK 170
AgGSTd1-3    YPQIFGKKVAGDPDRLRSMEQALEFLNTFLEGERFVAGGDDPTIADFSILASIATFDAAG 179

GSTd1-2      YDFSGYTSVYERYGRIQKVAPGADINREWAEAARPFFEKVKAPAQ 219
AgGSTd1-5    YDFSAYVNVLRWYKSMPELIPASDTNRSWAEAARPFFDKVKH--- 217
GSTd1-3      FDFSKYPNVQAWLERCKKNAPGYDLNQAGADEFKAKFLSGM---- 211
AgGSTd1-6    FDFAPYPNVAAWFARCKANAPGYALNQAGADEFKAKFLS------ 210
AgGSTd1-4    FDFAKYQHVAAWYENIRKEAPGAAINQAGIEEFKKYFEK------ 210
GSTd1-1      VDLSKYPNVASWYERLRKEAPGADINEAGCKEFAKYFEK------ 209
AgGSTd1-3    YDLRRYENIHRWYEQTGNIVPAADKNLAGAKIFGLYFRQK----- 219
```

Figure 7. 6 Multiple alignment of *GSTd1* from *Ae. aegypti* and *An. gambiae* (Ag).

Gray highlights indicate conserved sequences in three proteins. The arrow represents the conserved splicing site. Dashes are used to denote gaps introduced for maximum alignment

Table 7. 2 Percent identity of alternatively spliced *GSTd1* in *Ae. aegypti* and *An. gambiae.*

The highlights indicate the highest identity between the two species.

	Percent Identity						
	GSTd1-1	**GSTd1-2**	**GSTd1-3**	**AgGSTd1-3**	**AgGSTd1-4**	**AgGSTd1-5**	**AgGSTd1-6**
GSTd1-1	-	67.1	76.2	62.4	61.4	81.9	74.8
GSTd1-2		-	64.6	55.7	74.7	62.3	61.4
GSTd1-3			-	58.5	64.2	75.0	84.9
AgGSTd1-3				-	58.5	58.9	58.0
AgGSTd1-4					-	61.3	63.1
AgGSTd1-5						-	71.0
AgGSTd1-6							-

Figure 7. 7 Nucleotide sequence and putative amino acid sequence of *Ae. aegypti* *GSTs1* gene.

Primers used to confirm the expression of the two cDNAs are indicated.

```
ATGCCGGATTACAAGGTCTACTACTTCAACGTCAAGGCCTTGGGCGAGCCTCTGCGGTTCCTGCTGTCCTACGGCAATCTCCCGTTCGAT 90
——————>
GSTs1F
M P D Y K V Y Y F N V K A L G E P L R F L L S Y G N L P F D
Exon1

GATATCAGAATCACCCGCGAAGAATGGCCAGCTCTGAAACCAAGTGAGTCACGTCGGGAACACGTTTTTACACGTTTTTAGCTGTGCCTA 180
D I R I T R E E W P A L K P S
Exon1

ACCCTGCCCGATAACGAAACGTTTGTTTGAGTTTTCACCGGTAGCCATTACATCATCAGCCATTTCTTGTAAGAATTTATCAAAAAGGCC 270

TAATTACTTGTTGAAAGTCAGATTAGGATAATGATATTTTCATAAAGGTTAAATTTGCATAGGTCCTTTGGGGTCTCATGCACAAATTAC 360

GTCACGCTCCAAGGGGGAGGGGGGGGGGGGGGTGATCGAGCCAAGTGTGACAAGCCTTACAAAATTTTCGGAGGACTCATACAAAAAGTG 450

TGACAAAGAGGGGGGAGGGGGTCGAAAAAGTTGAAATTTAGCGTGACATAATTTGTGTACCATCCCTTTTGGAAAATTTTACAATAAATT 540

CATTATCTGTCTGCATGATTACCACTTATCAAGTACAGTCAATTCTTTATAGCTAGATATTACGTATTTATTGTCATAAAACCATAGTAA 630

ACGGTGGTTTTCATGGCTACCTTGAACAACAGTTGCACTGATTTTGTGTGCTATAACTAGATAATTCCTTCAATATCGAGTCGTAGAGAG 720

TTGACTGTAGTTAGACATTTGTAGACGTAATATTTAGATAAGGTTTTCTATTTTCGTCCATGTGTTACTCAATACATTCGGTTTGATTTG 810

AATTGTCATGGTTTGCGGTAAACAACTCACTCAATTTCAATTTTCCGATGATAACACTTGTCCCCTATTGACAATGATTTTCTGTTCTTT 900

AGCATGCTTATCGCGATAATCTCAGCCATCTCGACTGTAATACTAAATCTACTAGCTAATTATAACTCAAGAGCAAAGCGTGCCATGCTT 990

GTTGGGATTGATTGTCTTGTAGCTAATCATCTGTTGAGATGACATAGAGGCCTACTAGATGATATCACATTGTTTACCGCTTTGAAACTA 1080

AAAGTGGACGCAGCAAGGAAAACTATTATCTTATGACTTAAATTTTAATATTGGCTGGATTTTACAAAAAAACGAACCCCCTCACGACAT 1170

TCACCGACTCATCTACACCCCACATTCACAGTGTAAAACAAAATGGCAGCTTTCTCATCGCTTCATCATCCTGGTTTGCATCATCACATA 1260

GGTTTTACTAGCTTGTAACATGTTTGGTGGCAGCTTTCGGGGGAGGCACTGGTGTTATCGACGACGAAACCCCTCCTACGAACGAACCCA 1350

AACCAAACGATTTGCTTTTCTTTTCTCTCTTTTTCCCTCTTTTTTCGCGTTTTACAGCGATGCCCATGGGACAGATGCCGGTGCTGTCGG 1440
M P M G Q M P V L S
Exon2

TGGACGGCAAGAAGGTGCACCAATCGGTGGCCATGTCCCGCTATCTGGCCAAGCAGGTCGGCCTGGCCGGTGCCGACGATTGGGAAAATC 1530
V D G K K V H Q S V A M S R Y L A K Q V G L A G A D D W E N
Exon2

TCATGATCGACACCGTCGTCGACACCATCAACGATTTCCGTCTGAGTAAGGATCGATGAATTTGGATGAGTTCAGGGAAAAGGAACACGA 1620
L M I D T V V D T I N D F R L S
Exon2

GCGTTGCCATTGCTTTATAAGTTTTAAACGACTTCAAAAAGGTTTTATGTGACATTTTTATCAAGGATAAACCTTATTTACAGGTCTAGC 1710

TTTAAGAGACTTCTTTTGAAACGCTTTGTTTGGCGGTGTCATCATAGTAGCCTTGATTTTTTTAGTATGGACCAATGCACGAGTTCTTTT 1800

TCATGAATGCTTTCAAGAAAAATTTTCAGTTATTTTTCAATATTTTTACCATGATTTCTTGTAGAAATGTATAATAATTAATTGGAAATT 1890

ATGCTTTTCAAGAGCTCTCCGTTTTGAAAAAAAATGCTTATCGCATGTAAAATTGGTTCATAATGATCACAGTTCCCAGGGAGATTCATG 1980

AATTCTTCCTAATCTTATTGGCGTTACCTCTCAACTGGGAAGCCTGCTTTTCAGCTTAGTGTTCTTTTGAACACTAAGGAATTTAGGAAA 2070

TCTCGATAATTTGAGGTAAATTGATTAGATCTGATGTAATTGTGACTTTCACGTGTTTAGTTTTGTTTGAAAAAGACACGGTAAAAATTT 2160

GTATTCAAGACAAGAAAAAGTTTAAGTTAGAATGTTTTTTTTTTCCTTTAAAGTGCCCATCTCGTGATGTATGGACGGTTGGTAGTAAAC 2250

ATAATTACCTATCTTGAGACAAAATTTCATTCAAATATTTTGTTTTAAATTGACTTTAAGCTTTGAAAATCTTTTTTTTTCAGCGTAGG 2340

CCACACATTAAGTTTTTTCAGTACCGTAATCCGGGGTAACATTGATCAGCGGGGTAACATTGGTCGGTATGATCCTGCTCGTAAAAAGTT 2430

CGAATCATCATTTATTGATGAAATATTTTCAAAGCATGAATGGTGCCTCTCTTTCTTACATTCATAATCTAATGGAAATTGAGGTTTTTG 2520

TGAAAATAGCATTAAGTTCATGCGTAAATTTGTCAACATTTTGAATCAATGATTTTTATTTTTATGGATTACACTACCGAAGAAATGGTA 2610

TCGCCGAAAACGATTCCGTTGTCAAATCTTTCATAATTTTATTTAATTAAGCAACATTAACGAACTAATATTAAGAATTCAGAAAATCCT 2700

TAGGGAATTGCATACCTTTAGGCGTATTGCACGGTTCAAAGTGATTTTATTTTTTAAATAACTCAAATAGTAAATGACTTGTAAGAGTTT 2790

GGTCTTCATATTCGGCATCAGGGGCCATGATTTAAGTAAGCAACGACATTTTCAATATTTCCGAACGTTGTTTACATGGGTGATCAATGT 2880

TACCCCATATCAGCTAAATCAAAAAAAATCACTTAAAACATTTATTTAAACATACTTAAATCTTTTGAAAACCAAAATGCAGTGTATGGT 2970

CACGAGCGGTGGATGCCAGTACTTGTTTAAAAAATATAAAGTTGCGACATTTGCATTCTCTGATGAAAAATTTAAGAAATATCCCAAAA 3060
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ACTGATCAATGTTACCCTGTTACGGTACAACAATTTTTTAGGAGTGTATATACGATTATATCATTTTGAAAAAAAATCTGGTAAAAAAGT 3150

TCAGCCTTTTCGAATGATAGTCCAGATACATTTTGCAAAAACTAAGTATGCAAAATGGCTTACTTAGTCTATGTCTACACATCTATAAAG 3240

TTTCATTGAAATCTGAGAGGGTTCTGCCAACATCTATTCGAGATGGCGCGAAATGCGCCTAAAGATTCTTCATGAAAGTAATTCTACGTA 3330

TTGTCTAGAGATTGAAGGGATTTTTCAAGAATTCTATAAGAATTACTCAATTAAATTGTACATGAATTACTACAAGGCAGGGATTTATGT 3420

ATAGTAAATTTATAGGTTTATTTATTGGAAAGTCAATGCACATTAATTTATTTTTAAATGAAATGTTGGAAAATCCACTGAGAAATGAAA 3510

TTATGTGAATAAACATGTAATATTGCGTTCTTTTCAATGCTCCAAATATGTGCATTGATTTTAACCCAATTTTCAAAGAAAGTTTAGTTT 3600

CCATTTATTTAATTTTACAAACTTTTCGATTTACATGTCGTTTAATTTTCAATATTTTTTGCTGTGTACGCTATGAAAGTGAAAAACTAT 3690

ATTCTTCACGATAGCTAAAAACCTTTTTCGAACAAACACCACAACATAAGGCAACGCTCGTAAACTTTCCCGGAAAATGTAACGCAATGC 3780

TAAGCTTCCGCTCCCTTGTAGAAATCGCCGTTGTGTCCTACGAGCCCGATGATGACGTGAAGGAGAAGAAGCTGGTCACCCTGAACAGTG 3870
                         I  A  V  V  S  Y  E  P  D  D  D  V  K  E  K  K  L  V  T  L  N  S
                        |-------------------------------Exon3-------------------------------

AGGTCATTCCATTCTACCTGGAAAAGCTGGACGACATCGCCCGTGATAACAACGGCCACATGGCAAATGGCAAAGTAAATGCCAAGACAA 3960
E  V  I  P  F  Y  L  E  K  L  D  D  I  A  R  D  N  N  G  H  M  A  N  G  K
-------------------------------Exon3-------------------------------------|

TCGAGGAATCTGTTCATTTACTAATCCTAAAAATTTTACTTCCAGCTCACATGGCCGATATGTACTTTGTCGCCATCCTGGACTACCTG 4050
                                                L  T  W  A  D  M  Y  F  V  A  I  L  D  Y  L
                                               |--------------Exon4--------------------------

AACTACATGACCAAGTCCGACCTCGTGGCCAATCACCCGAACTTGCAGAGAGTGGTCGACAATGTCACGAGCATCGATTCGATCAAGGCC 4140
 N  Y  M  T  K  S  D  L  V  A  N  H  P  N  L  Q  R  V  V  D  N  V  T  S  I  D  S  I  K  A
-------------------------------Exon4-----------------------------------------------------

TGGATCGATAAGCGACCACAAACTGAGATCTAAACGACACCACACTCAGCCAAAAGGAACGACAACCTACCATTACTACAACATTCAACG 4230
 W  I  D  K  R  P  Q  T  E  I  .
-----------Exon4-------------|
          <-------------------
          |----GSTs1exon4R----|

GAGACTTTCACCACAGTTCATAATCTACACCATCTAAAGCGTTCCCGAACGTTTTTAGAAACATCAGTGCCATTTAAAGCATTGAGAATC 4320

AATCACATTAACATGCACAGAAACGCATTCACCAAATAAGACCAGTGAATATGCATTACACAACTTTGTTATAAGAAACGATAATAAAAC 4410

TTTAAAAAAATATGATCCAATAAATATCCCCTACACGTTTTATCTGGCATATCATCAGCAGAAATAAAAGACATGTTTTCCTTGCTGCAA 4500

ACCCTGTCTGAAACCGTGGCCCTCGATTGGTTTTCGTATTTCGCTGAGATAACATCTGAGAGCATCTCCTGTCGCTAAAAGCTTGTTTGT 4590

TTAATAAGTATAATTTATTTTTTGATATGATTGTCTGCCGCTGATAACAGCAGCGCCTTCCCGAAAATAACGTAAGTCTACCCGTTTCAC 4680

CCATGAATAGCCATAAGAAAGCTGCTTATCATCATCAATAACTGTTTTACACTTTTCCAAACACTTCTTCTTCAACGAAACATGCTGAAT 4770

TTTGACCAATCGCCAATCCAGCTTATATTAAAGTTTAACAAACATTTCTCTGCTTTCTCTCTCTCGCTTTCTTTCTTCCTCTGTCCCCCA 4860

TCCTCTCGTTTACAGCAATGCCCATGGGACAGATGCCGGTGCTGGAGGTCGACGGCAAGCGAGTCCACCAATCGCTGGCCATGTGCCGCT 4950
                  M  P  M  G  Q  M  P  V  L  E  V  D  G  K  R  V  H  Q  S  L  A  M  C  R
                 |--------------------------Exon5----------------------------------------

ACGTGGCCAAGCAGATCGGCCTGGCCGGGTCCGATCCGGTGGAGGAGCTGCAAATTGATGCGATTGTTGATACGATTAACGATTTCCGTC 5040
 Y  V  A  K  Q  I  G  L  A  G  S  D  P  V  E  E  L  Q  I  D  A  I  V  D  T  I  N  D  F  R
-------------------------------Exon5-----------------------------------------------------
                                                                       <--------------
                                                                       |--GSTs1exon5R--

TGAGTAAGTATTGGTTCGCGTGAATTGGGACAAAGCACTACTCATGAAGTCTATGTATTCTCAAAGACTGCTTGTTGCCAAAATATCTAT 5130
L  S
Exon|
----
GSTs|
```

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CGTCATTATATTTCTTAATGGGCCCAGGTAGCCGTAGCGGTAAACGCGCAGCTATTCCGCAAGACCAAGATAAAGGTTCGGATCCCTTTT 5220

CATAGAGGAAAGTTTCTTGATTTCTCTGGGCATAGAGTATCCTCGTGCTTGCCACACGATATACACATGCAAAAATGGTAAATAGGCAAA 5310

TAAAGCTTTCAGTTTAAATTTGTGGAAGTGCTCATTAGAACAATAAGCTGAGAAGCAAGCTACGTCCCAGTTGGAACGTAACATCAGAAA 5400

GAAGAAGAAATTCTTTAATACAGTCAACCCTTCATAACTCCATATTACGTATCTCGATACCGAGGTATAGAATCATAGTATAACATGGTT 5490

TTCCTGGCTTCTTAGAGGGTCTTTTGAATCATCGTAGTGGCACTCATTTTGTGTATCGGTGTATCCCTAATTCAATGGTCCCTTCATTGA 5580

ATCATAAAACGTTGGCTAGTTAATGGGCTGGAACATTAACTGCCACTTCAACTTAGTGACTTATAGACGTATTATAGTTATAGCTGCTCT 5670

ATTGTTCTTGAGCCGTTTGATTCGTTCATCATCCGCCTTCATCGGTACTAGCTATGCCCTCAACCATCTCGGTTTTATCGTAGTGCTACT 5760

TTCACCTTTCTATCATGATACCTCCATCCTTATCCAGACACTATGGTCACTAGCTGGTGGTGACCAATCGATTAAGTTTTCAGCTCAAAC 5850

GGATGTTTGGTTCTCCAGATTTGTGTTACAATTACTATTATGGGGTTGGTCTTTGTTTAAAAATCATTACTCTGTAATGAGTTGCTATTG 5940

CTTGCTCGAATGGTATTTCAGGCATAAACTTTTAAATTCTTTCCAGTTAACCAGGGTGAAAAGTTTTAAAACTATGCTTGAAAACAAATG 6030

GTACGAAATTTTTAGGAGAATTTCTTTTAAAAATTGTATTTCACCAAAATTTACAAATTTTGTTTGAAACATCTAGTAGATACTGCAAAT 6120

TTGTTCTTAGCCATATGAACTGTATATTTCTTCGTATTTTTTATATGCATCTTTCTGATGTTATTCAAATGTACGCAGACAAAATTCATT 6210

AAAAATAATAGAATACACATTTTACTTGCTGGAGTTATGTCAATAATATAATGTGTTCTGATCTCAAGAAAATAATATAACTTTTTGTGA 6300

CACATTTGACGATTTTCATAAAAAATCTCGTACAAAACGCTCAAAATATTTCAAAAATAATTTTTGCTAAGCTTTGATTAAGGGGCGCTG 6390

AAATGGAGCTACGCCAAGGGCGCCATGAAGCCACGCTACGGCTCTGTGCATAGGTCCATGGACCGATGCACGAGTTCACTAATTTGACGT 6480

TTAAGCGGTGCCGAATTCACTCGTTGCCATGGCCACATAAATAACACGGCACCGCTCAAAAGTCAAATAATAAACTCGTGTATCGGTCCA 6570

TAGTGCATATTACCCTAGGAACCCCTATATCAAGTGTCGGAAAAACGAAAGTGAAATTTGGATCGAAAACAAGGATAGAAAGAGATCAGG 6660

GCAAAATGAAAACTTTCGTGAAATTTAGTGAAAGCAATTTTATTAATTAGTGTCAATCGTTCTGATACATGAAAGGGCTCCCTCTCAATC 6750

ACACTAGCAAGAAAAAAAACTTCTCTAGGTTCGTTACTTTGCTCAAAATCTCGATTCTTCCACACCCTTGGGTGTTATATCCAAATTAGG 6840

ATATAACAATTTAAAACTGAGAAAAACTTAAAATCATTGCAATGGACAATTGAAGCAATATATTTAGTTTTTATCGTCAAGCAATTCATA 6930

TGCAATACAGATTGCATGATTGTGCGATATTTGCCAGAAAACCATTCGCCAGAAAACCATCCGCCAGAATGTACCATTTACCAGAAAACC 7020

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AGATGACCAAATGCAGAAATTGGTAGATGGATGATAGAAGCTTCAAACCCATTATGAAAAAAATACTACCTGAAGGATCTATTGAACGGT 7200

CTCGATGGATTATGAGGATCCTTTAGAATTTTGCAATAGGATCTACTGCGAGCAGGATAAAAAAAAATGACATTTGTGTCTATTTTGCTT 7290

AAAAAGAGACTTTAGATACTTTCTTTGCAATTGAGATGTCATACAATACACCACTAAACAATCCAATTTTATTATTCATTCCACAGAGAT 7380
                                                                                         I

TGCCATTGTTGCCTACGAGCCGGACGACATGGTAAAGGAGAAGAAGATGATCACCCTGACGAACGAAGTGATTCCCTTCTACCTGACCAA 7470
 A  I  V  A  Y  E  P  D  D  M  V  K  E  K  K  M  I  T  L  T  N  E  V  I  P  F  Y  L  T  K
------------------------------------------Exon6-------------------------------------------

GCTGAACGTGATCGCCAAGGAGAACAATGGCCACCTGGTGCTGGGCAAACCGACCTGGGCCGACGTGTACTTTGCCGGCATCCTGGACTA 7560
 L  N  V  I  A  K  E  N  N  G  H  L  V  L  G  K  P  T  W  A  D  V  Y  F  A  G  I  L  D  Y
------------------------------------------Exon6-------------------------------------------

TCTGAACTATCTGACCAAGACAGATCTGCTGACCAACTTCCCCCAACTGCAGGAGGTCGTGACCAAGGTGCTCGAGAACGAAAACGTCAA 7650
 L  N  Y  L  T  K  T  D  L  L  T  N  F  P  Q  L  Q  E  V  V  T  K  V  L  E  N  E  N  V  K
------------------------------------------Exon6-------------------------------------------

GGCGTACATCGAGAAGAGACCGGTGACTGAAGTCTAA 7687
 A  Y  I  E  K  R  P  V  T  E  V  .
-----------------Exon6--------------
```

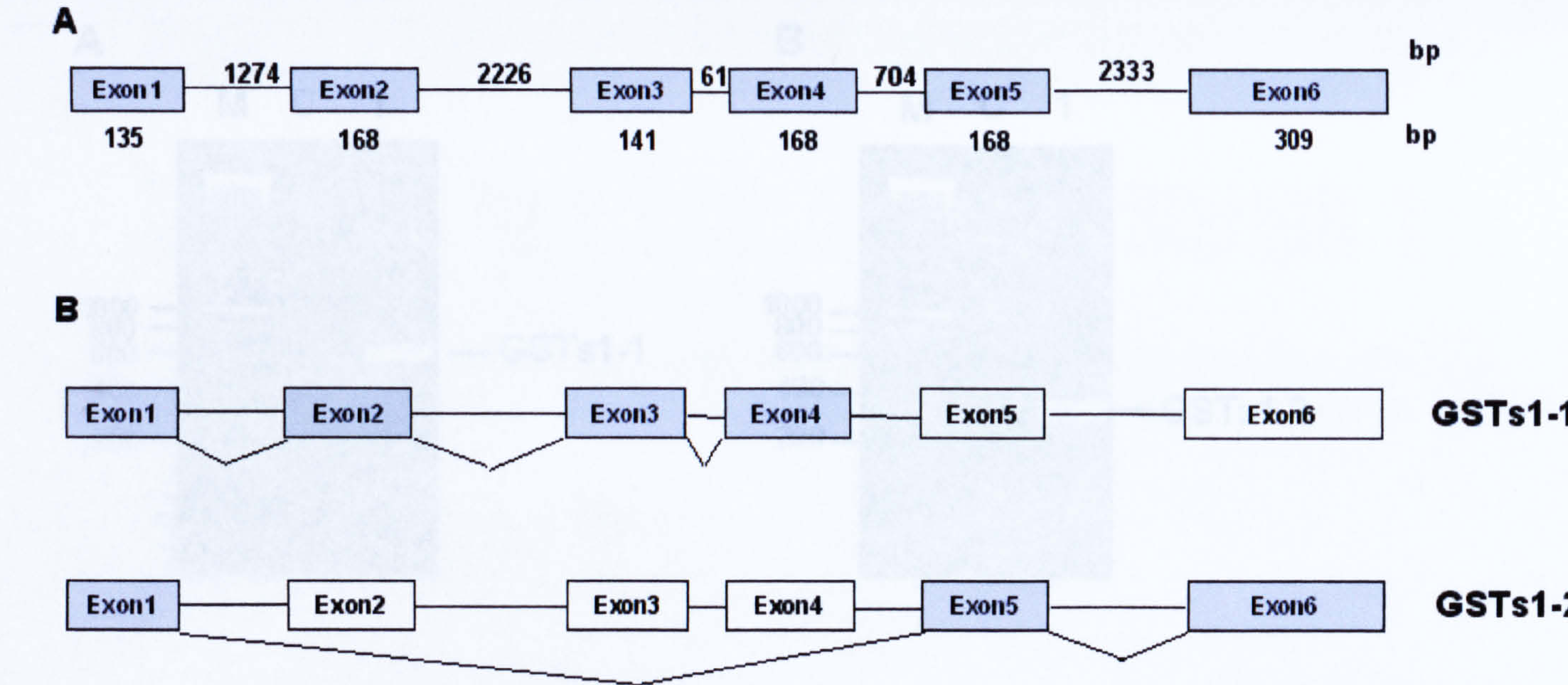

Figure 7. 8 *Ae. aegypti GSTs1* gene organization and alternative splicing.

(A) Exon-intron structure of *GSTs1* gene. Light grey areas indicate exons, whereas horizontal lines represent intron positions. Upper and lower numbers correspond to intron and exon size (bp), respectively. **(B)** Exon usages in the splice variants *GSTs1-1* and *GSTs1-2*. Light grey boxes indicate utilized exons. White boxes represent skipped exons.

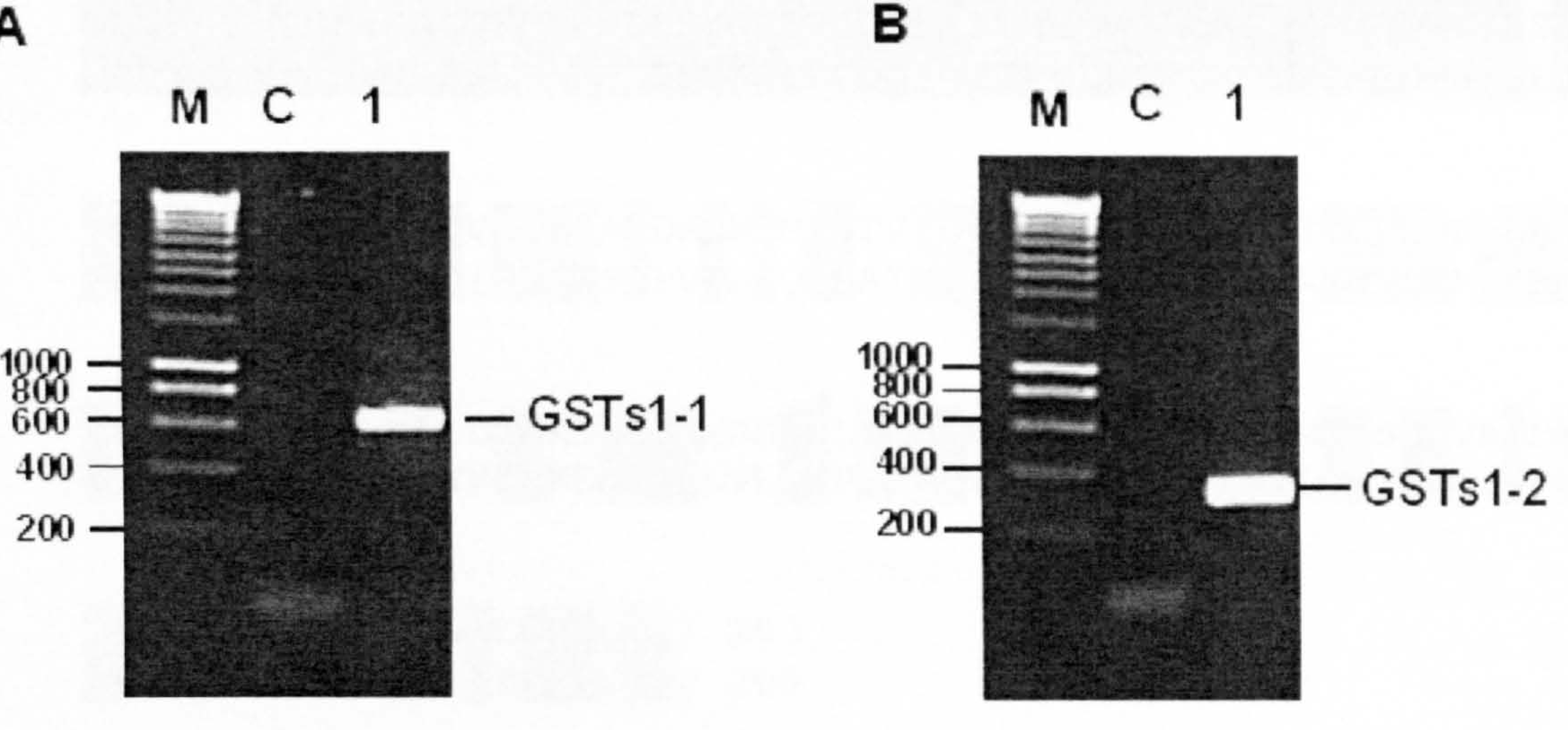

Figure 7. 9 Amplification of alternatively spliced *GSTs1-1* (A) and *GSTs1-2* (B).

PCR amplification was performed using two primer sets: GSTs1F primer and GSTs1exon4R primer (**panel A**) and GSTs1F and GSTs1exon5R primers (**panel B**). Lane M contains size standards (shown in base pairs). C is negative control. Lane 1 and 2 contain *Ae. aegypti* cDNA from adult mosquitoes.

```
GSTs1-1    MPDYKVYYFNVKALGEPLRFLLSYGNLPFDDIRITREEWPALKPSMPMGQMPVLSVDGKK 60
GSTs1-2    MPDYKVYYFNVKALGEPLRFLLSYGNLPFDDIRITREEWPALKPSMPMGQMPVLEVDGKR 60

GSTs1-1    VHQSVAMSRYLAKQVGLAGADDWENLMIDTVVDTINDFRLSIAVVSYEPDDDVKEKKLVT 120
GSTs1-2    VHQSLAMCRYVAKQIGLAGSDPVEELQIDAIVDTINDFRLSIAIVAYEPDDMVKEKKMIT 120

GSTs1-1    LNSEVIPFYLEKLDDIARDNNGHMANGKLTWADMYFVAILDYLNYMTKSDLVANHPNLQR 180
GSTs1-2    LTNEVIPFYLTKLNVIAKENNGHLVLGKPTWADVYFAGILDYLNYLTKTDLLTNFPQLQE 180

GSTs1-1    VVDNVTSIDSIKAWIDKRPQTEI 203
GSTs1-2    VVTKVLENENVKAYIEKRPVTEV 203
```

Figure 7. 10 Deduced amino acid sequences of spliced *GSTs1-1* and *GSTs1-2*.

Gray shading indicates the conserved amino acids in both splice variants. Vertical lines represent to the alternative spliced positions. Dashes are used to denote gaps introduced for maximum alignment.

```
GSTs1-1      ----------------------------------------------MPDYKVYYFNVKAL 14
AgGSTs1-2    ----------------------------------------------MPDYKVYYFNVKAL 14
GST1-2       ----------------------------------------------MPDYKVYYFNVKAL 14
AgGSTs1-1    ----------------------------------------------MPDYKVYYFNVKAL 14
DmGSTS1      MADEAQAPPAEGAPPAEGEAPPPAEGAEGAVEGGEAAPPAEPAEPIKHSYTLFYFNVKAL 60

                                                ↓
GSTs1-1      GEPLRFLLSYGNLPFDDIRITREEWPALKPSMPMGQMPVLSVDGKKVHQSVAMSRYLAKQ 74
AgGSTs1-2    GEPLRFLLSYGNLPFDDVRITREEWPALKPTMPMGQMPVLEVDGKKVHQSVAMSRYLANQ 74
GST1-2       GEPLRFLLSYGNLPFDDIRITREEWPALKPSMPMGQMPVLEVDGKRVHQSLAMCRYVAKQ 74
AgGSTs1-1    GEPLRFLLSYGNLPFDDVRITREEWPALKPTMPMRQMPVLEVDGKRVHQSLAMCRYVAKQ 74
DmGSTS1      PSPC-ATCSDGNQEYEDVAHPRR-VPALKPTMPMGQMPVLEVDGKRVHQSISMARFLAKT 118

GSTs1-1      VGLAGADDWENLMIDTVVDTINDFRLSIAVVSYEPDDDVKEKKLVTLNSEVIPFYLEKLD 134
AgGSTs1-2    VGLAGADDWENLMIDTVVDTVNDFRLKIAVVSYEPDDEIKEKKLVTLNNEVIPFYLEKLD 134
GST1-2       IGLAGSDPVEELQIDAIVDTINDFRLSIAIVAYEPDDMVKEKKMITLTNEVIPFYLTKLN 134
AgGSTs1-1    INLAGDNPLEALQIDAIVDTINDFRLKIAIVAYEPDDMVKEKKMVTLNNEVIPFYLTKLN 134
DmGSTS1      VGLCGATPWEDLQIDIVVDTINDFRLKIAVVSYEPEDEIKEKKLVTLNAEVIPFYLEKLE 178

GSTs1-1      DIARDNNGHMANGKLTWADMYFVAILDYLNYMTKSDLVANHPNLQRVVDNVTSIDSIKAW 194
AgGSTs1-2    DIARDNNGYLANSKLSWADIYFTAILDYLNYMTKSDLVANHPNLQRVVDNVTSIESIRSW 194
GST1-2       VIAKENNGHLVLGKPTWADVYFAGILDYLNYLTKTDLLTNFPQLQEVVTKVLENENVKAY 194
AgGSTs1-1    VIAKENNGHLVLGKPTWADVYFAGILDYLNYLTKTNLLENFPNLQEVVQKVLDNENVKAY 194
DmGSTS1      QTVKDNDGHLALGKLTWADVYFAGITDYMNYMVKRDLLEPYPAVRGVVDAVNALEPIKAW 238

GSTs1-1      IDKRPQTEI 203
AgGSTs1-2    IDKRPKTEI 203
GST1-2       IEKRPVTEV 203
AgGSTs1-1    IAKRPITEV 202
DmGSTS1      IEKRPVTEV 247
```

Figure 7. 11 Multiple alignment of *GSTs1* from *Ae. aegypti*, *An. gambiae* (Ag) and *D. melanogaster* (Dm).

Gray highlights indicate the conserved sequences in all three proteins. Arrow represents the positionof the first intron. Dashes are used to denote gaps introduced for maximum alignment.

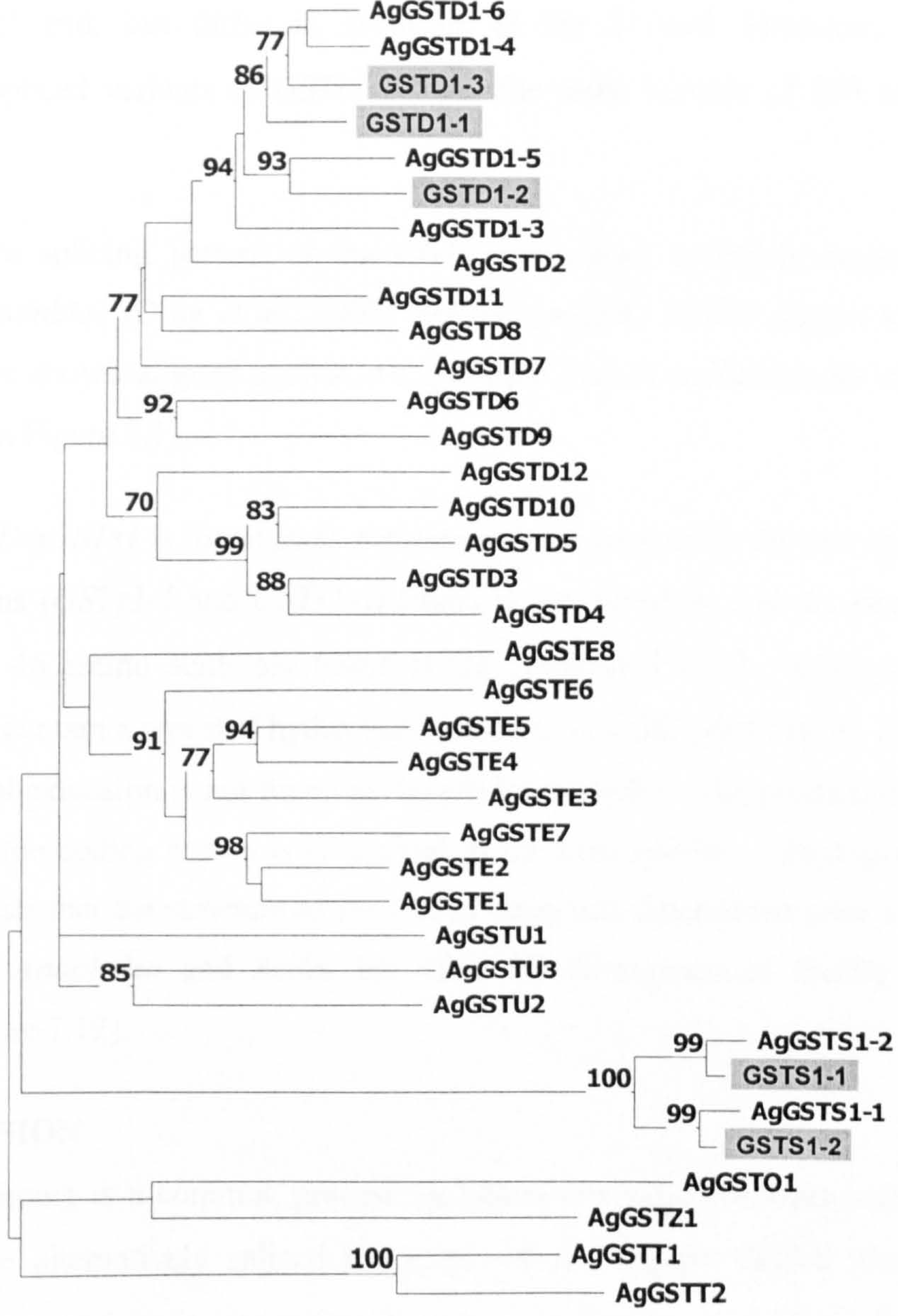

Figure 7. 12 Phylogenetic tree highlighting the relationships between *GSTd1* and *GSTs1* from *An. gambiae* and *Ae. aegypti.*

Amino acid sequences were aligned using ClustalW (www.ebi.ac.uk/clustalW) and a distance neighbor-joining tree was generated using TREECON. Nodes with distance bootstrap values (1000 replicates) of >70% are shown. GSTs from *Ae. aegypti* are highlighted in grey. Ag = *An. gambiae* and Dm = *D. melanogaster*.

Deduced amino acid sequences of *GSTs1-1* and *GSTs1-2* are shown in Figure 7.10. Both genes share common 5' coding exon, encoding the 45 amino acids at the N-terminal end, but differ in sequence at the 3' end. However, both alternatively spliced variants of *GSTs1* contain the same number of 203 amino acids.

The alternative splicing pattern of the GST sigma gene is highly conserved between *An. gambiae* (Ding *et al.*, 2003) and *Ae. aegypti. Aedes aegypti* sigma class GSTs are shown aligned against *An. gambiae* and *D. melanogaster* sigma GSTs shown in Figure 7.11.

Only a single *DmGSTs1* is found in *D. melanogaster* in contrast to the two spliced *GSTs1* isoforms (*GSTs1-1* and *GSTs1-2*) found in *An. gambiae* and *Ae. aegypti.* An additional 46 amino acids are found at the N-terminal of *D. melanogaster GSTs1*, which contain a repeated hydrophobic and acidic motif (Beall *et al.*, 1992). The N-terminal extension is not found in *Anopheles* or *Aedes*. The position of the first intron in the coding region is conserved in all three species. Phylogenetic analysis suggests that the structure of the *GSTs1* gene was determined prior to the split between *Anopheles* and *Aedes* but after the divergence of fruitfly and mosquito (Figure 7.12).

7.4 DISCUSSION

Alternative splicing is a common process by which one gene produces multiple proteins. Three alternatively spliced transcripts of *Ae. aegypti GSTd1* share a common 5' exon and have alternative 3' exons, leading to an increase in the diversity of the GST variants. A similar pattern is also found in the *GSTs1* gene. Amino acid variations located at the C-terminal can alter the binding capacity to different hydrophobic substrates (Mannervik & Danielson, 1988). As the 3' end of the gene encodes the majority of the residues in the substrate binding site this is a manner of increasing the substrate range of the GSTs.

Although, the alternatively spliced *Aedes* GST isoforms have not been biochemically characterized, in *An. gambiae AgGSTd1-5* and *AgGSTd1-6* showed differences in GST activity against CDNB, DCNB and peroxidase activity toward cumene hydroperoxide (Ranson *et al.*, 1997).

Sigma class GSTs has been widely studied in many organisms including cephalopods (Ji *et al.*, 1995), nematode *Onchocerca volvulus* (Kampkotter *et al.*, 2003), chicken (Thomson *et al.*, 1998), mouse (Kanaoka *et al.*, 2000), human and rat (Jowsey *et al.*, 2001). In *Drosophila*, *DmGSTs1* has been proposed to have a structural function rather than enzymatic function. *DmGSTs1* interacts with troponin-H through its N-terminal extension which contained the repeated hydrophobic/ acidic motif in the direct flight muscle (Beall *et al.*, 1992; Clayton *et al.*, 1998). However, recent studies have been demonstrated its protective role in oxidative stress (Singh *et al.*, 2001) by showing that it has activity against lipid peroxidation products (Agianian *et al.*, 2003). At present, several Sigma GSTs lack the N-terminal extension including in *An. gambiae*. In addition, mammalian Sigma GST exhibits prostaglandin D_2 synthase to catalyse the isomerisation of prostaglandin (Jowsey *et al.*, 2001; Thomson *et al.*, 1998).

The consistent use of a common 5'-exon and 3'alternatively splicing in *Aedes* and *Anopheles* suggested that alternative splicing of the gene evolved before the divergence of *Aedes* and *Anopheles*. Four *GSTd1* variants were detected in *Anopheles*, but only three *GSTd1* isoforms were identified in *Aedes*, indicating that one variant was lost or gained during its evolution.

Chapter 8

GENERAL DISCUSSION

8.1 The Extent of the *Aedes aegypti* GST family

Thirteen novel *Ae. aegypti* GSTs have been described in the preceding chapters. During the writing of this thesis sequencing of the *Ae. aegypti* genome progressed at a rapid rate and large numbers of WGS and EST sequences were released. These have now been searched to identify further putative GSTs.

Aedes GST genes were categorised into classes dependent on amino acid sequence identity and the phylogenetic relationship to *An. gambiae* GST genes (Ding *et al.*, 2003). In addition to the thirteen GST genes previously described, fourteen novel GST genes were identified from the sequence databases, twelve genes were from the EST database and another two putative GST sequences from the WGS database. Collectively, we have now identified twenty seven *Ae. aegypti* GST genes (Figure 8.1). One of these genes, *GSTe6* was translated from the genome sequence but contains a stop codon at position 67bp suggesting that this gene is probably a pseudogene. Excluding this stop codon, GSTe6 would encode a protein with 219 amino acids. The expression of these 14 novel GST genes was not verified further in this study.

The tree in Figure 8.2 shows the relationship of *Ae. aegypti* GSTs to other insect GSTs. Six GST classes were found in *Aedes* as in *Anopheles*. The largest classes are the insect-specific classes, Delta and Epsilon classes with nine and seven members respectively. Clear orthologs of each of the three unclassified GSTs were also found in *Aedes*. No orthologs of unclassified GSTs were found in *D. melanogaster*, suggesting that these GSTs may be specific to mosquitoes. In

addition single orthologs of the *Anopheles* Zeta and Omega GSTs were identified. Two additional members of the Theta class, *GSTt3* and *GSTt4* were identified, representing an expansion of Theta class in *Aedes* genes (4) relative to *Anopheles* genes (2). An ortholog of *GSTt4* is not found in *Anopheles*. *GSTt3* and *GSTt2* are paralogs and related to *AgGSTt2*, suggesting duplication of theta genes in the *Aedes* genome (65% bootstrap value). Four Theta GSTs are also found in *D. melanogaster*, only one of these has a clear ortholog in *Aedes* (*GSTt4* by bootstrap value 93%). Twenty seven *Aedes* GST genes were identified to date and this is very similar to the number identified in *An. gambiae* (28). However the number of cytosolic GST genes in *Ae. aegypti* may still be an underestimation as the full genome sequence of this species is not yet determined.

8.2 Characterisation of *Aedes* GST family

In the present study, the amount of transcript for four of the *Ae. aegypti* genes was quantified and one of these, *GSTe2,* was significantly over expressed in the insecticide resistant PMD-R strain, but was not over expressed in the parental line, indicating that insecticide selection increased *GSTe2* expression. In contrast, the transcription level of *GSTe4*, *GSTu3* and *GSTt2* are not associated with DDT and permethrin resistance. Furthermore, GSTE2 is also over expressed in the resistant strain of *Ae. aegypti* at the protein level. Characterisation of recombinant GSTE2-2 confirmed the role of this enzyme in DDT metabolism. Unlike its *Anopheles* ortholog, AeGSTE2-2 also exhibited glutathione peroxidase activity and thus, in addition to metabolising insecticides, AaGSTe2 may also confer insecticide resistance by protecting the insect from reactive oxygen species that are induced by insecticide exposure. Overexpression of GSTe2 is the only resistance mechanism that has been identified in this study. However, other unknown resistance mechanisms may be involved in resistance in the PMD-R strain.

Further investigation is needed to determine the mechanism of enhanced GSTe2 expression in the DDT/permethrin resistant PMD-R strain. Ding *et al.* (Ding *et al.*,

2003) suggested that the up regulation of Epsilon GST genes in *An. gambiae* is not due to gene amplification. Protein stability has not been reported to be involved in the up regulation of GST activity. Two possible mechanisms that have been associated with the over expression of GSTs in the insect species are discussed below. These are mutations in *cis* or *trans*-regulatory elements and post-transcriptional mechanisms, leading to enhanced stability of the transcript.

The *cis*-regulatory sequences upstream of structural genes play an important role in the transcription process. In *An. gambiae* the over expression of *GSTe2* is partly due to the deletion of the two adenosines in the promoter of the *GSTe2* gene in the DDT resistant strain (Ding *et al.*, 2005). Further investigation is needed to determine whether this mechanism is contributing to the over expression of *GSTe2* in *Aedes.* The promoter region of *GSTe2* will be sequenced and compared in both susceptible and resistant strain. If any differences are identified, site-directed mutagenesis will be performed and the promoter activity in different promoter sequences will be tested.

Trans-regulatory elements also regulate the expression of GST genes. In *Ae. aegypti,* it has been proposed that the regulation of GST is controlled by a *trans*-regulatory element, which generally suppressed the expression of this gene. The alteration of this element increased the expression of *GST*-2 in a DDT resistant strain (Grant & Hammock, 1992).

Posttranscriptional processing is also involved in the regulation of GSTs via mRNA stability and /or gene modification. Enhanced mRNA stability increases in the expression of human GST Pi class (Jhaveri *et al.*, 1997; Moffat *et al.*, 1997). In insects, the increase in *DmGSTD21* in *D. melanogaster* is influenced by the stabilisation of *GSTD21* mRNA (Tang & Tu, 1994). Modified regulatory genes might also affect the GST expression. The methylation of CpG islands in the regulatory sequences decreases the expression of human GST Pi associated with

prostate cancer (Lee *et al.*, 1994; Millar *et al.*, 1999). This mechanism has not been reported in insects.

Prior to this study, it was not assumed that the same GST would be involved in DDT resistance in different subfamilies of Culicidae, Anophelinae and Culicinae. In an analogous situation to the present study, two species of *Drosophila* have both developed the same mechanism of resistance to DDT. In this case, the up-regulation of a particular cytochrome P450 gene, *Cyp6g1* has been detected in multiple strains of *D. melanogaster* and *D. simulans* (Le Goff *et al.*, 2003). In both species of *Drosophila*, the increase in expression of *Cyp6g1* is caused by the insertion of a transposable element in the 5' regulatory region. Daborn *et al.* (Daborn *et al.*, 2002) investigated all *Drosophila* P450 genes and found that only a single *Cyp6g1* gene is over transcribed in DDT resistance strain through the world. In contrast, recently work by Pedra *et al.* (Pedra *et al.*, 2004) suggested that not only *Cyp6g1*, but several CYP450 genes are associated with DDT resistance in field populations and laboratory strains including *Cyp12d1*, *Cyp6a2* and *Cyp6w*.

Elevated GST activity has previously been associated with DDT resistance in DDT and permethrin resistant strain of *Ae. aegypti* from South America (Grant & Mutsumura, 1989). Phylogenetic analysis indicates that this gene is the ortholog of *AgGSTu3*, a unclassified GST in *An. gambiae* (Ding *et al.*, 2003). Enhanced expression of *GSTu3* caused by a mutation in a *trans*-acting factor was previously associated with resistance (Grant & Hammock, 1992). However, this GST is not over expressed in the PMD-R strain from Thailand. Furthermore DDT metabolism is also not associated with the recombinant GSTU3-3. These two results suggest that GSTu3 is not associated with DDT and permethrin resistance in *Ae. aegypti* mosquitoes from Thailand. The variation of the expression of this gene in different geographic regions, therefore, perhaps suggests differing routes of GST-based resistance in different strains of *Ae. aegypti.*

However, an alternative explanation is that Grant *et al.* (Grant & Hammock, 1992) were not studying a pure enzyme preparation. In this study, GSTu3 was investigated at the genetic level and by analysis of recombinant protein, which has been already sequenced and verified. In contrast, in the study by Grant *et al.* GST-2 was obtained by protein purification. As mentioned previously three GST isozymes were purified in *Ae. aegypti*. One of these, GST-2 was over expressed in the resistant strain (Grant *et al.*, 1991; Grant & Mutsumura, 1989). GSTu3 gene was retrieved from the GST-2 fraction by the N-terminal amino acid sequencing. Grant *et al.* (Grant & Hammock, 1992) suggested that GST-2 was over expressed at mRNA level by using a 123bp probe at N-terminal of GSTu3, which might cross hybridise which other GSTs. Elevated GST-2 was also observed at protein level by using the primary antibodies raised against this isozyme. But again, if the enzyme preparation contained multiple GSTs, this result does not implicate GSTU3 directly. At least 27 GSTs are present in *Ae. aegypti* database as described in this discussion, but only three isozymes were purified from *Ae. aegypti*. Therefore, the hypothesis that more than one GST was present in the GST-2 fraction should be studied further.

In summary, we identified 27 cytosolic GSTs in *Aedes aegypti*. In this report, the expression of four transcripts was quantified and only one of these, GSTe2 is associated with the DDT/Permethrin resistance in *Ae. aegypti*. This GST was over expressed at mRNA and protein level in PMD-R strain and it has the ability to metabolise DDT. Further study is still necessary to identify whether other GSTs are involved in GST-based resistance mechanism. In addition, other mechanisms, if any involved in DDT/permethrin resistance should be identified such as monooxygenases and *kdr*. However, the result in this study suggests the important role of GSTe2 in DDT resistance which has been reported previously in *Anopheles*. Further molecular studies would confirm its role in resistance, for example by using RNA interference technology to silence the over expression of

this gene. New inhibitors could be designed to down regulate GSTe2 expression. In the shorter term this gene can be used as a genetic marker for monitoring insecticide resistance in field populations. However, simplified assay needs to be developed.

Table 8. 1 *Aedes aegypti* GST gene members.

GST Classes	Gene name	No. of Transcripts	Protein Size	Gene size (bp)	TCs number	WGS scaffolds
Delta	*GSTd1*	3	209, 211 and 219	2771	TC52522, TC51556	AAGE01035830, AAGE01064758
	GSTd2	1	209	930	TC67297	AAGE01035830
	GSTd3	1	215	701	ND	AAGE01010054
	GSTd4	1	218	720	TC53163	AAGE01010054
	GSTd6	1	249	917	TC57303	AAGE01096081, AAGE01447518
	GSTd7	1	218	3438	TC55645	AAGE01231824, AAGE01181431, AAGE01527708
	GSTd11	1	222	739	TC67189	AAGE01096081
Epsilon	*GSTe2*	1	222	1233	TC54011	AAGE01019465
	GSTe3	1	222	725	TC54476	AAGE01184921
	GSTe4	1	224	812	TC65731	AAGE01092386
	GSTe5	1	221	795	ND	AAGE01025194, AAGE01061347
	GSTe6	1	219	820	TC64617	AAGE01184921, AAGE0427359
	GSTe7	1	220	724	TC67175	AAGE01025194
	GSTe8	1	220	858	TC56599	AAGE01394402

GST Classes	Gene name	No. of Transcripts	Protein Size	Gene size (bp)	TCs number	WGS scaffolds
Theta	*GSTt1*	1	229	805	TC58005	AAGE01037256
	GSTt2	1	227	1151	TC65938	AAGE01088537
	GSTt3	1	232	764	TC56273	AAGE01236813, AAGE01147755
	GSTt4	1	227	4951	TC62912	AAGE01053384, AAGE01059805
Sigma	*GSTs1*	2	203 and 203	7687	TC67856, TC63301	AAGE01051524, AAGE01631930, AAGE01044281, AAGE01133014
Omega	*GSTo1*	1	248	3790	TC62645	AAGE01008874
Zeta	*GSTz1*	1	233	9994	TC67856	AAGE01002747, AAGE0294778
Unclassified	*GSTu1*	1	231	770	TC61046	AAGE01101106
	GSTu2	1	218	784	TC57857	AAGE01028897
	GSTu3	1	218	3430	TC54577	AAGE011269409, AAGE01061301

GSTe6 gene contains stop codon leading to the short length of amino acid (22 amino acids), regardless this stop codon, GSTe6 would encode a protein with 219 amino acids. ND indicates to not detectable.

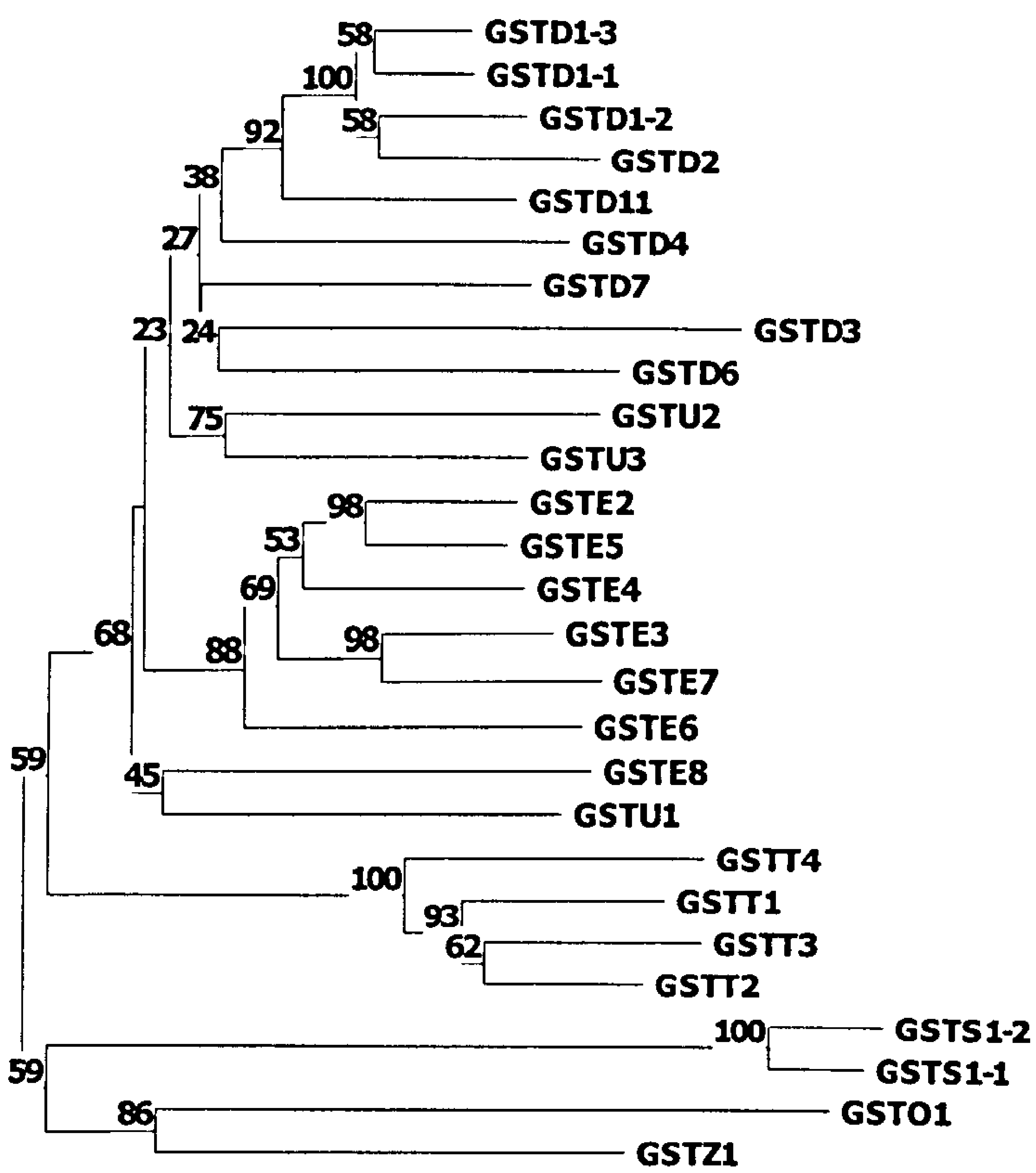

Figure 8. 1 Phylogenetic relationship of *Ae. aegypti* GSTs.

Amino acid sequences were aligned using ClustalW (www.ebi.ac.uk/clustalW) and a distance neighbour-joining tree was generated using TREECON. Nodes with distance bootstrap values (1000 replicates) of >20% are shown.

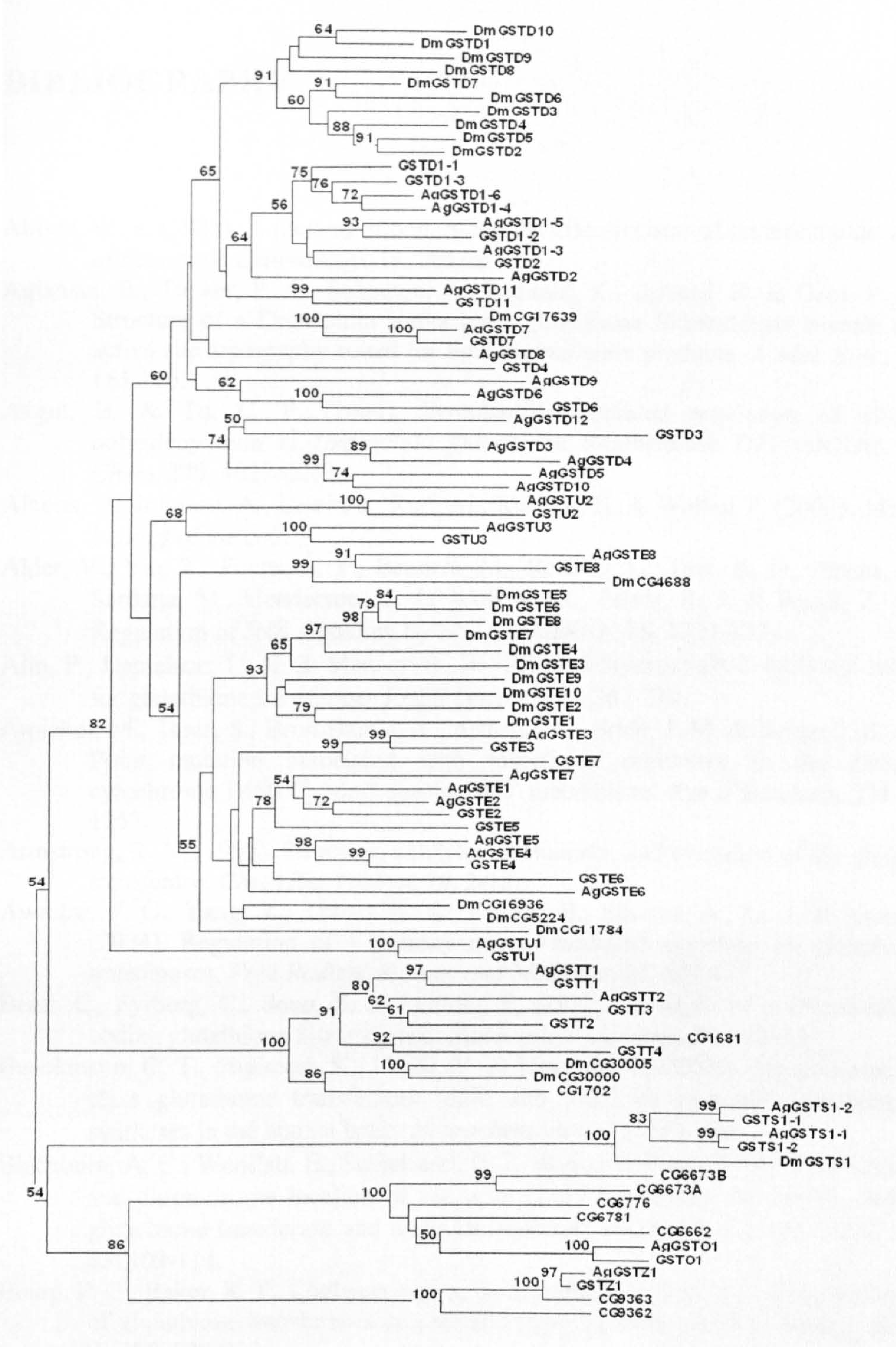

Figure 8. 2 Phylogenetic relationship of *Ae. aegypti* GSTs with *An. gambiae* and *D. melanogaster* GSTs.

Amino acid sequences were aligned using ClustalW (www.ebi.ac.uk/clustalW) and a distance neighbor-joining tree was generated using TREECON. Nodes with distance bootstrap values (1000 replicates) of >70% are shown. CG/Dm = *D. melanogaster*. Ag = *An. gambiae*.

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APPENDICES

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Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, *Aedes aegypti*☆

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Abstract

Glutathione transferases (GSTs) play a central role in the detoxification of xenobiotics such as insecticides and elevated GST expression is an important mechanism of insecticide resistance. In the mosquito, *Anopheles gambiae*, increased expression of an Epsilon class GST, GSTE2, confers resistance to DDT. We have identified eight GST genes in the dengue vector, *Aedes aegypti*. Four of these belong to the insect specific GST classes Delta and Epsilon and three are from the more ubiquitously distributed Theta and Sigma classes. The expression levels of the two Epsilon genes, a Theta GST and a previously identified *Ae. aegypti* GST [Grant and Hammock, 1992. Molecular and General Genetics 234, 169–176] were established for an insecticide susceptible and a resistant strain. We show that the putative ortholog of *GSTe2* in *Ae. aegypti* (*AaGSTe2*) is over expressed in mosquitoes that are resistant to the insecticides DDT and permethrin. Characterisation of recombinant AaGSTE2-2 confirmed the role of this enzyme in DDT metabolism. In addition, unlike its *Anopheles* ortholog, AaGSTE2-2 also exhibited glutathione peroxidase activity.
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Keywords: Glutathione transferase; Insecticide resistance; *Aedes aegypti*; *Anopheles gambiae*; DDT dehydrochlorinase; Pyrethroids

1. Introduction

The mosquito *Aedes aegypti* is the major vector of yellow fever, dengue and dengue haemorrhagic fever (DHF). Globally there are an estimated 50 million cases of dengue infection each year with 500,000 cases of DHF and over 24,000 deaths (WHO, 2002). Thailand suffers one of the highest rates of dengue and DHF infection in the world. In addition to the public health impact, DHF is leading to social and economic problems (Gubler, 1998). Effective vector control is essential to reduce the transmission of DHF, but this is hindered by the development of insecticide resistance in *Ae. aegypti.* In northern Thailand, populations of *Ae. aegypti* resistant to the organochlorine insecticide DDT and to pyrethroid insecticides have been isolated (Somboon et al., 2003). A colony of *Ae. aegypti* was established and a resistant line PMD-R selected. The molecular basis of insecticide resistance in this strain has now been investigated, with the goal of improving monitoring and management of insecticide resistance in this disease vector.

Abbreviations: GST, glutathione transferase; GSH, glutathione; CDNB, 1-chloro-2, 4-dinitrobenzene; DCNB, 1, 2-dichloro-4-nitrobenzene; CHP, cumene hydroperoxide; EST, expressed sequence tag, IPTG, isopropyl β-D-thiogalactoside; DDT, 1, 1, 1-trichloro-2, 2-bis-(*p*-chlorophenyl)ethane; DDE, 1, 1-dichloro-2, 2-bis—(*p*-chlorophenyl)ethane; HPLC, high-performance liquid chromatography; DDTase, DDT dehydrochlorinase; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); DTT, Dithiothreitol

☆The sequences described in this manuscript have been deposited to Genbank and have the following accession numbers: AY819709, AY819710, AY819711 and AY819712.

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DDT and pyrethroid insecticides share the same target site, the neuronal voltage-gated sodium channel (Soderlund and Knipple, 2003). Amino acid substitutions in the insecticide-binding domain of this protein can lead to resistance to both of these insecticide classes. One amino acid substitution occurs in subunit 6 of domain II of the sodium channel protein in *Ae. aegypti* populations from Thailand (Brengues et al., 2003; Prapanthadara et al., 2002) but the correlation between this allele and the resistance phenotype is, as yet, unknown. A second, highly prevalent, mechanism of insecticide resistance is an increase in the rate of insecticide metabolism. There are three major groups of enzymes involved in insecticide detoxification, carboxylesterases, cytochrome P450s and glutathione transferases (GSTs) (Hemingway et al., 2004). Elevated activity of these enzymes has been associated with resistance to DDT and/or pyrethroids in different mosquito species (Hemingway et al., 2004; Kasai et al., 2000; Prapanthadara et al., 2002; Vaughan and Hemingway, 1995). Preliminary biochemical characterisation of the DDT/pyrethroid resistant PMD-R strain from Thailand, found 10-fold higher levels of DDT metabolism compared to the susceptible strains, associated with elevated levels of total GST activity (Prapanthadara et al., 2002).

Multiple GSTs are found in all insect species. A subset of these enzymes is able to catalyse the glutathione dependent dehydrochlorination of DDT to the non-insecticidal product, DDE (Clark and Shamaan, 1984; Prapanthadara et al., 1995; Tang and Tu, 1994). GSTs are also implicated in resistance to other insecticide classes, through metabolism of the insecticide or its metabolites (Chiang and Sun, 1993), sequestration (Kostaropoulos et al., 2001) or by protecting against secondary toxic effects, such as increases in lipid peroxidation, induced by insecticide exposure (Vontas et al., 2001). Elevated GST activity has previously been associated with DDT resistance in the GG strain of *Ae. aegypti* (Grant et al., 1991) and expression of the GST subunit, GST-2, was correlated with the resistance phenotype (Grant and Hammock, 1992).

The majority of GSTs are cytosolic, dimeric enzymes with subunit sizes ranging from 17 to 28 kDa. There are at least six classes of cytosolic GSTs in insects: Delta, Epsilon, Omega, Sigma, Theta and Zeta (Ranson et al., 2002). Additional GSTs exist in insects that cannot be readily assigned to any of these classes (Ding et al., 2003). GST-2, previously isolated from *Ae. aegypti*, belongs to this 'unclassified' group of insect GSTs. The two largest insect GST classes, Delta and Epsilon, are both insect specific whereas the remaining classes are also found in other organisms. Elevated expression of Epsilon GSTs occurs in DDT resistant populations of the malaria vector, *Anopheles gambiae*. There are eight Epsilon GSTs arranged sequentially on chromosome 3R of *An. gambiae* in close association with the DDT resistance locus *rtdl* (Ding et al., 2003; Ranson et al., 2000). One of these, *AgGSTe2*, encodes an enzyme that possesses high DDT dehydrochlorinase activity (Ranson et al., 2001). We have identified several GST genes in *Ae. aegypti* by using a combination of homology based PCR and data mining of the incipient *Ae. aegypti* genome database (Severson et al., 2004). The expression of these newly identified genes and that of *GST-2* in insecticide susceptible and resistant *Ae. aegypti* were compared and those with elevated expression were characterised further.

2. Materials and methods

2.1. Mosquito strains

The PMD strain of *Ae. aegypti* was colonized from field caught material originating from Ban Pang Mai Dang, Mae Tang district, Chiang Mai Province in northern Thailand. A highly resistant line (PMD-R) was generated from the parental PMD colony by selection of one-day adults with either 4% DDT or 0.75% permethrin using standard WHO susceptibility kits (WHO, 1981). The resistance ratios of the parental PMD colony and the selected line, PMD-R were determined by comparison to New Orleans strain, a laboratory insecticide susceptible strain of *Ae. aegypti* originally collected from New Orleans, USA and kindly provided by the Centre for Disease Control (CDC), Atlanta, USA. The parental PMD strain had a resistance ratio of approx 2.5 to both DDT and permethrin. The LT_{50} value of the parental line was 124 and 19 min in DDT and permethrin, respectively. After selection, the resistance levels of this colony increased dramatically to a level at which 100% survival was observed after 7 h on both 4% DDT and 0.75% permethrin papers.

2.2. PCR amplification of *Ae. aegypti* GSTs

Genomic DNA was extracted using the LIVAK buffer extraction method as described previously (Collins et al., 1987). Total RNA was isolated using the TRI reagent (Sigma). After treatment with DNase RQI (Promega) to eliminate any contaminating genomic DNA, total RNA was re-extracted with TRI reagent. RNA was reverse transcribed to cDNA using an oligo $(dT)_{15}$ primer (Promega) and Superscript III reverse transcriptase (Gibco BRL). PCR primers were designed to amplify four putative *Ae. aegypti* GST genes whose sequence was retrieved from the TIGR database. Both genomic DNA and cDNA were used as templates in PCR reactions containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of forward and reverse primer and 2.5

Table 1
Sequences of oligonucleotide primers used to amplify full-length *Ae. aegypti* GSTs

Gene	Primer name	Primer sequence (5′–3′)	Product size (bp)		Number of introns (Intron size; bp)
			cDNA	Genomic	
GSTe2	GSTe2F	CTGCTCCACAATGACGAAGC	666	1230	2 (61 and 503)
	GSTe2R	TGCCTTTTGAGCATTCTTCTCC			
GSTe4	GSTe4F	GTTAATCTTCACCACTAGAAATG	672	812	2 (75 and 65)
	GSTe4R	CGAACAATGCAATTACTTCTT			
GSTt1	GSTt1F	ATGTCGAAGCTACGGTACTTTTAC	687	802	2 (53 and 62)
	GSTt1R	CAACTTCGGCTTCGGAACG			
GSTt2	GSTt2F	ATGGCAAACGGTCGCAAC	681	1150	2 (400 and 69)
	GSTt2R	CGTCGCAGTAGGCGCA			

Underlines indicate start or stop codons.

units of Taq DNA polymerase (Bioline, UK). The sequences of the primers used are shown in Table 1. The PCR products were ligated into a pGem T easy Vector (Promega), transformed into *Escherichia coli* JM 109 competent cells (Promega) and positive colonies identified by blue/white colony selection. Plasmids were purified using the QIAprep miniprep kit (Qiagen) and sequenced using a Beckman CEQ 8000 automated sequencer. The DNA sequence was determined for all three strains of *Ae. aegypti* used in the study.

2.3. PCR screening of an Ae. aegypti bacterial artificial chromosome (BAC) library

The *Ae. aegypti* NDL BAC library, was kindly provided by Dr. D. W. Severson (University of Notre Dame, USA) (Jimenez et al., 2004). A PCR-based plate pool screening approach, followed by column and row pools and individual colony PCR as previously described (Hong et al., 2003), was used to isolate BAC clones containing GST genes. DNA was extracted from the BAC clones using the plasmid purification kit (Qiagen) and 50 ng purified DNA used as a template in PCR reactions using the conditions outlined above.

2.4. Quantitative PCR

RNA was extracted from 3 replicate sets (10 mosquitoes per replicate) of 4th instar larvae, pupae, one-day old adult males or females from each of the three strains. The RNA was reverse transcribed to cDNA as described above. The number of copies of mRNA for each gene of interest was calculated by measuring the incorporation of the fluorescent dye SYBR Green I (Qiagen) into the double stranded PCR product and comparing this value to a standard curve produced from the PCR amplification of the same gene fragment from plasmids of known concentrations (ranging from 1 ng/μl to 1 fg/μl). The specific primers for each gene were designed to amplify products size less than 300 bp at the C-terminal end. The primers used in quantitative PCR reaction are shown in Table 2. An alternative primer was used for amplifying *GSTe2* from the susceptible strain as the primer binding sites encompassed a polymorphic nucleotide. The PCR consisted of 35 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s with fluorescence read at 82 °C. To normalize for variation in total cDNA concentration, the copy number of a ribosomal gene, S7 (accession AY380336) was similarly determined.

2.5. Western blot analysis

Batches of ten 4th instar larvae were homogenized in 0.1 M phosphate buffer pH 6.5 containing 10 mM DTT, and their protein content determined by the method of Bradford (Bradford, 1976). Fifty micrograms of protein from each homogenate were resolved on individual lanes of a 4–20% gradient Tris-HCl Ready Gel (Bio-Rad). Purified recombinant GSTE2-2 from *Ae. aegypti* and homogenates from the DDT-resistant ZAN/U strain of *An. gambiae* were also run as controls. The proteins were transferred to ECL hybond nitrocellulose membrane (Amersham Bioscience) using a Mini-Trans Blot Cell Assembly (Bio-Rad). The membrane was probed with a 1:5000 dilution of polyclonal antibody raised against the purified recombinant GSTE2-2 protein from *An. gambiae* (Ortelli et al., 2003). The bound antiserum was detected by incubation with a 1:50000 dilution of Peroxidase-labelled Anti-Rabbit Antiserum (Amersham) followed by visualisation using ECL Advance Blotting Detection Kit (Amersham Bioscience).

2.6. Expression of GSTE2-2 in vitro and protein purification

The full coding region of *GSTe2* was amplified from cDNA from the PMD-R strain of *Ae. aegypti* using the

Table 2
Primer sequences for quantification of mRNA expression

Gene	Primer name	Primer sequences (5′–3′)	cDNA size (bp)	Annealing/Detection Temp (°C)
GSTe2	GSTe2F2 GSTe2SusF2 GSTe2R	TATGTGCAAAAGGCTTACCAACTG TATGTGCAAAAGGCATACCAACTG TGCCTTTTGAGCATTCTTCTCC	270	60/84
GSTe4	GSTe4F2 GSTe4R	CAAGATTGACTACGCGTGCAAG CGAACAATGCAATTACTTCTT	283	60/84
GST-2	GST-2F2 GST-2R	ATCCTGGAAGCCCTAGGAAC TTAGAAAGGTTCCTCCAGCTTG	258	60/83
GSTt1	GSTt1F2 GSTt1R	GAGATTTCCGTTGCGGATCTG CAACTTCGGCTTCGGAACG	195	60/84
S7	aaS7F aaS7R	GCAAGCACGTCGTGTTCATCGGC GAACGTAACGTCACGTCCGGTCAG	296	60/87

GSTe2SusF2 is the forward primer for amplifying *GSTe2* from the New Orleans strain.

forward primer aaGSTe2BamF (5′-CGGAATTCG-GATCCATGACGAAGCTCATTTTGTACACG-3′) and the reverse primer aaGSTe2BamR (5′CGGAATTC-GGATCCTTATGCCTTTTGAG-CATTCTTCTCC-3′). These primers contain the initiation and stop codon, respectively, flanked by *BamHI* restriction sites (shown underlined). PCR amplification was performed using ProofStart DNA Polymerase (Qiagen). The PCR product was digested with *BamHI* restriction enzyme and ligated into the *BamHI* site of the pET3a vector (Novagen). After transforming into a non-expression host, *E. coli* JM109, the plasmid was purified and sequenced to verify the sequence integrity. The plasmids were then transformed into the expression host *E. coli* BL21 (DE3) pLysS (Novagen). A single colony containing recombinant plasmid was grown in 2 ml of LB-medium containing ampicillin (50 µg/ml) and chloramphenicol (50 µg/ml) at 30 °C overnight. This culture was used to inoculate 100 ml LB-medium and grown for a further 3 h. Expression was induced by adding of 0.4 mM isopropyl β-D-thiogalactoside (IPTG) and after an additional incubation at 30 °C for 3 h, bacterial cells were collected by centrifugation at 5000 g, 4 °C for 10 min. Pelleted cells were resuspended in lysis buffer containing 4.29 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, 0.137 mM NaCl and 0.1% Tween, pH 7.3. After snap freezing in liquid nitrogen, the cell lysate was thawed and incubated with 2 Units/ml of DNase RQI at room temperature for 20 min. to digest bacterial genomic DNA. After centrifugation to remove the cell debris, the soluble protein was collected for purification. The recombinant protein was purified using the T7•Tag Affinity Purification Kit (Novagen) according to the manufacturer's protocol. The enzyme purity and subunit size were resolved by SDS-PAGE. Protein concentration was determined using the Bio-Rad Protein Reagent (Bio-Rad) with bovine serum albumin for the standard protein (Bradford, 1976). The recombinant protein was concentrated using Microcon YM-30 columns (Amicon) collected in the presence of 40% (v/v) glycerol and 15 mM DTT and stored at −20 °C.

2.7. Recombinant GSTE2-2 characterisation

GST activity against 1-choro-2, 4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) was measured according to the method of Habig et al. (Habig et al., 1974). Glutathione peroxidase activity was determined at 340 nm by coupling the reduction of cumene hydroperoxide (CHP) by GSH to the oxidation of NADPH by oxidised GSSG with glutathione reductase as described previously (Simmons et al., 1989). DDT-dehydrochlorinase activity was determined by conversion of DDT to DDE detected by HPLC as described previously (Prapanthadara et al., 2000). Kinetic studies were performed by varying the concentration of CDNB (0.025–2 mM) and GSH (0.5–40 mM) at fixed concentrations of 15 mM GSH and 2 mM CDNB, respectively. The results were analysed by nonlinear regression analysis using GraphPad Prism 4 software.

3. Results

3.1. Identification of genes encoding GSTs in Ae. Aegypti

The database of expressed sequence tags (ESTs) maintained by The Institute for Genomic Research (TIGR) (www.tigr.org) was searched firstly using the keyword 'GST' and secondly by local BLAST searches using *An. gambiae* GSTs as the query sequences. Nine

putative GST sequences were retrieved and each of these was then searched against the non-redundant database at NCBI, using BLASTX, to confirm their identity as putative GST genes. Of the nine *Ae. aegypti* GSTs, two were classified as Delta, two as Epsilon, one as Sigma and two as Theta class, according to sequence similarities with other insect GSTs. The remaining two genes, which included the previously identified *GST-2*, were clear orthologs of 'unclassified' GSTs in *An. gambiae* (Ding et al., 2003). In the present study, we focused on the Epsilon and Theta class GSTs. Fig. 1 shows a phylogenetic tree of the entire family of *An. gambiae* cytosolic GSTs together with the nine *Ae. aegypti* GSTs. The assignment of the *Aedes* GSTs to the Delta, Epsilon, Sigma and Theta classes is clearly supported by this phylogeny. There are secure orthologs between the *Ae. aegypti* GSTs and *An. gambiae* GSTs. supported by bootstrap values of >85% (Fig. 1) for three of the four Epsilon and Theta genes and we therefore named these three *Aedes* GSTs *AaGSTe2*, *AaGSTe4* and *AaGSTt1*. The identity between the deduced amino acid sequences of these *Aedes* GSTs and their orthologs in *An. gambiae* range from 66.8 to 71.5% (Table 3). The phylogenetic relationship between the second *Ae. aegypti* Theta class GST and the *An. gambiae* Theta GSTs is less clear. Although it clearly clusters with *AgGSTt2* in Fig. 1 the support for this clade is low (61% of 1000 bootstrap replicates). The percentage amino acid similarity between these two GSTs is also low (51.1% (Table 3)) but given that, to date, only two Theta class GSTs have been identified in *Ae. aegypti*, we have tentatively named this GST as *AeGSTt2*. To further investigate the relationship between insect GSTs, we included all members of the Epsilon and Theta class from *Drosophila melanogaster* (Ranson et al., 2002; Sawicki et al., 2003) in the alignment and repeated the phylogenetic analysis using these classes. No clear orthologs were identified between *Ae. aegypti* and *D.*

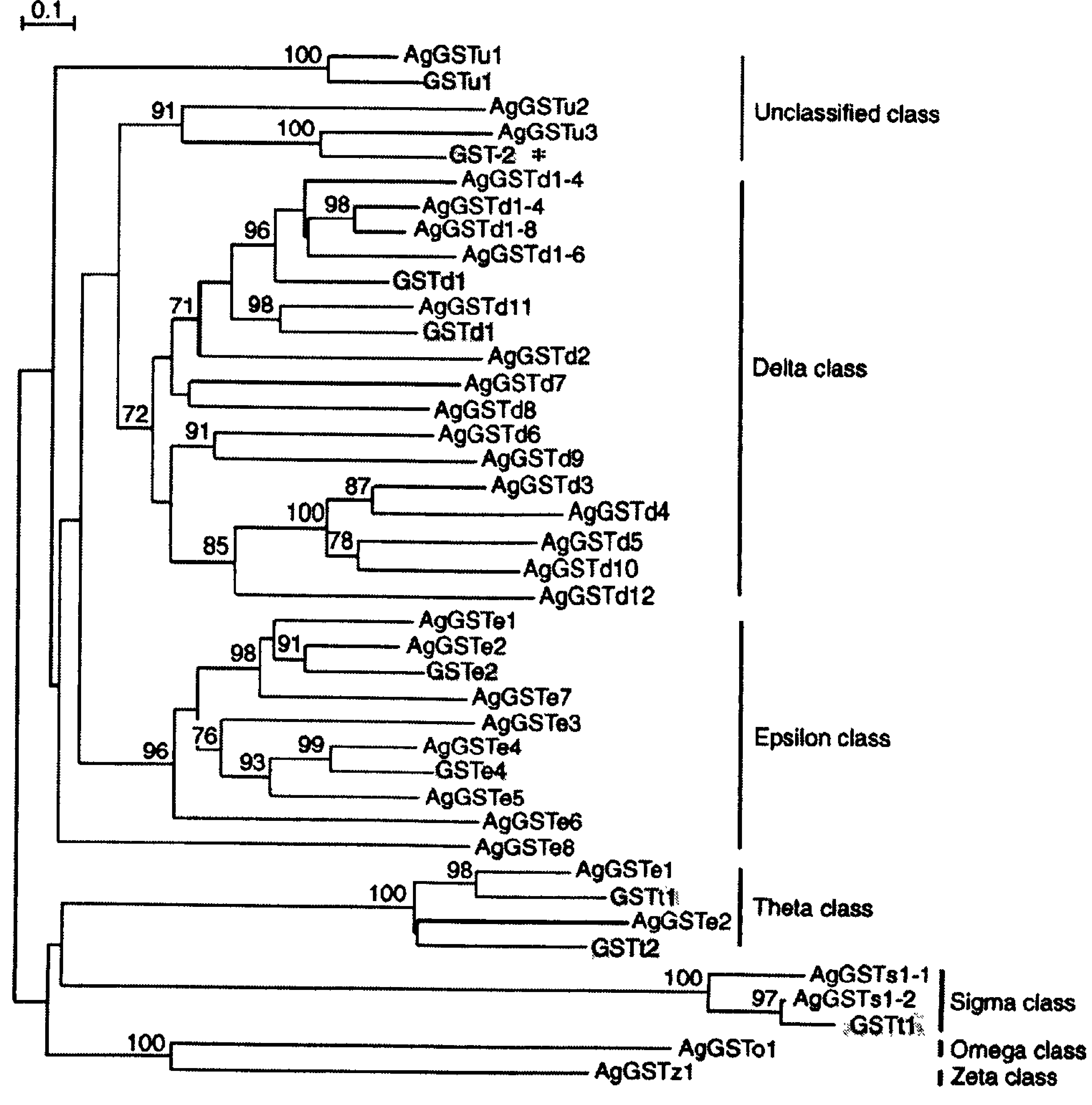

Fig. 1. Phylogenetic relationship of Epsilon and Theta GST class in *Ae. aegypti*, with all *An. gambiae* GSTs. Amino acid sequences were aligned using ClustalW (www.ebi.ac.uk/clustalW) and a distance neighbor-joining tree was generated using TREECON (Van de Peer and De Wachter, 1993). Nodes with distance bootstrap values (1000 replicates) of >70% are shown. GSTs from *Ae. aegypti* are highlighted in gray. Asterisk indicates GST-2 from *Ae. aegypti* identified previously (Grant and Hammock, 1992). Ag = *An. gambiae*.

Table 3
Percentage identities between mosquito GST protein sequences

	Percent identity							
	GSTe2	GSTe4	AgGSTe2	AgGSTe4	GSTt1	GSTt2	AgGSTt1	AgGSTt2
GSTe2	—	54.5	*71.5*	55.4	21.2	15.3	20.7	17.1
GSTe4		—	52.9	*71.0*	22.3	18.8	19.2	18.8
AgGSTe2			—	53.4	21.7	17.6	23.1	20.4
AgGSTe4				—	18.7	16.0	20.9	20.0
GSTt1					—	52.4	*66.8*	46.7
GSTt2						—	54.2	*51.1*
AgGSTt1							—	50.9
AgGSTt2								—

Percentage similarity was determined using the DNASTAR software. The italic numerals indicate the percentage similarity between putative *Ae. aegypti* and *An. gambiae* (Ag) orthologs.

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         ----------------------------------------------------------
GSTe2    MT---KLILYTLHVSPPCRAVELCAKALGLELEQKTVNLLTKEHLTPEFMK-MNPQHTVP 56
GSTe4    MG---KVQLYTAKLSPPGRAVELTAKAIGLDLDVHPINLIAGDHLKPEFVK-MNPQHTIP 56
GSTt1    MS---KLRYFYDLMSQPSRMLYIFLESTKIPYERCLVNLGKGEHLT-DKFKAINRFQKVP 56
GSTt2    MANGRNIRFYYDLISQPCRALYIFLEQNKIHYQKCPIALRKWEHTTPEYLQNVNRFGKVP 60

         ----------------------------------------------------------
GSTe2    -VLDDNGTIVCESHAIMIYLVSKYGKDDSLYSKELVKQAKLNAALHFESG----VLFARS 111
GSTe4    LIVDEDGTIVYDSHAIIIYLVSKYAKDDSLYPEDIATRAKINAALHFDSG----VLFARL 112
GSTt1    CIVDKNDLHLAESVAIVRYLAREYPFSDHWYPKDSQKRARIDEYLEWQQHNTRAVCATYF 116
GSTt2    AIVDGKNFKLAESIAILRYLAREFTVPDHWYPRDSRRRARVDEYLEWQHSNTRLHCAGYV 120

         ---C------------------------------------------I-----------
GSTe2    RFVFEPILFAGG---SEIPADRAEYVQKAYQLLEDTLVD---DYVVGNSLTIADFSCVSS 165
GSTe4    RFYLEPILYYGS---PDTPQDKIDYACKAYQLLNDTLVD---EYIVGNRMTLADLSCIAS 166
GSTt1    QYVWLRPKLMGTKVNPERAEEYKQKMEDCLDFIESDYLGGGNPFLVGNEISVADLFAACE 176
GSTt2    RYVWRGP-LRGETMDPRVAKRLKAEMVGCLDFIETNVLQRDVHFIAGDEISIADLVAACE 179

         ------------E-------------------A---------------------
GSTe2    VSSIMGVIPMDKAKFPKIYGWLDRLKALPYYEEANGSGAEQVAQFVLSQKEKN-AQKA   222
GSTe4    IASYHAIFPIDAAKYPKLAAWVQRLEKLPYYKGTNQEGAEELAAVYRDRLAQNRAGKK   224
GSTt1    IEQPKMAGFDPCVGRPKMTAWMARVREAT--NPHYDEAHKLVYRIAPDSVPKPKL---   229
GSTt2    IEQPKLAGYDARVGRPKLTAWMQRVKETT--QPDYDEAHKVLNKFAPTAT--------   227
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Fig. 2. The deduced amino acid sequence of *Ae. aegypti* GST Epsilon class and GST Theta class genes. Sequences shown are from the New Orleans strain. Four amino acid substitutions found in GSTE2 from PMD and PMD-R strain are shown in line 1 of the alignment. The amino acid sequences were aligned using ClustalW. Letters in bold indicate 100% conservation between the 4 sequences. Dashes are used to denote gaps introduced for maximum alignment. Vertical lines with asterisks indicate the intron positions. GenBank accession numbers for *AaGSTe2*, *AaGSTe4*, *AaGSTt1* and *AaGSTt2* are AY819710, AY819709, AY819711 and AY819712, respectively.

melanogaster but the topology of the tree supported the relationship between the *An. gambiae* and *Ae. aegypti* GSTs described above (Data not shown).

To confirm the sequence of the ESTs and to determine the structure of the genes from which they were derived, primers were designed to enable the amplification of the full coding region of each gene. The deduced amino acid sequences of the four *Ae. aegypti* GSTs are aligned in Fig. 2. The coding sequence of all four genes is interrupted by two introns (positions marked as vertical lines in Fig. 3). The *Ae. aegypti* Epsilon class GST genes, *GSTe2* and *GSTe4*, both contain two introns (Table 1) at identical positions. Introns are located at the equivalent position in *AgGSTe2* but the second intron is considerably longer in *Aedes* versus *Anopheles* (503 vs 90 bp) (Table 1). The location of the first intron is also conserved in *AgGSTe4* but the *Anopheles* gene does not contain the second intron found in *Aedes*. Similarly, the positions of the two Theta introns are conserved between *AaGSTt1* and *AgGSTt1* but *GSTt2* from *An. gambiae* contains only a single intron whereas its putative *Aedes* ortholog contains two.

Screening of the *Ae. aegypti* BAC library with primers specific to *GSTe4* identified a single positive clone

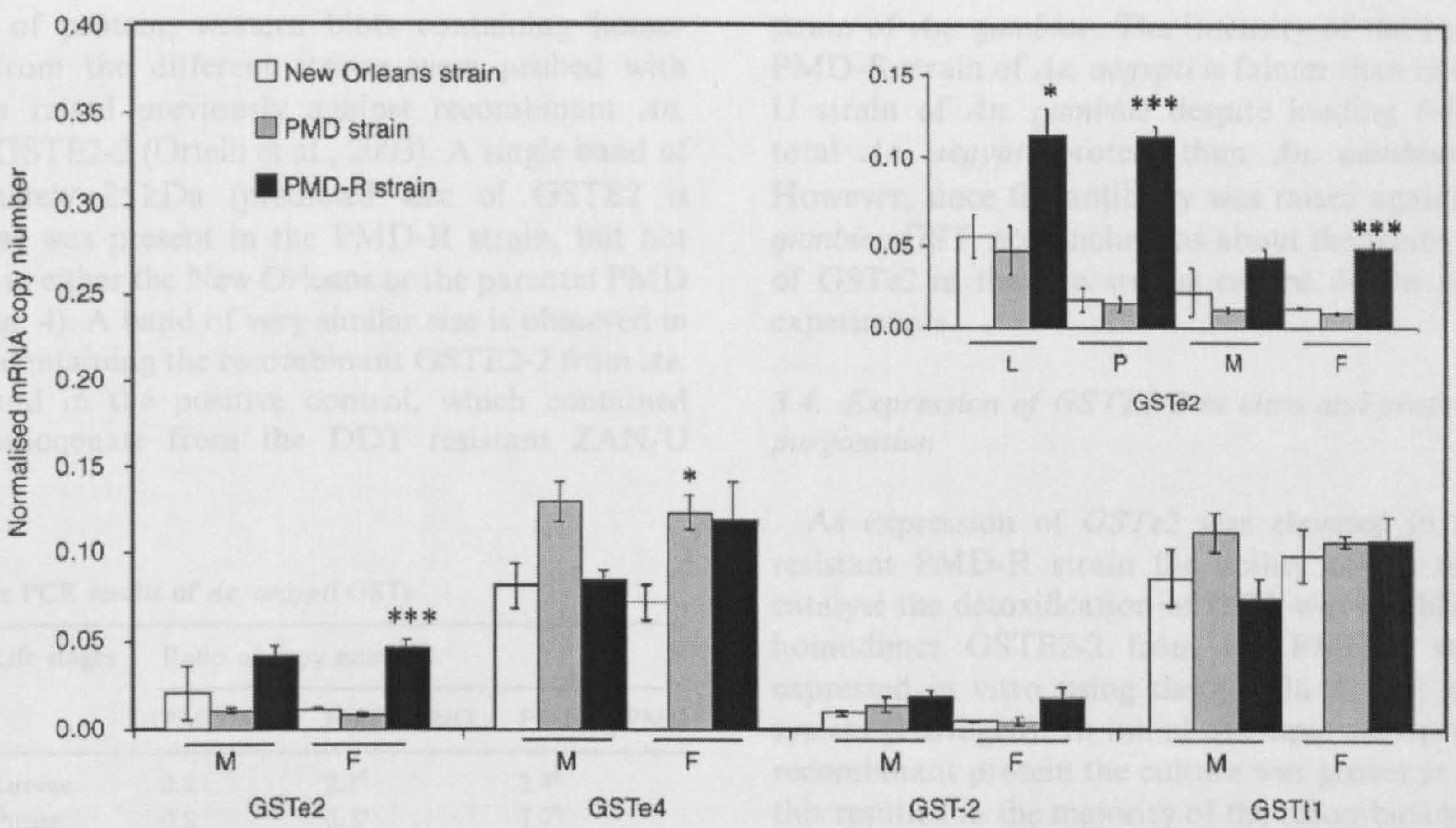

Fig. 3. Quantification of GST mRNA expression level in three strains of *Ae. aegypti*. Complementary DNA from three different biological replicates (ten mosquitoes each) was used as templates. Four life-stages were analysed; larvae (L), pupae (P), adult male (M), and adult female (F). Each sample was analysed in duplicate in each experiment and the results were averaged from three independent experiments. The mRNA copy numbers were determined by comparison with known concentrations of standard plasmids and normalised against the copy number of the ribosomal S7 transcript. Error bars indicate standard error of the mean. Statistically significant differences were evaluated with ANOVA test followed by pair-wise *t*-test ($p<0.001$ indicated by *** and $p<0.05$ as * relative to New Orleans strain.

(ND17A9). PCR screening of the BAC DNA isolated from this clone with primers designed for *GSTe2* gave a positive signal, whose identity was confirmed by DNA sequencing. The average insert size of the BAC clones in the NDL library is 122 kb (Jimenez et al., 2004) and this result indicates that the two Epsilon GSTs are in close proximity in the genome. Screening the BAC library with the Theta GST primers for both *GSTt1* and *GSTt2*, led to the identification of a single positive clone, ND2C20, again suggesting that the *Aedes* Theta GSTs are close together.

Each of the four GST genes described in this report were amplified and sequenced from the PMD, PMD-R and the New Orleans strain. Sequence polymorphisms resulting in amino acid substitutions were only observed in the Epsilon GSTs. A single glutamic acid to lysine (E89K) substitution was observed in GSTE4. Four conserved polymorphisms (F115C, V150I, A178E and E198A) were present in the C-terminal domain of GSTE2 from PMD and PMD-R strain (Fig. 2).

3.2. Quantitative analysis of Ae. aegypti GST expression

Elevated expression of Epsilon GSTs has been implicated in conferring resistance to DDT in *An. gambiae*. To determine whether the *Ae. aegypti* GSTs are also elevated in DDT resistant mosquitoes, the mRNA copy number of the *Ae. aegypti* Epsilon GST genes in different life stages of the three strains was determined. *GST-2* was also included in this analysis, since over expression of this gene had previously been associated with resistance to DDT in a strain of *Ae. aegypti* from Isla Verde, Puerto Rico (Grant and Mutsumura, 1989). Finally, a fourth gene, from the Theta class, not previously associated with insecticide resistance, was included as a control. The relative copy number of each gene was calculated by normalising with the ribosomal S7 gene. Expression of all four genes was detected in all life stages assayed (see Fig. 3, data for immature stages omitted). For *GSTe4*, *GST-2* and *GSTt1*, gene expression was not correlated with insecticide resistance status. However, expression of *GSTe2* was significantly highly in the DDT-resistant strain PMD-R in all life stages except adult male ($p=0.19$ in adult males, $p<0.001$ in pupae and adult female, and $p<0.05$ in larvae) when compared to the susceptible strain, and in all life stages ($p<0.001$ in pupae and adult female, $p<0.01$ in adult males and $p<0.05$ in larvae) when compared to the parental PMD strain (Table 4). In contrast no significant differences in *GSTe2* expression was detected between the parental PMD strain and the New Orleans strain in any life stage (Table 4).

3.3. Western blot analysis

To determine whether the elevation in *GSTe2* expression in the PMD-R strain translated into increased

amounts of protein, western blots containing homogenates from the different strains were probed with antiserum raised previously against recombinant *An. gambiae* GSTE2-2 (Ortelli et al., 2003). A single band of approximately 25 kDa (predicted size of GSTE2 is 24.74 kDa) was present in the PMD-R strain, but not observed in either the New Orleans or the parental PMD strain (Fig. 4). A band of very similar size is observed in the lanes containing the recombinant GSTE2-2 from *Ae. aegypti* and in the positive control, which contained crude homogenate from the DDT resistant ZAN/U strain of *An. gambiae*. The intensity of the band in the PMD-R strain of *Ae. aegypti* is fainter than in the ZAN/U strain of *An. gambiae* despite loading 5-fold more total *Ae. aegypti* protein than *An. gambiae* protein. However, since the antibody was raised against the *An. gambiae* GST, no conclusions about the relative amount of GSTe2 in the two strains can be drawn from these experiments.

Table 4
Quantitative PCR results of *Ae. aegypti* GSTs

Gene	Life stages	Ratio of copy number		
		PMD/NO	PMD-R/NO	PMD-R/PMD
GSTe2	Larvae	0.8	2.1[c]	2.4[c]
	Pupae	0.8	6.5[a]	7.7[a]
	Male	0.5	2.0	3.8[b]
	Female	0.7[c]	3.9[a]	5.3[a]
GSTe4	Male	1.6	1.0	0.6[c]
	Female	1.7[c]	1.6	1.0
GST-2	Male	1.4	1.9	1.3
	Female	1.0	3.2	3.2
GSTt1	Male	1.3	0.8	0.6
	Female	1.1	1.1	1.0

The GST transcript copy number was determined by normalizing with the transcript copy number of ribosomal S7 transcript. The ratio of the average copy number was calculated by comparing with the average copy number of New Orleans or PMD transcript. Statistically significant differences were evaluated with ANOVA test followed with pair-wise *t*-test ($p<0.001$ indicates by [a], $p<0.01$ illustrates as [b] and $p<0.05$ illustrates as [c] relative to New Orleans strain (NO) or PMD.

3.4. Expression of GSTE2-2 in vitro and protein purification

As expression of *GSTe2* was elevated in the DDT resistant PMD-R strain the ability of the enzyme to catalyse the detoxification of DDT was established. The homodimer GSTE2-2 from the PMD-R strain was expressed in vitro using the pET3a *E. coli* expression system (Novagen). In initial attempts at expressing the recombinant protein the culture was grown at 37 °C but this resulted in the majority of the recombinant GSTE2-2 being sequestered in insoluble inclusion bodies. Only by reducing the incubation temperature to 30 °C was sufficient soluble protein produced to enable purification (data not shown). The recombinant GSTE2-2 did not bind to an S-hexylglutathione affinity column, but was successfully purified by affinity chromatography using monoclonal antibodies against the T7 tag that is fused to the N-terminal end of the protein. This was typical in the recombinant GSTE2-2 from *An. gambiae*.

3.5. Substrate specificities and kinetic properties of recombinant GSTE2-2

The specific activities of GSTE2-2 with various substrates are shown in Table 5. The activities with

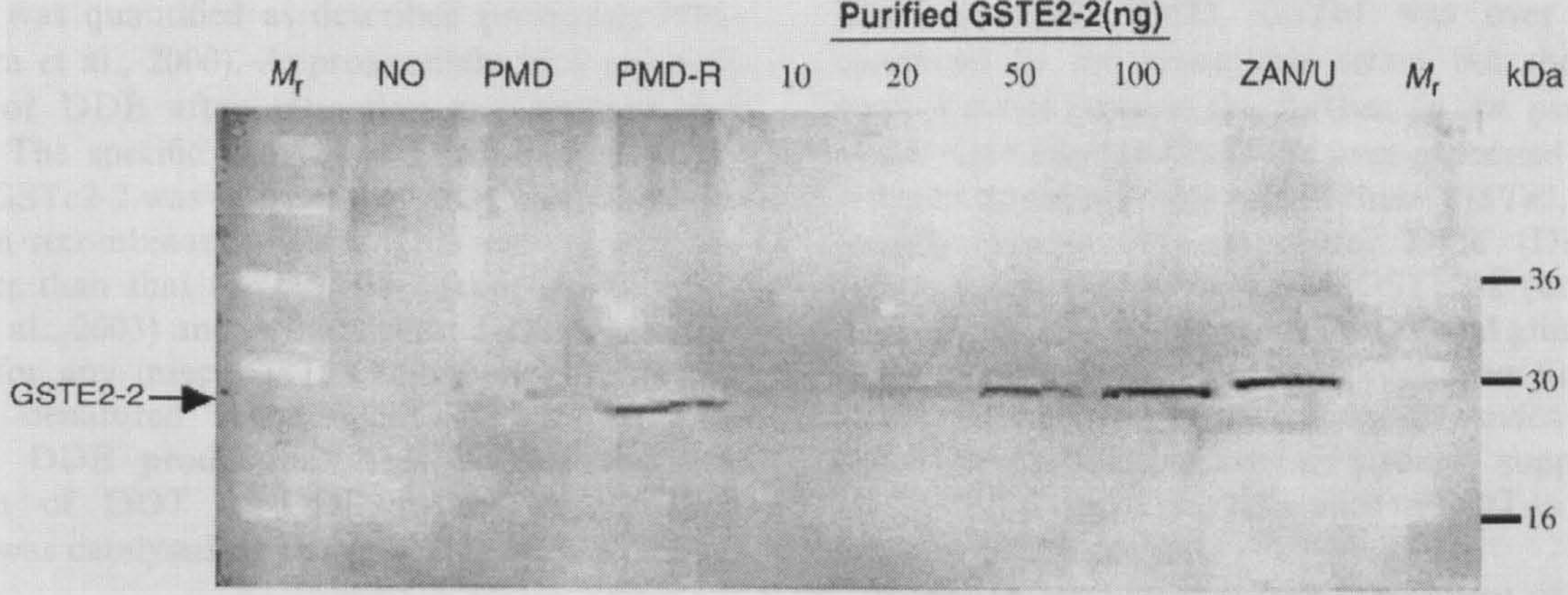

Fig. 4. Immunological cross reactivity with polyclonal antibody raised against recombinant GSTE2-2 from *An. gambiae*. Protein (50 μg) from 4th instar larvae from New Orleans (NO), PMD and PMD-R strains and purified recombinant GSTE2-2 (10, 20, 50 and 100 ng) were resolved on 4–20% Tris-HCl Ready Gel (Bio-Rad). Protein (10 μg) from *An. gambiae* (ZAN/U) was used as positive control. Proteins were transferred to a nitrocellulose membrane and probed with GSTE2-2 antibody, 1:5000 (Ortelli et al., 2003) for 1 h. Peroxidase labeled anti-rabbit antibody (1:50000; Amersham Pharmacia Biotech) was used as a second antibody. Proteins were visualised by enhancing the chemiluminescence using ECL Advance Blotting Detection Kit (Amersham Biosciences). Lane M_r indicates the molecular masses of standard protein. An arrow represents the expected size (24.7 kDa) of recombinant AeGST2-2.

Table 5
Substrate specificities and kinetic parameters for recombinant GSTe2-2 from *Ae. aegypti* and *An. gambiae*

	Specific activity of GSTe2-2	
	Ae. aegypti	*An. gambiae*
Substrate specificity		
CDNB (μmol/min/mg)	6.77±0.54	12.5±0.58
DCNB (μmol/min/mg)	2.89±0.46	5.87±0.24
CHP (μmol/min/mg)	0.11±0.01	ND
DDTase activity (nmol DDE/μg)	4.16±0.28	2.77
Kinetic parameters		
V_{max} (μmol/min/mg)	5.00±0.44	13.10±0.40
K_m^{GSH} (mM)	7.57±1.17	6.72±1.70
K_m^{CDNB} (mM)	0.18±0.04	0.07±0.01
k_{cat} (s^{-1})	4.12	10.84±0.33
k_{cat}/K_m^{GSH} ($s^{-1}mM^{-1}$)	0.54	1.61
k_{cat}/K_m^{CDNB} ($s^{-1}mM^{-1}$)	22.89	157.1
S-hexyl glutathione agarose binding	Unbound	Unbound

Three independent assays were performed. Results show mean±SD. Kinetic studies were determined by varying the concentration of GSH (0.5–60 mM) and the concentration of CDNB (0.025–2.0 mM) at fixed concentrations of CDNB of 2 mM and GSH of 15 mM, respectively. Substrate specificity and kinetic parameters of AgGSTe2-2 from *An. gambiae* are given for comparison (Ortelli et al., 2003). ND indicates not detectable.

CDNB and DCNB are within the range reported for other insect GSTs (Ortelli et al., 2003; Ranson et al., 1997; Sawicki et al., 2003; Vontas et al., 2002). CDNB is a better substrate for recombinant *An. gambiae* GSTE2-2 than for recombinant GSTE2-2 from *Ae. aegypti* as evidenced by the higher V_{max} and k_{cat} values and the lower K_m^{CDNB} of the *An. gambiae* enzyme. Only the *Ae. aegypti* enzyme displayed glutathione peroxidase activity as measured by the GSH-dependent reduction of cumene hydroperoxide.

DDT dehydrochlorinase activity of the recombinant GSTE2-2 was quantified as described previously (Prapanthadara et al., 2000). Approximately 98% recovery of DDT or DDE after extraction and analysis was obtained. The specific activity of DDT dehydrochlorinase for GSTe2-2 was 4.16 nmol of DDE formation per microgram recombinant protein. This activity was 1.5-fold higher than that for GSTE2-2 from *An. gambiae* (Ortelli et al., 2003) and is the highest DDTase activity reported for any insect GST. Control assays with no GSH or denatured recombinant enzyme had no detectable DDE production. This verified that the conversion of DDT to DDE in the experimental reactions was catalysed by the recombinant GSTE2-2.

4. Discussion

We have identified eight GST genes from the mosquito *Ae. aegypti* and conducted a preliminary phylogenetic analysis to establish their relationship with other insect GSTs. A complete examination will only be possible once the *Ae. aegypti* genome sequence is determined and the full extent of the GST gene family in this species is known. Nevertheless, several interesting points arise from this initial analysis. The topology of the tree in Fig. 1 suggests that the insect GST family had diverged prior to the split between *Aedes* and *Anopheles* mosquitoes, which occurred approximately 95 million years ago (Krzywinski et al., 2001), as secure orthologs for seven of the nine known *Ae. aegypti* GSTs can be readily identified (Note that both GSTd1 and GSTs1 are alternatively spliced in *An. gambiae* and all transcripts are included in Fig. 1). There have been at least two cases of intron loss or gain since the lineages diverged as both *GSTe4* and *GSTt2* in *Ae. aegypti* contain two introns in contrast to the single intron found in these genes in *An. gambiae*. Within the Epsilon and Theta classes, there are no clear orthologs between the mosquitoes and the fruit fly, *D. melanogaster*, supporting the earlier suggestion that these GST classes have radiated independently in the two families (Ranson et al., 2002).

The two Epsilon GST genes are present in a single BAC clone, indicating some degree of physical clustering in the genome as observed with the Epsilon GSTs in *An. gambiae* (Ortelli et al., 2003). In *Ae. aegypti*, the two Theta GSTs are also located within the same genomic region, in contrast to with *An. gambiae* where the two Theta GSTs are located at different ends of the X chromosome (Ding et al., 2003).

Transcription levels for four of the *Ae. aegypti* genes were quantified. *GSTe2* was significantly over expressed in the insecticide resistant PMD-R strain, but was not over expressed in the parental line, indicating that insecticide selection increased the *GSTe2* expression. While in adult PMD, *GSTe4* was over expressed compared to the susceptible strain but the selection strain did not increase this further. In *An. gambiae*, five of the eight Epsilon GSTs are over expressed in a DDT resistant strain but only one of these, *GSTe2*, encodes a protein capable of metabolizing DDT (Ding et al., 2003). When the recombinant GSTE2-2 protein from *Ae. aegypti* was incubated with DDT and glutathione, it efficiently catalyzed the dehydrochlorination of the insecticide to DDE in an enzyme dependent reaction. Collectively, these experiments strongly support a role for *GSTe2* in conferring resistance to DDT in the PMD-R strain of *Ae. aegypti*.

Although we note that true assignment of orthology cannot be confirmed until the full complement of the GST gene family is determined in *Ae. aegypti*, these results strongly suggest that the same GST is responsible for DDT resistance in both *An. gambiae* and *Ae. aegypti*. This is perhaps surprising given the extensive gene duplication and diversification that has occurred within

the GST family. Large enzyme families have a degree of redundancy or overlap and thus it may be expected that metabolic mechanisms of insecticide resistance would differ between different populations of the same species. Indeed, a previous report on the genetic mechanism of DDT resistance in *Ae. aegypti* associated elevated expression of *GST-2*, caused by a mutation in a *trans*-acting factor, with resistance (Grant and Hammock, 1992). This GST is not over expressed in the PMD-R strain from Thailand perhaps suggestive of differing routes of GST catalyzed DDT metabolism in different strains of *Ae. aegypti.* However, until the ability of GST-2 to metabolise DDT has been determined, its role in insecticide resistance via metabolism cannot be confirmed.

In an analogous situation to the present study, two species of *Drosophila* have both developed the same mechanism of resistance to DDT. In this case, the cytochrome P450 gene; *Cyp6g1* was up-regulated in multiple strains of *D. melanogaster* and *D. simulans* (Le Goff et al., 2003; Schlenke and Begun, 2004). In both species of *Drosophila*, the increase in expression of *Cyp6g1* was caused by the insertion of a transposable element in the 5′ regulatory region. Up-regulation of *GSTe2* in DDT resistant strains of *An. gambiae* is due, at least in part, to mutations in the *cis*-acting regulatory regions (Ding et al., 2003). The genetic basis of *GSTe2* overexpression in *Ae. aegypti* is currently unknown. Furthermore, the significance, if any, of the four amino acid substitutions found between the susceptible New Orleans strain, PMD and PMD-R strains from Thailand have not been investigated. However, an alignment between the two *Ae. aegypti* GSTE2 peptides and the GSTE2 subunit *An. gambiae* showed that the amino acid residue at three of these polymorphic sites was conserved between the susceptible strain of *Ae. aegypti* and the resistant strain *An. gambiae* GSTE2. Given that *An. gambiae* GSTE2-2 from resistant strain is able to metabolise DDT, these substitutions are presumably not essential for DDTase activity. Only the alanine to glutamic acid substitution at position 178 was found uniquely in the PMD strain of *Ae. aegypti.* A serine is present at this position in *An. gambiae*.

In addition to DDT resistance, the PMD-R strain has high levels of resistance to permethrin. This may be due to a mutation in the sodium channel protein, which is the target site for both DDT and pyrethroid insecticides producing a *kdr* phenotype. An alternative explanation is that the elevated levels of GSTE2 confer resistance to both permethrin and DDT. Interestingly, unlike its *Anopheles* counterpart, the recombinant GSTE2-2 enzyme from *Ae. aegypti* exhibits levels of glutathione peroxidase activity comparable with other insect GSTs that are involved in the conjugation of lipid peroxidation end products (Singh et al., 2001; Vontas et al., 2001). Therefore, in addition to its role in insecticide detoxification, GSTE2-2 may provide an important defense mechanism against oxidative stress and may thereby provide a second line of defense against the toxic effects of insecticides.

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pET-3a-d Vectors

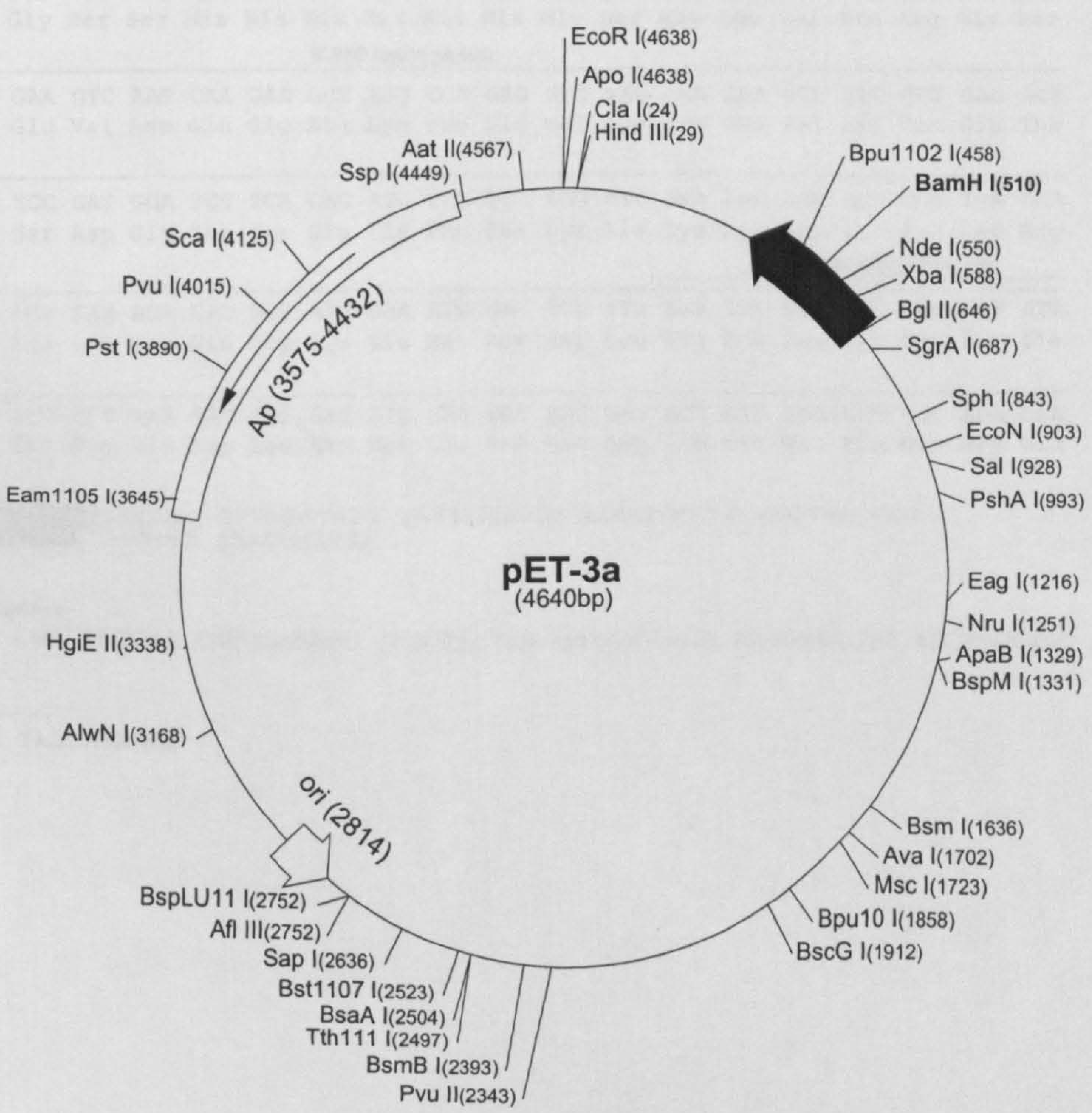
	Cat. No.
pET-3a DNA	69418-3
pET-3b DNA	69419-3
pET-3c DNA	69420-3
pET-3d DNA	69421-3

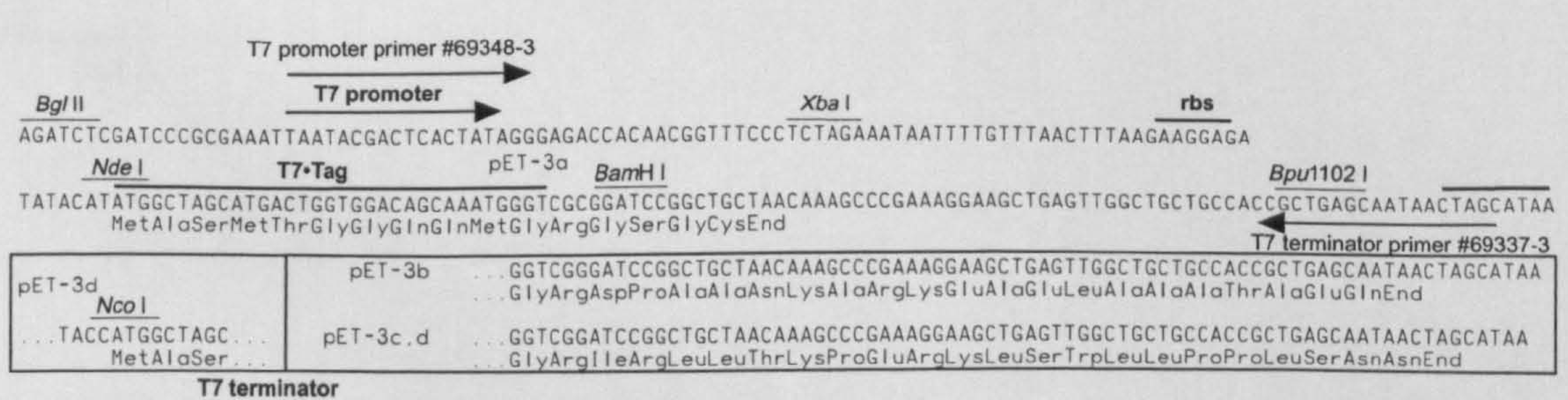
The pET-3a-d vectors carry an N-terminal T7•Tag® sequence and *Bam*H I cloning site. These vectors are the precursors to many pET family vectors; the pET-23a-d(+) series corresponds to pET-3a-d but incorporates several additional features. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below.

pET-3a sequence landmarks

T7 promoter	615-631
T7 transcription start	614
T7•Tag coding sequence	519-551
T7 terminator	404-450
pBR322 origin	2814
bla coding sequence	3575-4432

The maps for pET-3b, pET-3c and pET-3d are the same as pET-3a (shown) with the following exceptions: pET-3b is a 4639bp plasmid; subtract 1bp from each site beyond *Bam*H I at 510. pET-3c is a 4638bp plasmid; subtract 2bp from each site beyond *Bam*H I at 510. pET-3d is a 4637bp plasmid; the *Bam*H I site is in the same reading frame as in pET-3c. An *Nco* I site is substituted for the *Nde* I site with a net 1bp deletion at position 550 of pET-3c. As a result, *Nco* I cuts pET-3d at 546. For the rest of the sites, subtract 3bp from each site beyond position 551 in pET-3a. *Nde* I does not cut pET-3d.





pET-3a-d cloning/expression region

```
121  ATAGGCGCCA GCAACCGCAC CTGTGGCGCC GGTGATGCCG GCCACGATGC GTCCGGCGTA GAGGATCGAG ATCTCGATCC

                   T7 promoter                  lac operator
201  CGCGAAATTA ATACGACTCA CTATAGGGGA ATTGTGAGCG GATAACAATT CCCCTCTAGA AATAATTTTG TTTAACTTTA

                                                              HisG epitope
      RBS                                    Polyhistidine region
281  AGAAGGAGAT ATACAT ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC GGC AGC GGC CTG GTG CCG CGC GGC AGC
                       Met Gly Ser Ser His His His His His His Gly Ser Gly Leu Val Pro Arg Gly Ser

                                                            SUMO fusion protein
354  GCT AGC ATG TCG GAC TCA GAA GTC AAT CAA GAA GCT AAG CCA GAG GTC AAG CCA GAA GTC AAG CCT GAG ACT
     Ala Ser Met Ser Asp Ser Glu Val Asn Gln Glu Ala Lys Pro Glu Val Lys Pro Glu Val Lys Pro Glu Thr

426  CAC ATC AAT TTA AAG GTG TCC GAT GGA TCT TCA GAG ATC TTC TTC AAG ATC AAA AAG ACC ACT CCT TTA AGA
     His Ile Asn Leu Lys Val Ser Asp Gly Ser Ser Glu Ile Phe Phe Lys Ile Lys Lys Thr Thr Pro Leu Arg

                                                                         SUMO forward priming site
498  AGG CTG ATG GAA GCG TTC GCT AAA AGA CAG GGT AAG GAA ATG GAC TCC TTA AGA TTC TTG TAC GAC GGT ATT
     Arg Leu Met Glu Ala Phe Ala Lys Arg Gln Gly Lys Glu Met Asp Ser Leu Arg Phe Leu Tyr Asp Gly Ile

570  AGA ATT CAA GCT GAT CAG ACC CCT GAA GAT TTG GAC ATG GAG GAT AAC GAT ATT ATT GAG GCT CAC AGA GAA
     Arg Ile Gln Ala Asp Gln Thr Pro Glu Asp Leu Asp Met Glu Asp Asn Asp Ile Ile Glu Ala His Arg Glu

642  CAG ATT GGT GGT  PCR product  AGACAAG CTTAGGTATT TATTCGGCGC AAAGTGCGTC GGGTGATGCT
     GTC TAA CCA CCA               TCTGTTC GAATCCATAA
     Gln Ile Gly Gly
                    ▲
                    SUMO cleavage site

701  GCCAACTTAG TCGAGCACCA CCACCACCAC CACTGAGATC CGGCTGCTAA CAAAGCCCGA AAGGAAGCTG AGTTGGCTGC

              T7 reverse priming site
781  TGCCACCGCT GAGCAATAAC TAGCATAACC
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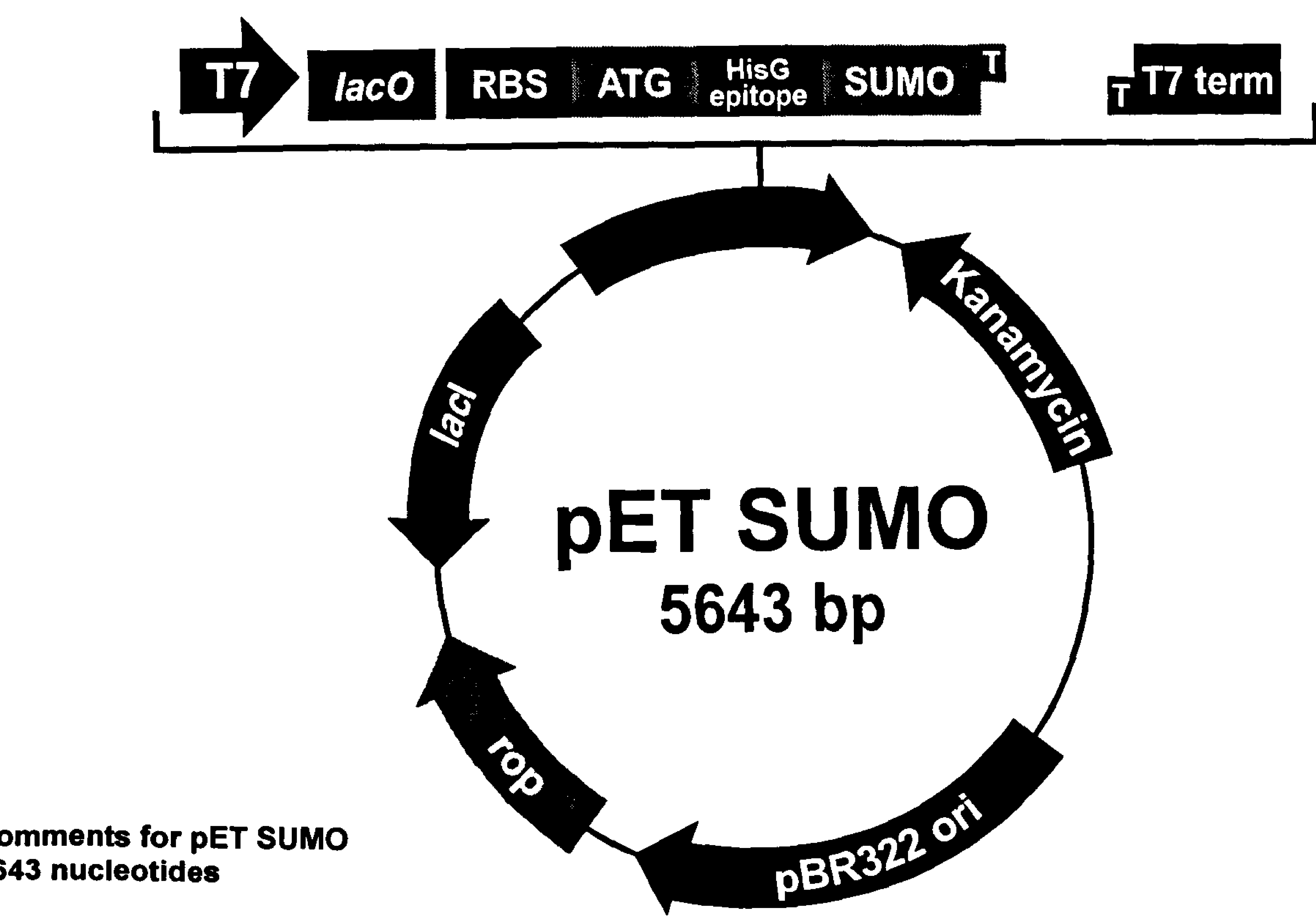

Comments for pET SUMO
5643 nucleotides

T7 promoter: bases 209-225
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288
Initiation ATG: bases 297-299
HisG epitope: bases 309-329
SUMO ORF: bases 360-653
SUMO forward priming site: bases 549-571
TA Cloning site: bases 653-654
T7 reverse priming site: bases 783-802 (C)
T7 terminator: bases 744-872
Kanamycin resistance gene: bases 1431-2246 (C)
pBR322 origin: bases 2342-3015
ROP ORF: bases 3383-3574
lacI ORF: bases 4383-5474 (C)

(C) = complementary strand