The Evolutionary Dynamics of Insecticide Resistance in the Whitefly, *Bemisia tabaci*

Christopher Mark Jones MSc.

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Abstract

The whitefly *Bemisia tabaci* (Hempitera: Aleyrodidae), is one of most destructive insect pests of agriculture and horticulture worldwide. *B. tabaci* has an extensive host-plant range, transmits several plant viruses and is a highly invasive species. Managing *B. tabaci* is therefore extremely problematic and expensive, with a heavy burden placed upon insecticides. Despite a broad spectrum of insecticidal chemistry available for whitefly control, resistance is widespread and insecticide resistance management (IRM) programmes have been introduced to prolong the longevity and efficacy of these compounds. In particular, resistance is commonly associated with two morphologically indistinguishable and invasive populations, known as the B and Q biotypes. The identification of these biotypes using molecular-based diagnostics has become a key feature of IRM programmes. The development of a high-throughput real-time PCR assay which was able to discriminate between B and Q biotypes is described in Chapter 3 of this project. Two major mechanisms of resistance, target-site modification (i.e. pyrethroids and the sodium channel) and enzyme detoxification (i.e. P450-based metabolism of neonicotinoids) have been widely studied in resistant B and Q biotypes of *B. tabaci*. The evolutionary origins of two 'knockdown resistance' mutations associated with pyrethroid resistance are described in Chapter 4. Furthermore, the neonicotinoid, imidacloprid, is one of the most successful insecticides registered for *B. tabaci*; however, reports of resistance are rapidly increasing. The molecular characterisation of imidacloprid resistance in *B. tabaci*, and in particular, the association of a P450-enzyme (*CYP6CM1*) with age-specific resistance, is reported in Chapter 5. The advancement in our understanding of the molecular mechanisms underlying insecticide resistance is essential to improve management strategies implemented against this pest.

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Dedication

This PhD thesis is dedicated to Professor Christopher Curtis who sadly passed away during its duration. Chris was the first person I ran to tell on receiving the news I was successful in obtaining the PhD.

"Don't get it right, get it written"

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Chapter 1 General introduction

1.1 Bemisia tabaci

The sweetpotato or cotton whitefly, *Bemisia tabaci* Gennadius (Hempitera: Aleyrodidae), was first described in 1889 from Greek tobacco (Gennadius 1889). Whiteflies are phloem sap-feeding insects and belong to the order Hemiptera alongside aphids, mealybugs, psyllids and scales. There are approximately 1500 described whitefly species (Family: Aleyrodidae) (Martin *et al.* 2000; Mound & Halsey 1978). However, only *B. tabaci* and the greenhouse whitefly *Trialeurodes vaporarium* are considered significant agricultural pests on a worldwide scale although many other whitefly species (e.g. *Bemisia afer* and *Trialeurodes abutiloneus*) are competent pests of various field and vegetable crops (Martin *et al.* 2000). Indeed, *B. tabaci* has become one of the most formidable and widespread insect pests of agriculture and horticulture over the past 30 years.

The life cycle of *B. tabaci* can take from approximately 2-3 weeks up to several months to complete depending on host and temperature conditions (Inbar & Gerling 2008). Following egg hatch on the leaf or plant surface, the crawler stage (N1) emerges before moulting into the sessile second-instar-nymph (N2). Two additional moults occur leading to a fourth-instar nymph (N4). The N4 instar is commonly referred to as the 'pupa', and this practice will be followed here despite whiteflies lacking any form of holometabolism (Byrne & Bellows 1991). *B. tabaci* displays arrhenotokous parthenogenesis whereby maternal whitefly eggs require fertilisation in order to develop into diploid females (2N) and unfertilised eggs develop into haploid male offspring (1N) (Byrne & Devonshire 1996; Crowder *et al.* 2010). This form of haplo-diploidy is also observed in the orders Hymenoptera (bees and wasps) and Thysanoptera (thrips) (Heimpel & de Boer 2008).

Unlike most whitefly species, which exhibit a narrow host range, *B. tabaci* is highly polyphagous infesting cultivated, non-cultivated, annual and perennial host species (Mound & Halsey 1978). A figure of between 500-600 host-plant species is commonly cited (Naranjo & Ellsworth 2009) but this is likely to be an underestimate and new host species are continuously being reported (Simmons *et al.* 2008). Among the most economically significant field crops affected by *B. tabaci* are cotton, cassava, and

field vegetables, including melon and sweetpotato. *B. tabaci* was historically restricted to tropical and sub-tropical climates. The expansion of the horticultural industry and increase in commercial trade has allowed *B. tabaci* to thrive in temperate regions (e.g. Asia, Europe and North America) where glasshouses provide a wealth of suitable host plants and environmental conditions. Among the key protected crops affected by whiteflies are tomatoes, fruit/vegetables and ornamentals such as poinsettias. The magnitudes of whitefly outbreaks tend to vary annually according to temperature, host-plant availability and the abundance of natural enemies (Naranjo & Ellsworth 2009). Their ability to readily disperse, colonise new hosts and reproduce rapidly makes *B. tabaci* an exceedingly tough pest to manage and/or eradicate (Blackmer *et al.* 1995; Brown *et al.* 1994; Denholm *et al.* 1998; Palumbo *et al.* 2001).

Figure 1.1 Adults whiteflies, *Bemisia tabaci*

B. tabaci causes much of its damage by direct feeding on phloem sap and, as a result, crop yield losses of up to 50% have been reported (Horowitz *et al.* 2003). Whiteflies also excrete honeydew which reduces the capability of leaves to fully photosynthesise and serves as a substrate for black-sooty fungal moulds (Inbar & Gerling 2008). This is particularly problematic for the cotton industry when the quality and value of lint used downstream of harvesting is reduced by 'sticky cotton' (Henneberry *et al.* 2000). Furthermore, *B. tabaci* is an extremely competent vector of several plant-viruses with only the whitefly genera *Bemisia* and *Trialeurodes* being capable transmitters (Jones 2003). The most significant viruses vectored by *B. tabaci* belong to the Begomoviruses (Family Geminiviridae) for which *B. tabaci* is the sole vector. Begomoviruses have a single stranded DNA (ssDNA) genome and the virus-vector relationship evolved only fairly recently (Brown 2007). The number of begomovirus species has, however, increased rapidly with their occurrence seemingly in a state of flux across the world. Damaging signs and symptoms on crops caused by the Begomoviruses include leaf-yellowing, mosaics, stunting, curling and vein-thickening (Jones 2003). For example, in East and Central Africa, there is currently an epidemic of cassava mosaic virus (CMV) and cassava brown streak virus affecting the vital staple crop cassava (Legg *et al.* 2006; Pearce 2007). CMV alone has caused an estimated 43% loss in cassava production in those countries where the virus is prevalent (Legg *et al.* 2006).

Throughout the majority of the 20th century, *B. tabaci* remained a relatively sporadic pest, with localised outbreaks reported from vegetables and cotton. During this time, *B. tabaci* underwent several taxonomic revisions (Perring 2001). In a classic study by Bird (1957), the presence of two different 'host races' of *B. tabaci* were discovered on the island of Puerto Rico; a polyphagous *Sida* race found on a variety of plants and a monophagous *Jatropha* race (Bird *et al.* 1970). Similarly, in Brazil, it was noticed that populations of native *B. tabaci* were incapable of colonising cassava, in contrast to African populations (Costa & Russell 1975). Together with the sudden rise of *B. tabaci* as an agricultural pest, this work stimulated a much greater and broader interest in whitefly biology and systematics. It soon became clear that *B. tabaci* consists of several morphologically indistinguishable groups that vary in their biology, biochemistry and genetic composition. These groups were subsequently termed 'biotypes' and the number of biotypes reported grew rapidly. Based on its

ability to induce a physiological disorder in cucurbits known as squash silverleaf disorder, coupled with mating and crossing studies, Perring (1993) elevated the so-called B biotype to a separate species, *Bemisia argentifolli*. This taxonomic change received a mixed response, with many experts viewing it as premature. It is only recently, that we are starting to realise the extent of reproductive incompatibility and genetic diversity within *B. tabaci*, which is likely to trigger a more radical revision of taxonomic nomenclature in the near future (Dinsdale *et al.* 2010).

The 1980s saw an unprecedented increase in the frequency and severity of whitefly occurrence and damage in the Americas, Africa and central Asia. During this period, a number of non-native plant viruses emerged causing significant damage to cotton, cucurbits, lettuce, pepper and tomato (Brown & Bird 1992). This whitefly-associated virus epidemic was correlated with the geographical expansion of the B biotype (Bedford *et al.* 1994; Brown 2007). The B biotype first invaded Florida in the mid-1980s and subsequently displaced the indigenous *B. tabaci* biotype over a short period of time (Brown *et al.* 1995). Whitefly numbers are estimated to have risen 300-fold from the 1970s to 1980s (Wilser *et al.* 1998). In 1988, Israel experienced much higher numbers of whiteflies than previously (Horowitz *et al.* 2007) while, in Sudan, *B. tabaci* suddenly transformed from a secondary pest to a primary pest of cotton during the early 1980s (Dittrich *et al.* 1985). From Florida, the B biotype dispersed west across the US, and by 1991-1992 caused severe economic damage to the cotton, melon and vegetable industry in southwest Arizona, southern California and north-west Mexico (Ellsworth *et al.* 2001; Naranjo & Ellsworth 2009). The 1992 outbreak reduced cotton yield by 50% and the damage to lint wreaked havoc with market prices (Ellsworth *et al.* 2001). *B. tabaci* is commonly transported on plant material shipped over long distances. As one of the top hundred invasive species (Boykin *et al.* 2007; www.issg.org) strict restrictions are placed on any cargo which may potentially carry *B. tabaci*. Despite this, the past 15 years has seen the rise of a second invasive *B. tabaci* biotype known as the Q biotype. The speed and extent of the worldwide spread of the Q biotype has mirrored that of the B biotype and it is arguably regarded as even more threatening. The ascent of *B. tabaci* as a major pest is therefore due to a combination of factors including micro-evolutionary change, changing agronomic practices and increased monocultures, repeated insecticide use and depletion of natural enemies, and the globalisation of trade in plant produce.

1.2 *Integrated pest management*

Since the 1960s, agricultural production has matched population growth and this is largely due to the 'green revolution' brought about by plant breeding, coupled with dramatic increases in pesticide and fertiliser use. This period also saw a greater focus on the concept of Integrated Pest Management (IPM) which incorporates biological, cultural, physical and chemical means to control insect pests (Pretty *et al.* 2008). Insecticides are only used when necessary and, on the whole, yields are maintained or improved while providing ecological benefits and preventing the development of insecticide resistance.

Research into IPM strategies to counter *B. tabaci* outbreaks has accelerated since the pest became global and significant. During the mid-1990s, an IPM program was introduced and incorporated to control *B. tabaci* on Arizona cotton following the devastating whitefly outbreaks of 1991-1992 (Palumbo 2001). The strategy employs biological control, sound sampling, sensitive detection thresholds and selective insecticide spraying. Foliar insecticide treatment has been reduced by 70% and saved over $200 million in terms of *B. tabaci* control and the cost of cotton yield in Arizona (Naranjo & Ellsworth 2009). Successful examples such as this are, however, few and far between. IPM is logistically difficult to implement and when outbreaks are acute and severe, the most common and arguably most effective response is to spray chemical insecticides.

1.3 *Chemical control of insect pests*

The application of chemicals to control pests dates back centuries. Compounds showing insecticidal activity often occur naturally as exemplified by the pyrethrins. These compounds are found in the flowers of the pyrethrum daisy, *Tanacetum cinerariaefolium* and are thought to have been used in China as long ago as the 1st century AD (Davies *et al.* 2007; Glynne-Jones 2001). One major outcome of the 'green revolution' was the advent of large-scale commercial production of synthetic insecticides, commencing with the organochlorine DDT (dichloro-diphenyl-trichloroethane). The insecticidal properties of DDT were recognised by Paul Müller in 1939 and it soon became a 'magic bullet' for insect pests of agriculture and human health (Mellanby 1992). The World Health Organisation's (WHO) campaign for the

global eradication of malaria was based predominantly on the effectiveness of DDT against mosquito populations carrying the malaria parasite, *Plasmodium sp.* The campaign successfully eradicated malaria in Europe and the number of malaria cases in India and Africa dropped substantially. DDT soon received negative publicity due to its highly lipophilic nature, persistence in the environment and accumulation in fatty tissues of non-target species (Carson 1962; Mellanby 1992). At Rothamsted Research, Michael Elliott and colleagues pioneered the development of synthetic pyrethroids as photo-stable analogues of natural pyrethrins with high insecticidal activity, low mammalian toxicity and a vulnerability to environmental degradation (Elliot 1980). Pyrethroids became a highly successful class of insecticide for pests of agriculture and human health. Numerous other insecticide groups have been discovered and commercialised, despite a steady increase in cost and time of development within prevailing regulatory frameworks.

1.4 Insecticide resistance

The repeated use of an insecticide against any target insect inevitably leads to insecticide resistance. There are numerous definitions of insecticide resistance given in the literature, but by far the most useful and enlightened is provided by Denholm (1990): 'the development of insecticide resistance reflects a progressive increase of genes conferring biochemical or physiological traits that improve the ability of insects to withstand insecticide exposure'. Resistance to all major insecticides has been reported from a wide range of insect pests of medical and agricultural importance. There are over 500 resistant species of arthropod (Denholm *et al.* 2002) and this figure continues to increase. As Sawicki & Denholm (1984) point out, 'resistance represents a parallel evolutionary phenomenon in several extremely diverse taxonomic groups, and aside from its economic significance; it provides an ideal model for investigating how organisms can respond to large-scale exposure to xenobiotics'. In other words, insecticide resistance is of both academic and practical interest. It is widely known as perhaps the best example of organisms adapting in response to environmental stresses over a short period of time. Genes associated with resistance can be selected for and their impact on phenotype studied. Insecticide resistance varies in magnitude and distribution within and between insect taxa and both the biology of the insect and selection pressure exerted by the insecticide greatly influence how resistance manifests itself within the population.

Insecticide resistance has been studied extensively at the ecological, toxicological, physiological, and molecular levels. It is often a multi-factorial phenomenon involving several mechanisms that may interact additively, multiplicatively or in a more complex manner. In general, two major mechanisms attract the most attention; (i) target-site resistance and (ii) metabolic detoxification. These two types of mechanism are discussed briefly below:

1.5 Target-site resistance

Mutations in the genes encoding the insecticide target-protein (point mutations causing amino acid substitutions) confer conformational changes to the protein structure reducing/preventing the insecticide from acting/binding. This commonly occurs in trans-membrane proteins or in enzymes within the insect nervous system, to which the different types of insecticide target. In some cases, more than one mutation in the target protein can act concordantly together to give much stronger resistance. Target-site resistance often gives cross-resistance to compounds with the same mode of action but rarely between two insecticide classes.

1.6 Metabolic detoxification

The metabolic detoxification or sequestration of an insecticide occurs when there is an increase in the production or activity of specific enzyme families. In response to xenobiotic compounds entering the body of the insect, increased levels of enzyme break down or sequester the insecticide, thereby preventing it from reaching the target site. Enzyme levels are either up-regulated through gene expression (i.e. through modifications to the transcriptional machinery by transposable elements or changes in the sequence of the promoter region) or due to an increase in gene copy number. There are three large enzyme families involved in insecticide detoxification in addition to the plethora of metabolic functions they perform naturally; (i) P450-dependent monooxygenases, (ii) carboxylesterases and (iii) glutathione transferases (GSTs). These three gene families are discussed below:

1.7 P450-dependent monooxygenases

P450-dependent monooxygenases (also known as cytochrome P450s or mixed function oxidases) are one of the oldest and largest gene superfamilies. P450-enzymes play a diverse role in insects from the synthesis and degradation of development hormones to the detoxification of foreign chemicals such as insecticides (Feyereisen 1999). P450s are encoded by a large number of *CYP* genes, of which, between 46 and 143 have been recorded in individual insect species (Feyereisen 2006). This number can vary widely between closely related species. For example, the P450 gene family in the peach-potato aphid *Myzus persicae* is 40% larger than that in the pea-aphid *Acryrthosiphon pisum* (Ramsey *et al.* 2010). The dengue-transmitting mosquito *Aedes aegyptii* has 132 *CYP* genes compared with 106 in *Anopheles gambiae* (Feyereisen 2006). The largest groups of *CYP* genes in insects fall into the *CYP4* and *CYP6* subfamilies. Various individual *CYP* genes have become familiar targets for establishing the molecular basis of insecticide resistance. The involvement of P450s in insecticide resistance is often diagnosed *in vitro* with application of the P450-inhibitor, piperonyl-butoxide (PBO). Although correlations between P450 activity and reduced insect mortality are indicative of resistance, due to the multiplicity of P450 genes and their broad specificity, this does not necessarily provide a definitive causal link (Feyereisen 1999).

1.8 Carboxylesterases

The qualitative and/or quantitative change in carboxylesterases in response to insecticide exposure is a common resistance mechanism in many insects (Devonshire *et al.* 1998; Karunaratne 1994). The involvement of esterases in resistance has been well-known for some time with for example, the increased production of two esterases (E4 and FE4) responsible for broad resistance in *M. persicae* as a result of gene amplification (Devonshire & Moores 1982; Field *et al.* 1993). Inhibitors of esterases, such as *S,S,S*-tributyl phosphorothioate (DEF), can prolong the activity of certain insecticides by acting as synergists and therefore indicate the presence of resistance. Furthermore, esterase activity can be measured using model substrates (i.e. 1-napthyl acetate) in fluorometric assays (Devonshire *et al.* 1998).

1.9 Glutathione transferases (GSTs)

GSTs are a large family of multifunctional enzymes involved in the detoxification of a wide range of xenobiotic compounds including insecticides. They principally act by catalysing conjugation reactions with reduced glutathione resulting in more water-soluble and excretable metabolites. GSTs can also metabolise insecticides by catalysing a dehydrochlorination reaction. Individual GSTs have broad substrate specificity and this functional diversity is enhanced by gene duplications, post-transcriptional modification, alternative splicing and amino acid substitutions in insects (Ranson *et al.* 2002). GSTs have been associated with resistance to many insecticide classes including DDT and organophosphates (Ranson *et al.* 2001) but have yet to be shown to metabolise pyrethroids (Enayati *et al.* 2004). Vontas *et al.* (2001) did however show that elevated GSTs protect populations of the brown planthopper, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) from the oxidative stress caused by pyrethroids. Following the silencing of the Epsilon class of GSTs using RNA interference, *Ae. aegypti* became more susceptible to pyrethroids suggesting an as yet, undefined role for GSTs in pyrethroid resistance (Hilary Ranson *pers. comm.*). For a more detailed review of GSTs and insecticide resistance see Enayati *et al.* (2004).

1.10 Cuticular resistance

In addition to metabolic and target-site mechanisms, resistance can arise from reduced penetration of the insecticide through the cuticle. Reduced penetration of insecticide or the involvement of cuticular proteins in resistant insects has been demonstrated in the peach-potato aphid (*M. persicae*), malaria mosquito (*Anopheles gambiae*), the cotton bollworm (*Helicoverpa armigera*), red flour beetle (*Tribolium castaneum*) and German cockroach (*Blatella germanica*) (Ahmad *et al.* 2006; Puinean *et al.* 2010; Valles *et al.* 2000; Walter & Price 1989). The up-regulation of cuticular proteins is likely to be selected for by insecticides which act through direct contact or are applied as a spray/foliar treatment.

1.11 The detection and monitoring of insecticide resistance

Assessing the magnitude and frequency of insecticide resistance requires accurate techniques and much research is dedicated to standardising these for different insect species. Tests should be rapid, simple, sensitive and yield repeatable results to discriminate between temporal and geographical differences in toxicity against field populations (Denholm 1990). The most commonly used and well-established 'whole-organism' bioassay for whiteflies is the leaf-dip bioassay for adults and immature life-stages (Cahill *et al.* 1995), however, other methods do exist (Cahill & Hackett 1992; Prabhaker *et al.* 1996). Biochemical assays have been used for many years and often complement toxicological data. The majority of such assays assess the enzymatic activity of GSTs, esterases or P450s using a number of model substrates and fluorometric changes are recorded depending on the affinity of the enzyme for the substrate. Biochemical assays are also available to detect resistant genotypes in target-site enzymes such as acetylcholinesterase (AChE) in organophosphate- or carbamate-resistant insects (Byrne & Devonshire 1993).

The field of molecular biology has advanced rapidly in recent years. The number of genes discovered which have direct or indirect associations with insecticide resistance has multiplied rapidly and these advances have arguably provided the most significant progress in understanding insecticide resistance. From a practical perspective, molecular techniques offer an advantage over biochemical and toxicological methods of resistance detection, as they can detect and track the presence of single resistance alleles within insect populations (Denholm *et al.* 1990). Historically, discerning the genotype of resistant insects had to be done using discriminating concentrations of insecticide in bioassays (e.g. dieldrin resistance in houseflies; Sawicki & Farnham 1968). The inability to detect heterozygotes is a considerable handicap since this genotype is a key determinant of selection rates, particularly when resistance genes are rare. The discovery of several target-site mutations and SNPs associated with insecticide resistance has led to the development of rapid DNA-based diagnostics which can potentially pre-empt the emergence of insecticide resistance before operational problems are experienced in the field or greenhouse.

1.12 Insecticide resistance management

As mentioned previously, insecticide resistance management (IRM) is essential to maintain the effectiveness and life-span of insecticides. This is particularly pertinent as new compounds with unique modes of action are costly and time-consuming to develop. IRM is based fundamentally on the premise that resistance in an insect to a specific insecticide can be avoided or delayed by alternating insecticides with different modes of action. A starting point for IRM is the assumption that resistance to the selecting insecticide will also render compounds with a similar mode of action ineffective, so insecticides with a contrasting mode of action must be alternated. However, this assumption must be treated with care as some target-site mutations (i.e. MACE in *M. persicae*) and many metabolic enzymes are capable of broad substrate specificity. IRM is a proactive approach designed to prevent the build-up of resistance alleles. Sawicki & Denholm (1987) describe the principle aims of IRM as, '(to) conserve susceptibility through strategies aimed at either overcoming resistance to currently used compounds or preventing the development of resistance to existing or new pesticides'. Forming an effective IRM strategy is not straightforward as resistance is complex and only certain factors such as spray application, timing and dose can be controlled. There are a handful of 'general' tactics and theoretical models central to the concept of IRM and these are reviewed in detail elsewhere (see Curtis 1985; Denholm & Rowland 1992; Roush 1989). In summary;

a. Insecticides with different modes of action can be mixed in the same formulation. This strategy is designed to overpower insects carrying resistance alleles.
b. Insecticides can be alternated on a temporal basis. This is based on the assumption that frequencies of resistance alleles to each compound decline in the absence of the selecting insecticide. This tactic primarily preserves susceptible genotypes.
c. Insecticides can be applied as a spatial mosaic in which adjacent areas are treated with different chemicals. Few successful examples of this strategy exist and it is largely ignored as an IRM tactic today.

As the major investors in research and development for new insecticides, the agrochemical industry has taken a strong and proactive approach to IRM. The

industry's global Insecticide Resistance Action Committee (IRAC) was formed in 1984 to co-ordinate actions of individual companies and to work with the public sector on the design and implementation of management strategies (http://www.irac-online.org/). IRAC classifies compounds based on their mode of action (MoA) and suggests which sequence or alteration of MoA should be used depending on the pest.

1.13 Insecticides for whitefly control

Insecticides remain the primary method for whitefly control. There are currently twelve classes of insecticide registered for use against whiteflies (http://www.irac-online.org/), although availability varies from country to country. These groups either target the nervous or muscular system, or interfere with life-stage development or respiration. Organochlorines, such as DDT, dieldrin and endosulfan, were widely used against whiteflies during the 1970s and 1980s and repeated use quickly led to resistance (Dittrich *et al.* 1990). Moreover, due to concerns with their persistence, only endosulfan remains registered for whitefly control.

Table 1.1 Insecticide mode of action groups available for whitefly control

Mode of action (MoA)	Insecticide class
Nerve and muscle targets	
Acetylcholinesterase (AChE) inhibitors	Organophosphates Carbamates
GABA-gated chloride channel inhibitors	Cyclodiene organochlorines (e.g. Endosulfan)
Sodium channel modulators	Pyrethroids/pyrethrins
Nicotinic acetylcholine receptor (nAChR) agonists	Neonicotinoids
Selective Homopteran feeding blockers	Pymetrozine
Growth and development targets	
Juvenile hormone mimics	Pyriproxyfen
Inhibitor of chitin biosynthesis (Type 0)	Benzoylureas
Inhibitor of chitin biosynthesis (Type 1)	Buprofezin
Inhibitor of lipid synthesis	Tetronic acids (e.g. Spiromesifen, Spirotetramat)
Respiratory targets	
Inhibitors of mitochondrial ATP synthase	e.g. Diafenthiuron
Mitochondrial complex I electron transport inhibitors	e.g. Tolfenpyrad, Pyridaben

*Table modified from IRAC poster 'Aphids, Whiteflies and Hoppers – Insecticide Mode of Action Classification' (IRAC 2010).

The pyrethroids, organophosphates and carbamates (all neurotoxins) were progressively introduced as whitefly outbreaks became more difficult to manage (Dittrich *et al.* 1990; Horowitz & Ishaaya 1996). This inevitably led to the emergence of resistance in many areas. A small number of more novel classes of insecticide have been registered for whitefly control over the past 20 years. These include the neonicotinoids, insect growth regulators (IGRs), diafenthiuron and feeding blockers (i.e. pymetrozine) and are often either used in rotation with each other or along with more conventional compounds to maintain their effectiveness as part of IRM strategies. The mode of action, historical application and summary of resistance in *B. tabaci* to some of the key insecticides widely used for *B. tabaci* are discussed in turn below;

1.14 Organophosphates & carbamates

Organophosphates and carbamates inhibit acetylcholinesterase (AChE) responsible for catalysing the hydrolysis of the neurotransmitter acetylcholine in insect synapses (Fournier & Mutero 1994). OPs and carbamates are analogues to acetylcholine in that they enter and bind to the active site of AChE, however they are chemically different to acetylcholine and act by inactivating the enzyme through phosphorylation (OPs) or carbamylation (carbamates) of a serine residue in the catalytic centre that results in repetitive firing of the postsynaptic nerve. The repeated use of OPs and carbamates for controlling *B. tabaci* has led to resistance in many areas (Cahill *et al.* 1995; Denholm *et al.* 1996; Dittrich *et al.* 1990; Roditakis *et al.* 2009).

Insensitive AChE and increased levels of esterases have both been associated with OP and carbamate resistance (Byrne & Devonshire 1993; Denholm *et al.* 1998; Dittrich *et al.* 1990). The *ace-2* gene encodes the main synaptic AChE in Dipteran flies while *ace-1* encodes this enzyme in other insects (Fournier 2005). There are several mutations present in *ace* genes which are associated with OP or carbamate insensitivity (Fournier 2005; Weill *et al.* 2002). Most mutations confer a similar pattern of resistance however G119S, in two sub-species of mosquitoes, and S331F are associated with higher levels of resistance towards carbamates than OPs (Russell *et al.* 2004). Recently, Alon *et al.* (2008) isolated full-length sequences of *ace1* and *ace2* from OP-resistant and susceptible strains of the B biotype of *B. tabaci*. The *ace1*

gene contained the amino acid change F331W in the resistant phenotype. Furthermore, two carboxylesterase genes (*coe1* and *coe2*) were isolated and *coe1* was shown to be overexpressed in the OP-resistant strain (Alon *et al.* 2008). The results suggest that both target-site and metabolic mechanisms of OP resistance occur in the *B. tabaci* B biotype.

1.15 Pyrethroids

The voltage-gated sodium channel is the principal target for DDT and pyrethroids. Pyrethroids are the synthetic analogues of the insecticidal esters (pyrethrins) found in the flowers of *Tanacetum cinerariaefolium* (Davies *et al.* 2007; Elliot 1980). They are generally grouped into two forms based on the presence (type II) or absence (type I) of an α-cyano group. In general, type I pyrethroids (i.e. permethrin) are fast-acting due to their ability to provide a sub-lethal but debilitating effect known as 'knockdown'. Type-II compounds (i.e. deltamethrin) give greater mortality at lower concentrations but do not induce a knockdown as quickly as type-I.

Pyrethroid resistance in *B. tabaci* is widespread but the pattern and magnitude varies (Cahill *et al.* 1995; Cahill *et al.* 1996; Denholm *et al.* 1996; Dittirch *et al.* 1990). Resistance is often due to a combination of increased esterase/P450 activity and the presence of target-site mutations within the sodium channel (Alon *et al.* 2006; Byrne *et al.* 2000; Dittrich *et al.* 1990; Luo *et al.* 2010; Morin *et al.* 2002). Resistance to pyrethroids in *B. tabaci* is discussed in greater detail in Chapter 4.

1.16 Neonicotinoids

Neonicotinoid insecticides, like naturally occurring nicotine, act as agonists of post-synaptic nicotinic acetylcholine receptors (nAChRs) (Jeschke & Nauen 2008). There are seven commercially available neonicotinoids and these can be grouped according to whether they possess an open-chain or a 5/6 ring structure (open chain compounds = acetamiprid, nitenpyram, clothianidin and dinotefuran; 5/6 ring compounds = imidacloprid, thiamethoxam and thiacloprid). Imidacloprid (Bayer CropScience) was the 'forerunner' neonicotinoid and is perhaps the most successful insecticide in the history of crop protection. The success of this insecticide was largely due to its versatility, broad target-spectrum and the lack of resistance.

Furthermore, some of the more recent neonicotinoids possess a comparable efficacy with imidacloprid and in some cases, provide a different spectrum of activity that increases the flexibility of the group as a whole.

Neonicotinoids have been incorporated into a wide range of IPM programmes to control *B. tabaci* in field and protected crops (Horowitz *et al.* 2007; Palumbo 2001). Despite their widespread use, resistance to neonicotinoids took some time to develop in insects (Nauen & Denholm 2005). Unlike other insecticide classes, such as the pyrethroids, neonicotinoids lack the ester bond cleaved by esterases. This is perhaps the why resistance has taken so long to develop in insects such as aphids, for which elevated esterases have been a significant cause of resistance to other types of insecticide chemistry. *B. tabaci* was the first species to tolerate field-level doses of neonicotinoids and over the past 15 years, resistance has quickly spread within and across biotypes (Karunker *et al.* 2009; Nauen & Denholm 2005). As a result, *B. tabaci* became the primary species to investigate the biochemical and molecular mechanisms of neonicotinoids (and in particular imidacloprid) resistance. This is discussed in further detail in Chapter 5.

1.17 Selective feeding blockers

Pymetrozine affects the nerve controlling the salivary pumps of sucking pests and is effective against whiteflies, aphids and planthoppers (Harrewijn & Kayser 1997; Ishaaya *et al.* 2007; Polston & Sherwood 2003). The anti-feedant property of this insecticide causes starvation and eventually death. Resistance to pymetrozine has been reported from adult *B. tabaci* populations in southern Spain (Elbert & Nauen 2000). In reciprocal selection experiments, resistance to pymetrozine was shown to be due to the same mechanism as resistance to neonicotinoids (Gorman *et al.* 2010), which has implications for the deployment of these insecticides in IRM strategies.

1.18 Insect growth regulators

The insect growth regulators (IGRs) for whitefly control can be generally split into two classes; a) inhibitors of chitin biosynthesis (e.g. buprofezin) or b) a juvenile hormone mimic (i.e. pyriproxyfen). The need for a greater diversity of insecticide chemistry following the outbreaks of *B. tabaci* during the 1980s and early 1990s,

together with the emergence of resistance was countered in part by changing to IGRs (Horowitz & Ishaaya 1996). The IGRs provided a unique mode of action and desirable environmental profile and, in addition, offered an alternative group of compounds to use in conjunction with the neonicotinoids as part of IRM programmes.

The juvenile hormone mimic pyriproxyfen has been used for *B. tabaci* control since 1991 and is one of the main agents for controlling *B. tabaci* in IRM programmes on cotton fields in Israel and from 1996 in the southwestern USA (Horowitz & Ishaaya 1996; Dennehy & Williams 1997). Pyriproxyfen affects all preimaginal stages of whiteflies, influencing embryogenesis, metamorphosis and adult formation. It acts by inhibiting whitefly egg hatch (ovicidal), either directly or transovarially, and by suppressing adult emergence, reducing the number of nymphs due to pupal (N4) mortality (Ishaaya & Horowitz 1992). The vapour phase and translaminar activity of pyriproxyfen enhances the suppression of embryogenesis as the mortality is not necessarily based on contact. Strong resistance to pyriproxyfen has been observed after successive treatments (Horowitz *et al.* 1994). In Israel, where only a single application per season is recommended, susceptibility has been maintained in some regions but in other geographically isolated populations, resistance gradually led to disuse of the compound (Horowitz *et al.* 2003; Horowitz *et al.* 2005). Crosses between a pyriproxyfen susceptible and resistant strain of *B. tabaci* revealed that resistance is partially dominant when bioassaying the F_1 heterozygous females (Horowitz *et al.* 2003). This level of dominance was sufficient for heterozygous females to survive field exposure (Horowitz *et al.* 2003).

Pyriproxyfen is often used in rotation with buprofezin. Buprofezin is a thiadiazine inhibitor of chitin synthesis in a number of hemipteran pests (Ishaaya *et al.* 1988). The incorporation of N-acetyl-[D-H^3] glucosamine into chitin is inhibited, which subsequently interferes with cuticle formation (Kanno *et al.* 1981) although the mode of action is not completely understood. Buprofezin has no direct effect on adult longevity or oviposition, acting through inhalation by nymphs (Ishaaya *et al.* 1988). Buprofezin is a major compound in areas where resistance to more conventional insecticides is present (Horowitz *et al.* 2007). Resistance has been reported from cotton (Dennehy *et al.* 1999; Horowitz *et al.* 1999) and in glasshouses from geographically widespread regions (Cahill *et al.* 1996; Elbert & Nauen 2000;

Horowitz *et al.* 1994). Toscano *et al.* (2001) reported increased susceptibility from populations from California and Arizona.

An additional chitin synthesis inhibitor and commonly used IGR is the benzoylphenyl urea novaluron. Novaluron is most toxic to whitefly crawlers (N1) and 2nd instar nymphs (N2) acting by both ingestion and contact (Ishaaya *et al.* 1996; Ishaaya *et al.* 2007). There is no cross resistance between novaluron and the neonicotinoids (Ishaaya *et al.* 2003) and therefore novaluron is considered an important component of IRM programmes (Ishaaya *et al.* 2007).

1.19 Other insecticides for whitefly control

Diafenthiuron, an inhibitor of oxidative phosphorylation and mitochondrial ATP synthesis (Ruder & Kayser 1993), is used as an alternative to pyriproxyfen when levels of resistance reach unacceptable levels (Ishaaya *et al.* 2007). The tetronic and tetramic acid derivatives (i.e. spirotetramat and spiromesifen), inhibit the lipid synthesising enzyme acetyl Coenzyme A carboxylase. These have shown good efficacy against both B and Q biotypes (Grávalos *et al.* 2009; Prabhaker *et al.* 2009) and demonstrated no cross resistance to imidacloprid-, bifenthrin- and pyriproxyfen-resistant *B. tabaci* (Prabhaker *et al.* 2009). Together with the recent development and current commercialisation of Cyazypyr™, an anthranilic diamide which activates ryanodine receptors in insect muscles (Schuster *et al.* 2009), these chemical insecticide classes with novel modes of action will be an important component of future IRM programmes (Nauen 2009).

Biologically-derived compounds are gradually being incorporated for control of *B. tabaci* (Horowitz *et al.* 2007). Fungal-based products (mycoinsecticides) provide some control of whiteflies in greenhouses and field crops but they are slow to act, have poor adulticidal activity, a high cost, a limited shelf-life and depend on favourable environmental conditions (Faria & Wraight 2001). Fermentation components of the soil actinomycete *Streptomyces avermitilis* (i.e. abamectin) are effective against *B. tabaci* (Horowitz & Ishaaya 2004; Luo *et al.* 2010; Wang *et al.* 2007) but are vulnerable to resistance and should be used in rotation with other effective insecticides.

1.20 Insecticide resistance management in B. tabaci

In *B. tabaci*, there are two well-cited examples of IRM programmes established to tackle this pest. These come from the cotton industry in Israel and from southern USA. Following the emergence of resistance in *B. tabaci* to OPs and pyrethroids during the 1980s, IRM strategies were devised to restrict the use of these conventional insecticides and introduce compounds with different modes of action such as the IGRs (i.e. buprofezin and pyriproxyfen), neonicotinoids and diafenthiuron. In Israel, insecticides with alternate chemistries were used in rotation and allocated for use in distinct periods of the growing season (Ishaaya & Horowitz 1995). The strategy was highly successful between 1988 and 1996 although pyriproxyfen had to be replaced with neonicotinoids (acetamiprid and imidacloprid) in 1997 due to high levels of pyriproxyfen resistance (Horowitz *et al.* 2007). The strategy has reduced the level of insecticide use and delayed the onset of resistance in Israeli cotton. Resistance levels have however increased during the 20 years since inception (Horowitz *et al.* 2007) demonstrating that although IRM strategies can delay the onset of resistance, they are not necessarily 'resistance-proof'. During the early 1990s, there were fears over the survival of the cotton industry in Arizona following the whitefly outbreaks (Naranjo & Ellsworth 2009). The continued use of broad spectrum insecticides led to a comprehensive search for suitable alternatives. In 1994, a synergistic formulation of the pyrethroid fenpropathrin with the OP acephate provided effective control of *B. tabaci* in Arizona (Denholm *et al.* 1998). Acephate is an esterase inhibitor and therefore restored the efficacy of pyrethroids even though the use of either compound alone would offer little or no protection. Resistance however soon followed and, following the example of Israel, a unique emergency application was made for the registration of the IGRs, buprofezin and pyriproxyfen, for use on cotton (Denholm *et al.* 1998). The current day chemical arsenal for *B. tabaci* control includes the IGRs along with acetamiprid (neonicotinoid) and spiromesifen and these are integrated in an IRM strategy as part of a larger IPM program which has successfully turned *B. tabaci* into a manageable pest in this area (Naranjo & Ellsworth 2009).

1.21 The molecular basis of insecticide resistance

At first, much of our knowledge on the molecular basis of resistance stemmed from those genes encoding the insecticide target-site. These were primarily identified and cloned in the fruitfly *Drosophila melanogaster* or the housefly *Musca domestica* (Loughney *et al.* 1989; Williamson *et al.* 1993) and focussed mainly on the voltage-gated sodium channel (target of DDT and pyrethroids), acetylcholinesterase (target of OPs and carbamates) or the γ-amino butyric acid (GABA)-gated ion channel (target for cyclodienes) (ffrench-Constant *et al.* 1993; Soderlund *et al.* 2003; Weill *et al.* 2002). Point mutations, gene amplifications/duplications, splice variants, transcriptional changes have all been characterised from various insects resistant to a spectrum of insecticides. As a result, the spread and origin of resistance genes, their response under selection pressure and their impact on phenotype have been widely investigated. For instance, it is now known that some target-site mutations or gene amplifications arise independently in taxonomically isolated insects at the level of subspecies, haplotype, or biotype (Alon *et al.* 2006; Andreev *et al.* 1999; Anstead *et al.* 2004). On the other hand, other such resistance-associated mutations have only a single origin (Field *et al.* 1994; Raymond *et al.* 1991). The spread of resistance genes is heavily influenced by mutation rate, selection and migration of the insect (ffrench-Constant *et al.* 2004). In the latter, insect biology can have a significant impact as is the case for example, in *Tribolium castaneum* or *B. tabaci* as resistance alleles spread across the world through the trade of grain or plants respectively (Andreev *et al.* 1999; Anthony *et al.* 1995). It is often stated that studies investigating the spread of resistance alleles are of paramount importance when devising insecticide resistance management strategies (ffrench-Constant *et* al. 2004) but there appear to be few examples when such information is translated into practical insecticide resistance management.

The molecular basis of metabolic resistance is more complex due to the large number of genes within the three main detoxification enzyme families (P450s, carboxylesterases, GSTs) and in many instances, the overlapping substrate specificity each enzyme has for the same compound. For example, some P450 enzymes show specificity for either type I or type II pyrethroids (Scollon *et al.* 2009). Many *CYP* genes are over-expressed in response to insecticide selection and in some cases the same gene is up-regulated in response to different insecticides (Daborn *et al.* 2001).

The insertion of transposable elements and the over transcription via gene regulation and/or gene amplification also play active roles in increasing enzyme activity (Daborn *et al.* 2002). The improvement in gene expression platforms (e.g. microarray) allows gene expression levels to be compared between resistant and susceptible populations of insects. In *D. melanogaster*, a microarray slide containing all known P450s revealed over-transcription of *CYP6G1* in DDT-resistant strains leading to further characterisation of this enzyme (Daborn *et al.* 2002). The *Anopheles* 'detox chip' contains approximately 290 probes specific for known detoxification genes (David *et al.* 2006) implicating several *CYP* genes in resistance. Larger genomic arrays contain up to 44,000 cDNA probes allowing a broader look at the genes up or down regulated in resistant insects with, for example, the discovery that cuticular genes play a key role in neonicotinoid resistance in *M. persicae* (Puinean *et al.* 2010). Following the sequencing of the *D. melanogaster* and *An. gambiae* genomes, the advent of next generation sequencing technology led to the publication of the pea-aphid *Acyrthosiphon pisum* (pea-aphid) (International Aphid Genomics Consortium, 2010). Researchers now have the necessary tools to pick the most likely candidate genes linked to resistance. In *B. tabaci*, this has already been achieved in neonicotinoid resistant populations (Karunker *et al.* 2009; Puinean *et al.* 2010). However, despite the progress made in identifying some key enzymes involved in metabolic resistance, no simple, high-throughput and cheap diagnostic-based assays yet exist to inform IRM programmes.

1.22 Summary of the project

Insecticide resistance is a multi-faceted and varied topic. The biology, invasiveness and genetic diversity of *B. tabaci* enhances this complexity when studying and attempting to manage insecticide resistance in this species. The work presented in this thesis covers a number of areas relevant to understanding the origins, spread and management of insecticide resistance. Chapter 2 describes the general materials and methods used throughout all experiments. Chapter 3 details the development of a DNA-based diagnostic for the two most economically important and threatening biotypes. In Chapter 4, the origin, spread and nature of two target-site mutations present in the whitefly sodium channel and association with pyrethroid resistance is investigated. Finally, in Chapter 5, the correlation of a P450 encoding gene, *CYP6CM1*, with imidacloprid is reported. Each of these areas of research provides

new information and it is hoped that the results presented will advance our understanding of insecticide resistance and consequently inform studies relevant to insecticide resistance management.

Chapter 2 General materials and methods

A general description of the materials and methods used in more than one chapter are outlined below. Those methods more specific to an area of research are described separately in the relevant chapter.

2.1 Insect rearing

All live *B. tabaci* strains used in experiments were reared at Rothamsted Research on cotton plants (*Gossypium hirsutum* cv. Linda) at 28 ± 2°C under a 16 hour photoperiod.

2.2 Leaf-dip bioassay for insecticide resistance

Insecticide resistance in adult whiteflies was measured using an established leaf-dip bioassay (Cahill *et al.* 1995). Leaf discs (40mm wide) were cut from intact cotton (*Gossypium hirsutum* cv. Linda) leaves and immersed for 10 seconds in solutions of insecticide diluted to the required concentration with distilled water containing 0.01% Agral (Syngenta, UK). Control leaf discs were dipped in the dilutant only. Leaf discs were placed adaxial surface down onto a bed of agar (2%) held in a small, plastic Petri dish and allowed to dry. Adult whiteflies (mixed) were aspirated onto each leaf and the dish closed using a plastic lid. All concentrations were assayed in triplicate with approximately 20 adults placed in each dish. Bioassays were incubated at 28°C and mortality scored after 48 hours for DDT and pyrethroids and after 72 hours for neonicotinoids. Individuals were scored as dead if absolutely no movement was observed from the insect.

Dose-response data were subject to probit analysis using PoloPlus© statistical software (LeOra Software, Berkeley, CA). Data are firstly corrected for control mortalities, percentage mortalities are then converted to a probit scale and median (LC_{50}) values estimated.

2.3 Genomic DNA Extraction

Individual whiteflies were ground in 1.5 ml sterile Eppendorf tubes using a liquid nitrogen-chilled blue homogeniser. Genomic DNA (gDNA) was extracted following homogenisation in 200 µl of DNAzol® (Invitrogen UK) at a fifth scale of the supplier's recommended protocol. The DNA was re-suspended in either 20 µl of Tris-EDTA buffer (10 mM Tris-HCl pH8, 0.1 mM EDTA) or 10-20 µl of sterile distilled water, depending upon the quantity required.

2.4 Total RNA Extraction

Live insects (adults or immature stages) were collected in sterile 1.5 ml eppendorf tubes and frozen in liquid nitrogen. Total RNA was extracted from whiteflies using TRIzol® reagent (Invitrogen UK) at between one-half and one-tenth scale of the suppliers recommended protocol, depending on the starting material. RNA was re-suspended in 15-30 µl of diethylprocarbonate (DEPC)-treated water. All RNA samples were quantified using the ND-1000 spectrophotometer (NanoDrop Technologies Inc.). RNA integrity was checked using agarose gel electrophoresis as described in section 2.6 of this chapter. 1 µl of RNA was mixed with 4 µl of RNA-loading dye solution (95% formamide, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.025% ethidium bromide, 0.5 mM EDTA; Fermentas Germany) and 3 µl of sterile distilled water, heated to 75°C for 5 minutes and quickly chilled on ice prior to loading. This allowed the 18S and 28S subunit structures to be clearly seen under UV light.

2.5 Standard PCR Protocol

All PCR reactions were done in 0.2 ml sterile plastic tubes using a thermal cycler with a hot lid (Genius, Techne; GeneAmp PCR System 2700, Applied Biosystems). gDNA (1-3 µl) was mixed with 12.5 µl of Hot Start Taq Polymerase which includes both PCR buffer and dNTPs (Promega, Madison, WI, USA), 1 µl of forward and reverse primer (100 µM) and a 25 µl total volume was made up with sterile distilled water. Temperature cycling conditions were 95°C for 2 minutes followed by 25-30 cycles of 95°C for 30 seconds (denaturation), 50°C for 30 seconds (annealing) and 72°C for 60 seconds (extension) unless otherwise stated. A blank negative control of 1 µl sterile

distilled water was used in all reactions (i.e. replacing the DNA suspension with SDW and maintaining all other PCR conditions).

2.6 Agarose gel electrophoresis

Agarose gels were made from heating molecular biology grade agarose (Helena Biosciences Europe) in 100 ml of 1x TBE buffer (0.89 M Tris Borate pH8.3 and 20 mM Na_2EDTA) to make 1.2-1.4% gels. The agarose was left to set in trays containing combs to create wells at the top of the gel. PCR products (5 µl) were mixed with 2 µl of 6x loading-dye (10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA; Fermentas, Germany), loaded into the wells within an electrophoresis tank filled with 1x TBE buffer and run at 80V for between 1 and 2 hours. A GeneRuler™ (Fermentas, Germany) 1kb DNA ladder (5 µl) was loaded into two flanking wells to ascertain DNA band size. Gels were post-stained in ethidium bromide solution (0.5 µl/ml) for 20 minutes at room temperature and de-stained for 20 minutes in distilled water. DNA fragments were visualised on the Gene Genius Bio Imaging System (Syngene, Cambridge, UK) and analysed using GeneSnap (Syngene).

2.7 Purification of PCR products

Prior to DNA sequencing, the PCR products were purified by ethanol precipitation. PCR products (20 µl) were mixed with an equal amount of 5M ammonium acetate (NH_4OAC) and 2 volumes of 100% ethanol, left at room temperature for 15 minutes and centrifuged for 20 minutes at 13,200 rpm. The supernatant was removed, and the pellet washed with 180 µl of 75% ethanol and centrifuged for 5 minutes. The pellet was air-dried in a speed-vac for 2 minutes and the DNA re-suspended in 15 µl of sterile distilled water.

2.8 DNA Sequencing

All sequencing reactions were performed in 0.2 ml sterile plastic tubes on a thermal cycler (GeneAmp PCR System 2700; Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit™ (Applied Biosystems). Purified PCR products (2-4µl) were mixed with 4 µl BigDye Terminator v1.1 reaction mix, 4 µl BigDye Terminator 5x

Buffer, 1 µl of a gene specific oligonucleotide and sterile distilled water to make up a 20 µl total reaction volume. Thermal cycling conditions were 96°C for 1 minute followed by 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 60°C for 3 minutes 50 seconds. Sequencing reactions (20 µl) were mixed with one-third volume of 3M NH_4OAC and 8-volumes of 100% ethanol, left at room temperature and centrifuged at maximum speed for 20 minutes. The pellet was washed with 75% ethanol and air dried for 2 minutes. This removed unincorporated dye terminators before sequencing. All samples were sent as dry pellets to GeneService (Dept. of Biochemistry, University of Oxford) for sequencing.

2.9 cDNA synthesis

First strand cDNA was synthesised from total RNA using Superscript II Reverse Transcriptase (Invitrogen UK). In each reaction, RNA was added to 1 µl oligo$(dT)_{20}$ (500 µl/ml) or a gene specific primer, 1 µl dNTP Mix (10 Mm) and sterile distilled water to make up a 12 µl volume. The quantity of RNA added to the reaction varied between samples in order to standardise the resultant amount of cDNA synthesised. The mix was heated to 65°C for 5 minutes to denature the RNA and quickly chilled on ice. 4 µl of 5X First-Strand Buffer, 2 µl 0.1 M DTT and 1 µl RNaseOUT® (Invitrogen UK) were added to the mix, gently vortexed, and incubated at 42°C for 2 minutes. RNaseOUT ® protects mRNA from RNase enzymes and improves cDNA yield. Finally, 1 µl of Superscript II RT enzyme was added and the final reaction volume incubated at 42°C for 50 minutes in a thermal cycler (Genius, Techne; GeneAmp PCR System 2700, Applied Biosystems). The reaction was inactivated by heating at 70°C for 15 minutes.

2.10 TaqMan® PCR

TaqMan® is the term describing real-time PCR chemistry which uses fluorescent dye-labelled oligonucleotide probes to quantify the PCR product. Each probe contains a reporter and a quencher dye and is designed to anneal to a specific sequence between unlabelled forward and reverse primers. Cleavage of the probe by the 5′ nuclease activity of Taq Polymerase results in emission of fluorescence by the reporter dye, allowing the quantity of the PCR product to be monitored in real-time. Allele-specific probes, with different reporter dyes attached to the 5′ end, emit

fluorescence at various wavelengths. The technique allows the detection of single nucleotide polymorphisms.

Each PCR reaction was composed of gDNA (1-5 µl), SensiMix DNA kit (12.5 µl; Quantace Ltd, Neutral Bay, Australia), 900 nM of each primer and 200 nM of each probe, and the total volume (25 µl) was made up with sterile water. Real-time PCR was performed on a Rotor-Gene 6000™ (Corbett Research) using temperature cycling conditions of 10 min at 95°C followed by 40 cycles of 95°C for 10 s and 60°C for 45s. The increase in VIC and FAM reporter dyes was monitored in real time using the Rotor-Gene 6000™ software.

Chapter 3 A high throughput molecular diagnostic assay for discriminating B and Q biotypes

3.1 Introduction

The taxonomic status of *B. tabaci* is complicated and remains unresolved. This is due to the existence of numerous morphologically identical populations that differ in host-plant and geographical ranges and capacity to vector plant viruses (Bedford *et al.* 1994; Markham *et al.* 1994). Differences in these traits, coupled with a number of biochemical and DNA-based markers, have led to *B. tabaci* being regarded as a species complex composed of several biotypes (reviewed by Perring 2001) which were originally characterised on the basis of differences in non-specific esterase binding profiles from polyacrylamide gel electrophoresis (PAGE) (Brown *et al.* 1995; Byrne & Devonshire 1993; Costa & Brown 1991). Maxwell (1980) defines biotype as "an individual or population that is distinguished from the rest of its species by criteria other than morphology". Other insects that have evolved cryptic sibling populations include the malaria mosquito, *Anopheles gambiae* (Coetzee *et al.* 2000), the brown planthopper, *Nilaparvata lugens* (Claridge & Denhollander 1983), and blackflies of the genus *Simulium sp.* (Xiong & Kocher 1991).

There is increasing evidence to suggest that at least some biotypes of *B. tabaci* should be elevated to species status. Sequencing of DNA markers such as the mitochondrial cytochrome oxidase I (mtCOI) gene and the internal ribosomal spacer I (ITS-1) has greatly enhanced our understanding of the genetic framework underlying *B. tabaci* (Berry *et al.* 2004; Boykin *et al.* 2007; De Barro *et al.* 2000; De la Rua *et al.* 2000; Frohlich *et al.* 1999; Ueda *et al.* 2009). In one comprehensive phylogenetic analysis, Boykin *et al.* (2007) demonstrated high genetic diversity between geographically distinct samples and re-classified biotypes of *B. tabaci* into twelve 'major genetic races'. Microsatellite analysis suggests that gene flow is limited between some of these genetic races (De Barro 2005) and this is supported by the existence of pre- and post-zygotic barriers resulting in reproductive incompatibility (Bedford *et al.* 1994; Crowder *et al.* 2010; De Barro & Hart 2000; Liu *et al.* 2007; Maruthi *et al.* 2004). Dinsdale *et al.* (2010) have subsequently proposed that there are 24 distinct species of *B.tabaci* based on mtCOI sequence divergence. Although

the support for *B. tabaci* being named a cryptic species complex composed of morphologically identical species is increasingly strong, for convenience and in accordance with the literature, the term biotype is used here to be consistent with the bulk of previous literature.

The upsurge in whitefly outbreaks in the Americas during the 1980s was subsequently associated with insects that exhibited a different esterase electromorph pattern from that of the indigenous American A biotype (Costa & Brown 1991). This was named the B biotype (also described as *B. argentifolii* Bellows & Perring (Bellows *et al.* 1994)) and is thought to originate from the eastern Mediterranean basin and/or Asia Minor (Boykin *et al.* 2007; De Barro *et al.* 2000). The B biotype is now globally widespread and a highly significant crop pest, displaying high fecundity, broad host range and strong resistance to insecticides compared with the majority of other biotypes.

The Q biotype, which was first reported in the Iberian Peninsula (Guirao *et al.* 1997), is now also a global pest with reports in many areas around the Mediterranean and the Middle East (Dalmon *et al.* 2008; De Barro *et al.* 2000; Horowitz *et al.* 2003; Simon *et al.* 2003; Zanic *et al.* 2005), the United States (Dennehy *et al.* 2005), Central America (Bethke *et al.* 2009; Martinez-Carrillo & Brown 2007), New Zealand (Scott *et al.* 2007) as well as parts of China (Chu *et al.* 2006; Hsieh *et al.* 2007; Lee *et al.* 2008; Ueda & Brown 2006; Zhang *et al.* 2005). The rapid spread of B and Q biotypes to new countries and continents is thought to be a consequence of the expanding global trade in ornamental plant material. The spread of the Q biotype is of particular concern as it characteristically displays even stronger and broader resistance to the spectrum of insecticides available for whitefly control than biotype B (Horowitz *et al.* 2005; Nauen & Denholm 2005; Roditakis *et al.* 2009).

A number of DNA-based techniques have been designed to complement or improve upon biochemical methods for diagnosing biotypes (Bosco *et al.* 2006; Cervera *et al.* 2000; Guirao *et al.* 1997; Khasdan *et al.* 2005; Lima *et al.* 2002; Moya *et al.* 2001). These techniques can be applied to insects air-dried or stored in ethanol, but often require either post-amplification, enzymatic or hybridisation procedures which can be time-consuming and labour-intensive. Direct sequencing of the mtCOI gene is

currently the most common method for diagnosing the biotype status of whitefly populations (Brown & Idris 2005; De la Rua *et al.* 2006; Shatters *et al.* 2009) but this requires multiple processing steps and good quality DNA and is relatively costly.

Polymerase chain reaction (PCR)-based allelic discrimination assays which use fluorescent dye-labelled probes, provide a fast and reliable way for detecting single nucleotide polymorphisms (SNPs). These have been used, for example, to detect mutations associated with insecticide resistance (Anstead *et al.* 2004; Anstead *et al.* 2008; Bass *et al.* 2007; Daborn *et al.* 2004; Morgan *et al.* 2009) and members of the malaria mosquito *Anopheles gambiae* species complex (Bass *et al.* 2008). Since the B and Q biotypes are widely distributed and can co-exist, a rapid but reliable means of discriminating between them would be a valuable complement to insecticide resistance monitoring programmes and laboratory studies investigating their competitive interaction.

3.2 Materials and methods

3.2.1 Insect strains

The *B. tabaci* strains used were either already being reared at Rothamsted or were obtained specifically for this purpose. The biotype status of each strain was initially determined by comparing banding patterns of non-specific esterases on polyacrylamide gel electrophoresis. Reference B biotype strains were PIRGOS (Cyprus, 2003), GUA-MIX (Guatemala, 2004), GRB (USA, 1997) and MEX-2 (Mexico, 2004). Reference Q biotype strains were CHLORAKA (Cyprus, 2003) and CRT-1 (Crete, 2006). Field samples of previously-unknown biotype were collected and transported in 90-100% ethanol for biotype analysis (Table 3.1).

Table 3.1 Strain, origin, host-plant, source and biotype of *Bemisia tabaci* field collections.

Strain name	Country of origin	Host plant[1]	Source	Biotype status[2]
BRA04	Brazil	Cotton	Syngenta	B
BRA06	Brazil	Cabbage	Syngenta	B
TAI EP1	Taiwan	Eggplant	Syngenta	B
TAI EP2	Taiwan	Eggplant	Syngenta	B
TAI TOM	Taiwan	Tomato	Syngenta	B
TAI CF	Taiwan	Cauliflower	Syngenta	B
UNDERBORG	South Africa	-	Rothamsted	B
GRB	USA	-	Rothamsted	B
EGYPT-1	Egypt	-	Syngenta	B
YESHA	Israel	-	Rothamsted	B
ALM-1	Spain	-	Rothamsted	Q
NZ-1	New Zealand	Poinsettia	Canterbury Ag & Sci Cent., NZ	Q
NZ-2	New Zealand	Poinsettia	Canterbury Ag & Sci Cent., NZ	Q
LFNU	Spain	-	Rothamsted	Q
JAP-TOMATO	Japan	Tomato	Syngenta	Q
JAP-MELON	Japan	Melon	Syngenta	Q
BRITS	South Africa	-	Rothamsted	Non B/Q
PAK1	Pakistan	-	Syngenta	Non B/Q
PAK9	Pakistan	-	Rothamsted	Non B/Q
IND-A	India	-	Syngenta	Non B/Q
IND-B	India	-	Syngenta	Non B/Q

[1]Host plant given where known; [2]Confirmed by B/Q TaqMan® PCR

3.2.2 DNA extraction

Genomic DNA was extracted from individual whiteflies (mixed-sex) following the protocol outlined in Chapter 2.3 and re-suspended in 15 µl of sterile-distilled water.

Following extraction, the quality and quantity of gDNA was checked using the ND-1000 spectrophotometer (NanoDrop Technologies Inc.).

For high-throughput DNA extraction, individual *B. tabaci* of unknown biotype were placed in a 96-well plate containing 30 µl of a 10% sucrose buffer and ground with 3 repetitions of 30 clockwise and 30 anticlockwise turns of a multi-homogeniser (Burkard Scientific). Each plate contained two blank controls containing sucrose only and one B and Q biotype positive control. The homogenate was transferred to a 96-well PCR plate, boiled for 10 minutes in a thermal cycler and spun at 5,000rpm (Sigma).

3.2.3 PCR amplification of mtCOI

A 241bp region of the mtCOI gene was amplified to confirm the target SNPs and validate the known biotypes tested in the B/Q TaqMan® assay. The forward and reverse oligonucleotide primers used to PCR amplify this region were mtCOIPf (5'-TGGCCTTTGATTTACAGGATT-3') and mtCOIPr (5'-CATGATATAAAATTGAGACACC-3'). The PCR conditions were as described in Chapter 2.5 and the products separated on a 1.5% agarose gel. Each amplicon was sequenced using the reverse primer mtCOIPr2 (5'-TGGAAATCAATAGATAACTC-3') following the protocol described in Chapter 2.8.

3.2.4 The B/Q TaqMan® allelic discrimination assay

A nucleotide alignment of 76 mtCOI gene sequences from a range of *B. tabaci* B and Q biotypes available in the National Centre for Biotechnology Information (NCBI) database (GenBank) revealed a number of SNPs that discriminate between these biotypes. The target SNP chosen for this assay was within a conserved region of the mtCOI gene sequence to facilitate the design of TaqMan® probes and primers. Two primers and two fluorescent dye-labelled probes were designed and manufactured by Applied Biosystems (Foster City, CA, USA). The forward primer BEMBQ-SNP1F (5'-GCCTTTGATTTACAGGATTTTTATTTTTATTTACTATAGGT-3') and the reverse primer BEMBQ-SNP1R (5'-GAAATCAATAGATAACTCCTCCTACAATAGCA-3') were unmodified PCR primers. The probe SNP1V2 (5'-ATGCAGACACACATC-3') was labelled with the reporter dye VIC at the 5' end for detection of B biotype individuals, and the probe

SNP1M2 (5'-ATGCAAACACACATC-3') was labelled with 6-FAM at the 5' end specific for the Q biotype. The design of the allelic discrimination assay is shown in Figure 3.1. Both probes contained a non-fluorescent quencher dye (NFQ) and minor groove binding (MGB) groups at their 3' ends. The NFQ dye suppresses VIC or FAM fluorescence until the probe is broken down during PCR, while the MGB groups increase the stability of matched duplexes, thereby resulting in a more accurate allelic discrimination (Afonina *et al.* 1997). Following the successful design and validation of the assay, primers and probes were acquired separately, and diluted in sterile distilled water to the required concentrations.

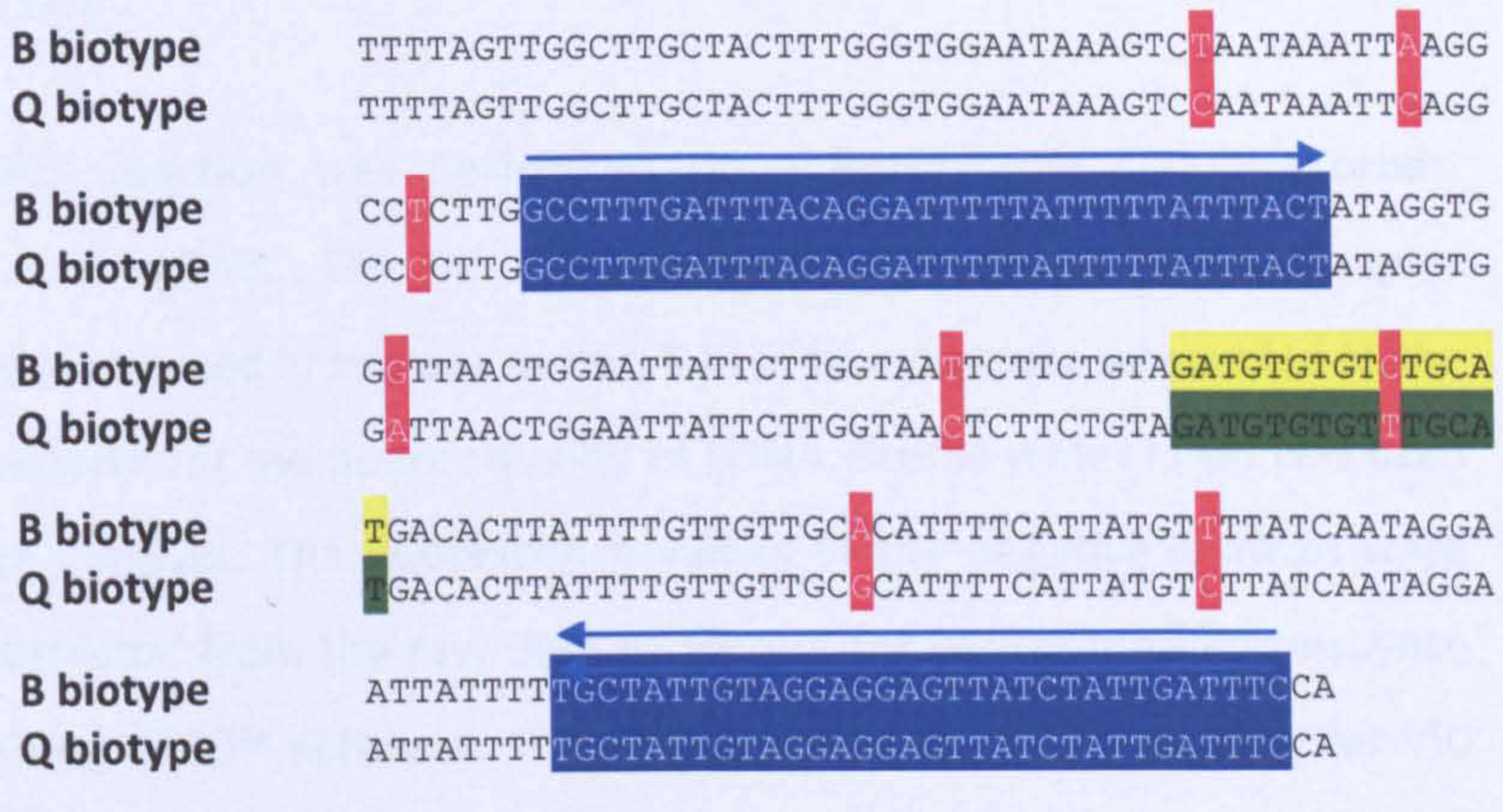


Figure 3.1 B and Q biotype mtCOI sequence and design of TaqMan® allelic discrimination assay

TaqMan primers (blue) and probes (VIC = yellow; FAM = green) are highlighted. SNPs between B and Q biotypes are in red.

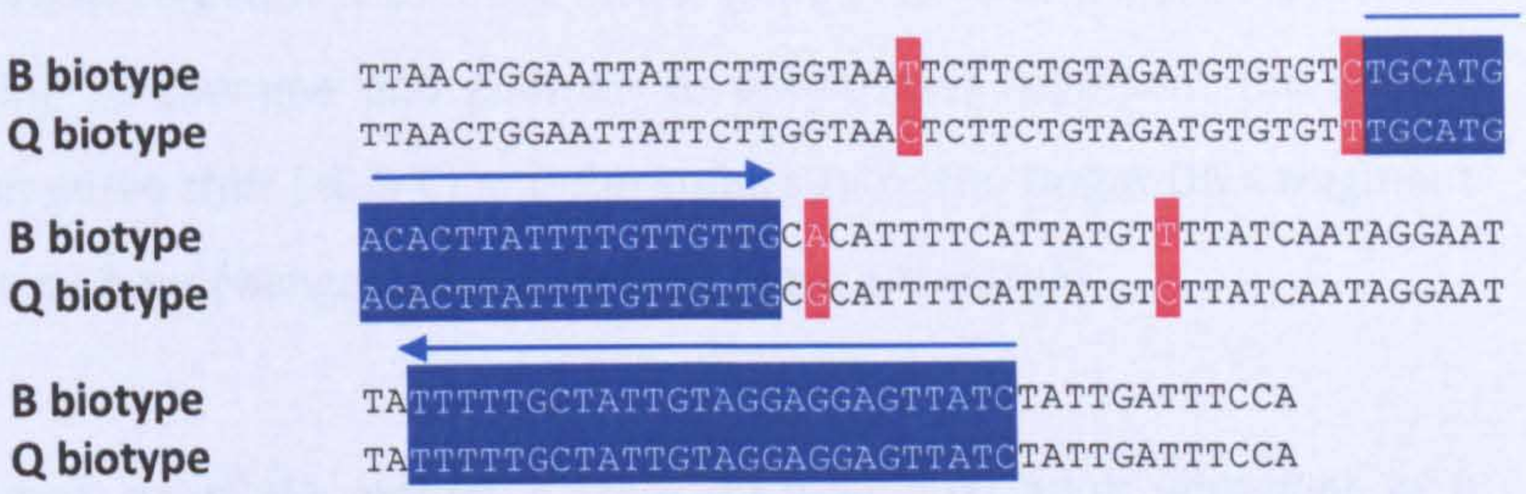


Figure 3.2 HRM design to discriminate B and Q biotypes of *B. tabaci* from mtCOI

Oligonucleotide primers are highlighted in blue surrounding the two SNPs targeted in the HRM assay.

3.2.5 TaqMan® PCR

Each real-time PCR reaction was performed on a Rotor-Gene 6000™ (Corbett Research) using 1 µl of gDNA. The PCR conditions follow those outlined in Chapter 2.10. For whiteflies extracted in sucrose buffer, 5 µl of homogenate was added to the reaction to compensate for the poorer quality of gDNA. Sterile water (1 µl) was used for blank negative controls. The fluorescence values of the negative controls were averaged and subtracted from the raw data to correct for background fluorescence using the Rotor-Gene 6000™ software. The endpoint values of fluorescence for VIC and FAM were then plotted against each other in bivariate scatter plots that gave a clear clustering of the samples and enabled easy scoring of the B and Q biotypes.

3.2.6 High resolution melt analysis

The HRM assay was designed following previous recommendations (Liew *et al.* 2004). Two SNPs were targeted within the mtCOI gene sequence (Figure 3.2). These mutations (cytosine to thymine and guanine to adenosine) represent the largest differences in melt curve shift (>0.5°C) and the small size of the target DNA fragment (83bp) allowed these base changes to be detected more accurately.

HRM was performed on gDNA extracted from 20 individual adult whiteflies of B (GUA-MIX) and Q (CRT-1 and CHLORAKA) biotype status. The initial pre-amplification of the target sequence was performed using the forward primer BEMBQF1 (5'-TGCATGACACTTATTTTGTTGTTG-3') and the reverse primer BEMBQR1 (5'-GATAACTCCTCCTACAATAGCAAAAA-3'). Each HRM reaction was composed of gDNA (1 µl), SensiMix HRM (12.5 µl) and EvaGreen dye (1 µl) (Quantace Ltd, Neutral Bay, Australia), 200 nm of each primer and the total reaction volume (25 µl) was made up with sterile water. Sterile water (1 µl) was used for blank negative controls and one known B and Q biotype were included as positive controls. The HRM was performed on the RotorGene 6000™ (Corbett Research) and the standard thermal cycling conditions for the pre-amplification phase were 10 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds and 60°C for 15 seconds. The temperature was increased from 65°C to 90°C in 0.1°C increments every 2 seconds for the melt curve analysis. The increase in EvaGreen fluorescence was monitored in real-time on the

Rotor-Gene 6000™ and the genotype of each sample was scored by examination of the melt curve and normalised difference plots.

3.3 Results

3.3.1 The B/Q TaqMan® allelic discrimination assay

A number of conserved SNPs exist within the mtCOI gene and discriminate between the B and Q biotypes. Direct sequencing of a 281bp region of the mtCOI gene from individuals of known biotype status confirmed the consistency of these SNPs (Figure 3.3).

Figure 3.3 Mitochondrial cytochrome oxidase I sequence from six strains of *B. tabaci.*

SNPs discriminate between B (blue) and Q (red) biotypes within the 137bp sequence.

The TaqMan® allelic discrimination PCR assay was designed to target a single SNP (Figure 3.1). When DNA from a B biotype adult is tested, the B-selective probe anneals to its mtCOI target sequence and is then cleaved in the PCR by the 5' nuclease activity of Taq DNA polymerase, thereby releasing the VIC dye away from its quencher and resulting in an increase in VIC fluorescence. Conversely, when DNA from a Q biotype adult is tested, only the FAM-labelled Q selective probe anneals, resulting in cleavage of this probe and an increase in FAM fluorescence. The relative fluorescence of these two dyes is measured in real time during the PCR (Figure 3.4), with the endpoint values corrected for background and plotted against each other in a bivariate scatter plot (Figure 3.5).

An initial analysis of individual adult whiteflies from the six laboratory strains of known biotype status (two Q and four B biotypes) is given in Figure 3.5. The assay worked extremely well with the B and Q biotypes grouping into two distinct clusters. Following validation, field samples from around the world of unknown biotype were tested and consistently grouped into two clear clusters. The distribution and biotype status of these samples are given in Figure 3.6. All individuals from the same field sample conformed to the same biotype. The number of failed reactions was minimal (<5% of the total) and these were likely to be due to the presence of a biotype other than B or Q or due to poor quality DNA.

To improve the high-throughput nature of the assay, DNA was extracted from insects of known biotype boiled in a sucrose solution. Very few failed reactions were observed and this allowed the biotype status of 92 individuals in 96-well plates (2 blank negative controls, 1 B and Q biotype positive control) to be processed and confirmed within 2 hours (Figure 3.5). As a result, this method was used for laboratory population dynamic experiments investigating the competitive interaction between B and Q biotypes.

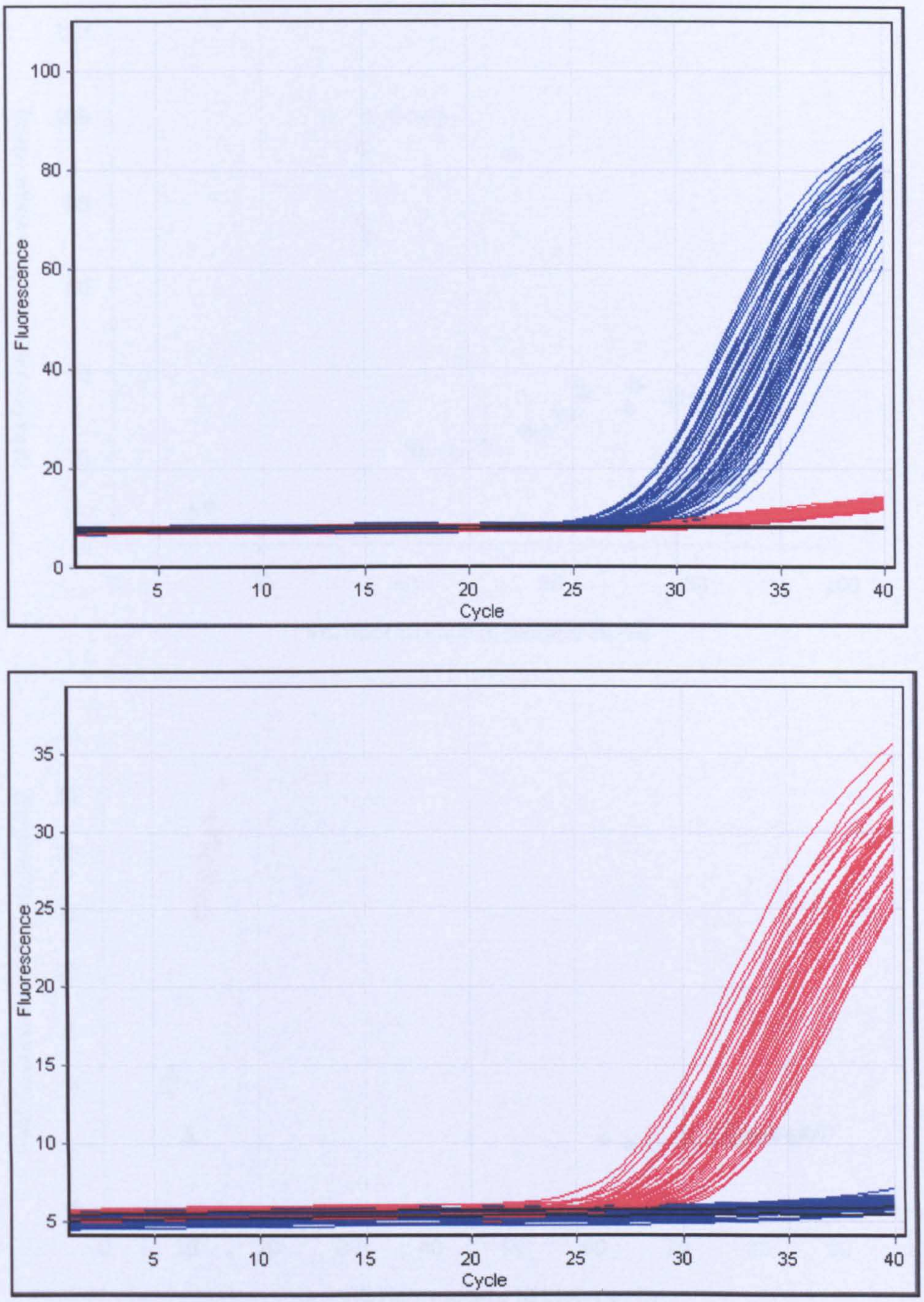
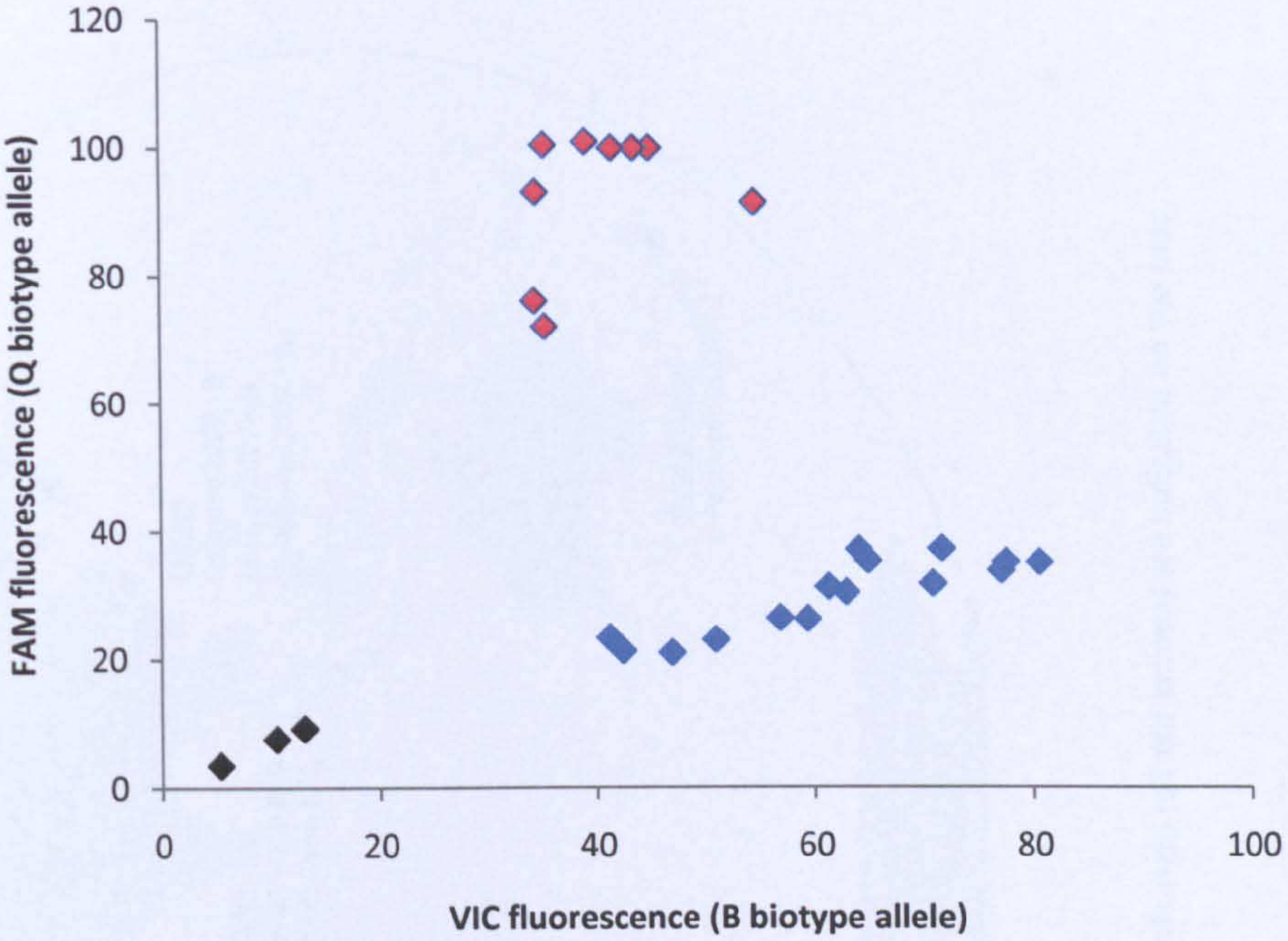


Figure 3.4 Example of real-time PCR fluorescence profiles for B and Q biotype detection.

An increase in VIC (A) and FAM (B) fluorescence specific for the B biotype (blue) and Q biotype (red) mtCOI alleles over 40 PCR cycles is shown.



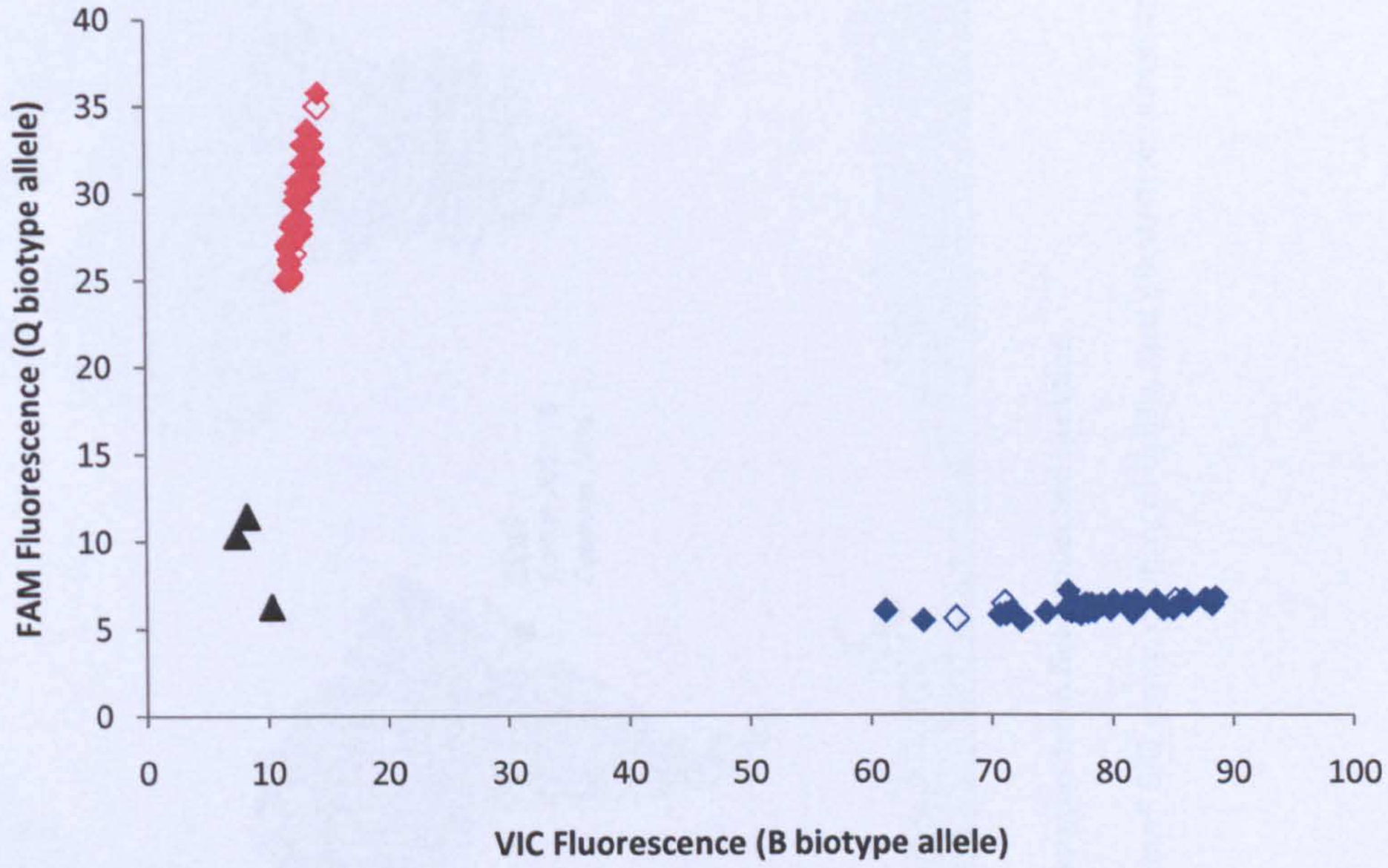


Figure 3.5 Scatter plot analysis of fluorescence data from the B/Q TaqMan assay

A, adults of known biotype from the laboratory strains show tight clustering according to their respective increases in VIC (blue, B biotype) or FAM (red, Q biotype) fluorescence. Negative controls (water) are represented in the 'blank' cluster. **B**, an example analysis of 92 individuals homogenised in sucrose buffer. Three each of water controls (blank), known B types (open diamonds) and known Q types (open triangles) were also included.

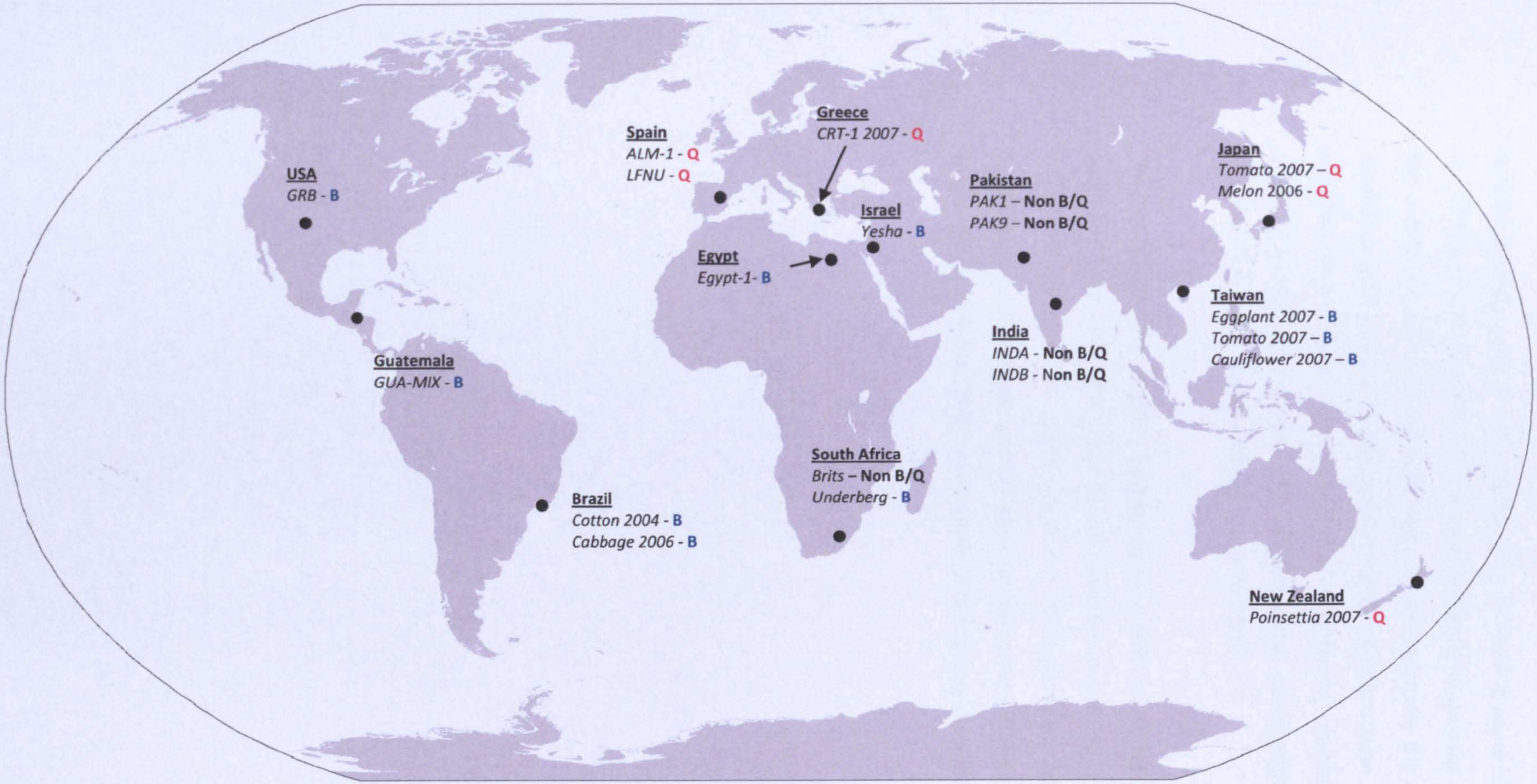


Figure 3.6 Global distribution of *B. tabaci* biotypes from field collected samples.

The biotype status as confirmed by the TaqMan® B/Q assay, country of origin, host plant/strain name and year of origin (where known) are indicated on the map.

3.3.2 A HRM assay for discriminating B and Q biotypes

In HRM analysis, a small region of the gene of interest containing the target SNPs is amplified and monitored in real-time as third-generation dyes are incorporated into the dsDNA. Under an incremental temperature increase, the emitted fluorescence decreases as the dye is released following the disassociation of dsDNA into single stranded DNA (ssDNA). The behaviour of the DNA is recorded and the difference between genotypes is scored with machinery that allows high optical and thermal precision (i.e. Rotor-Gene 6000™) (Liew *et al.* 2004).

To complement the TaqMan biotype assay, a HRM assay was developed to target two SNPs within the mtCOI that discriminate between B and Q biotypes (Figure 3.2). A total of 40 individual adults (20 B and 20 Q biotypes) were tested. Following amplification of the target gene in each sample, clear differences between the melt curve phases of B and Q biotypes existed, allowing easy discrimination (Figure 3.7).

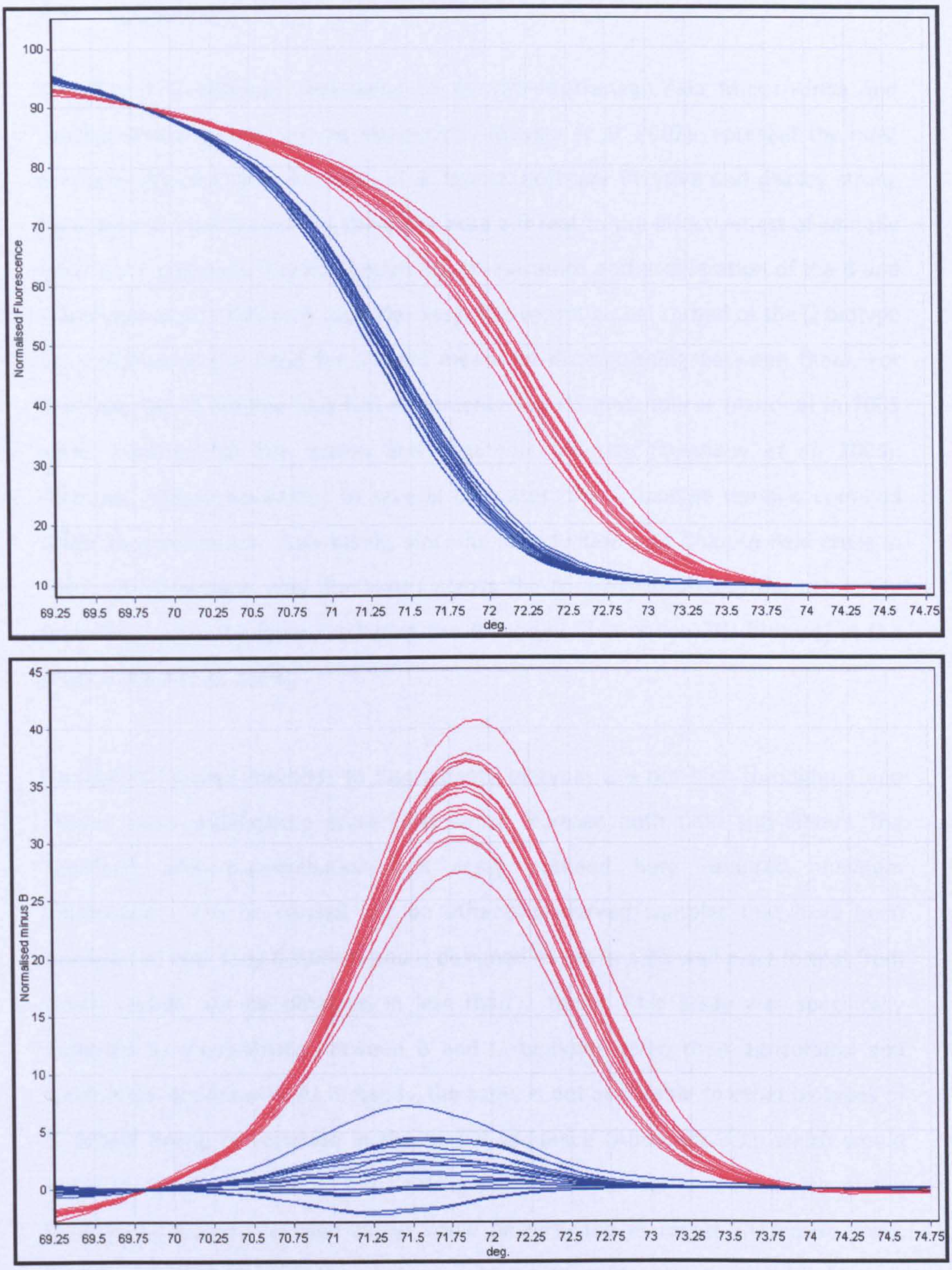


Figure 3.7 High Resolution Melt (HRM) analysis for B and Q biotypes

A) The temperature shift between genotypes during HRM analysis. Each line represents a different sample analysed; blue lines represent B biotypes and red lines represent Q biotypes. **B)** Melt curve analysis plotted as the normalised difference in fluorescence of the Q biotype compared with the B biotype as the temperature of the reaction increases.

3.4 Discussion

The B and Q biotypes (belonging to the Mediterranean/Asia Minor/Africa and Mediterranean genetic groups respectively (Boykin *et al.* 2007) represent the most economically-important biotypes of *B. tabaci.* Both are invasive and display strong resistance to insecticides and therefore pose a threat to the effectiveness of whitefly control programmes. The establishment, co-existence and proliferation of the B and Q biotypes across different countries vary. The recent global spread of the Q biotype has heightened the need for a rapid means of distinguishing between them. For example, the Q biotype was first documented in US glasshouses (Arizona) in 2005 amid concerns for the cotton and vegetable industry (Dennehy *et al.* 2005). However, despite spreading to several US states, the Q biotype remains confined solely to glasshouses. Conversely, since its introduction into Chinese field crops in 2003, the Q biotype now dominates across the country, displacing the previously prevailing non-Q biotypes (including the B biotype and native ZHJ biotype) in the process (Chu *et al.* 2006).

Current PCR-based methods to discriminate biotypes are not high throughput and require post-amplification procedures which increase both time and labour. The TaqMan® allelic-discrimination PCR assay outlined here required minimum optimisation, can be carried out on ethanol-preserved samples that have been transported over long distances and is designed to run in a 96-well plate format from which results can be obtained in less than 2 hours. This assay was specifically designed to discriminate between B and Q biotypes given their agricultural and economical importance. As it stands, the assay is not applicable to other biotypes of *B. tabaci* owing to variation in the mtCOI sequence (Appendix 3.1), which would result in either a failure of the flanking primers to anneal or a mismatch of the fluorescent probes. TaqMan assays could be designed to target alternative SNPs, thereby enabling discrimination between other biotype/genetic race combinations.

One potential drawback of using a mitochondrial rather than nuclear marker is its inability to detect potential genetic introgression between B and Q biotypes. However, successful mating between these biotypes is known to be extremely rare, if it occurs at all (Rothamsted Research, unpublished data). Biotypes are defined

according to differing biological characteristics; any single SNP present within the nuclear or mitochondrial genome is therefore most likely to correlate with, rather than determine, biotype status. Many of the current molecular biotype diagnostic assays rely on such a correlative approach. Although the SNP chosen in this assay consistently separated B and Q biotypes from 76 sequences in the available database, verification of the target SNP by occasional sequencing of the mtCOI gene is essential. Failed reactions must also be treated with caution before interpretation as the presence of an alternative biotype. In the case of a negative result, the quality of the gDNA and integrity of the control genotypes should be checked and verified before sequencing the mtCOI gene to determine the biotype status.

TaqMan® PCR is a highly sensitive SNP detection technique. The assay required approximately only one-tenth of a whitefly, offering the opportunity of combining the biotype diagnostic work with finding other traits associated with SNPs, such as insecticide resistance mutations. Successful genotyping with TaqMan® PCR using very little DNA has been achieved (Dr. Chris Bass *pers. communication*) and therefore the B/Q assay was adapted to work on whiteflies homogenised in a sucrose buffer. This destructive approach does not allow other molecular approaches to be used on the samples of interest but permits several hundred insects to be processed by one operator in a single day. It therefore provides a very valuable new research tool and was used to investigate the interactions between Chinese B and Q biotypes in laboratory field simulators treated with or without the neonicotinoid imidacloprid (Luo *et al.* unpublished data). Over 5-6 generations, it was shown that under selection pressure from the insecticide, Q biotype numbers increased whereas without exposure to the insecticide, the B biotype dominated. The results from this experiment suggest that the recent predominance of the Q biotype in China (Chu *et al.* 2006) is a result of its ability to withstand insecticides, compared with the B biotype and native biotype ZHJ.

An additional assay, based on HRM analysis, was designed to discriminate between B and Q biotypes. HRM has been used to detect *kdr* mutations in *An. gambiae* (Bass *et al.* 2007). In the melt curve and normalised difference plots it was relatively easy to score the B and Q biotypes (Figure 3.7). Although HRM can be performed in a 96-well plate format and does not require relatively expensive fluorescent probes compared

with the TaqMan® assay, it requires significant optimisation, high DNA quality and relies on the consistent PCR amplification of the target gene to a plateau stage prior to the melt curve analysis. For these reasons, HRM was not employed as a regular technique to score the biotype of field or laboratory insects; however it is a potentially useful tool to complement other molecular biotype diagnostics and could be adapted to detect mutations associated with insecticide resistance in *B. tabaci*.

Chapter 4 Distribution and evolutionary dynamics of knockdown resistance alleles in *Bemisia tabaci*

4.1 Introduction

DDT and synthetic pyrethroids target the *para*-type voltage gated sodium channel (Na_V) channel in the insect nervous system. The Na_v channel is a transmembrane protein consisting of four internally homologous domains (I-IV) each containing six membrane-spanning segments (S1-S6) (Catterall *et al.* 2007; Davies *et al.* 2007). The Na_V channel mediates the flow of sodium ions across the nerve cell membrane enabling the transmission of nerve impulses. Pyrethroids and DDT bind to and hold the Na_V channel in an open conformation, resulting in the repetitive firing of the action potential leading to insect paralysis and death.

Point mutations in the gene sequence encoding the Na_V channel have been identified that cause target-site insensitivity to pyrethroids and DDT. This form of resistance is referred to as 'knockdown resistance' or *kdr* and was first observed in DDT-resistant houseflies (Busvine 1951). The *kdr* allele is usually recessive and therefore can persist at low frequencies in insect populations. A second recessive trait, known as super-*kdr*, which confers much stronger resistance to pyrethroids co-exists with *kdr* in some insect species. Genetic mapping linked *kdr* and *super-kdr* to the Na_v channel in houseflies (*Musca domestica*), the tobacco budworm (*Heliothis virescens*) and the German cockroach (*Blatella germanica*) (Dong & Scott 1994; Taylor *et al.* 1993; Williamson *et al.* 1993). Subsequently, the *kdr* mutation L1014F in domain IIS6 and the super-*kdr* mutation M918T in the IIS4/S5 linker were associated with moderate and high levels of resistance respectively (Dong 1997; Williamson *et al.* 1996). Several *kdr* mutations have now been reported in a wide range of agriculturally and medically important insect pests (reviewed in Davies *et al.* 2007; Hemingway *et al.* 2004; Soderlund & Knipple 2003).

Electrophysiology and modelling studies have identified putative amino acid residues critical for insecticide binding within the sodium channel. Using a homology model based on the crystal structure of the rat brain potassium channel, O'Reilly *et al.* (2006) predicted that the binding sites for pyrethroids and DDT occur within a

hydrophobic cavity formed by the IIS4-S5 linker and the IIS5 and IIIS6 helices. Many of the *kdr* mutations associated with resistance are found within this region. The 'O'Reilly model' predicts that the T929 residue (present on the IIS5 helix) forms a particularly close interaction with DDT and pyrethroids as a result of hydrogen bonding. *In vitro* expression of the T929I mutation, originally discovered in the diamondback moth *Plutella xylostella* (Schuler *et al.* 1998), rendered the Na_V channel highly insensitive to all compounds (type-I/type-II pyrethroids and DDT).

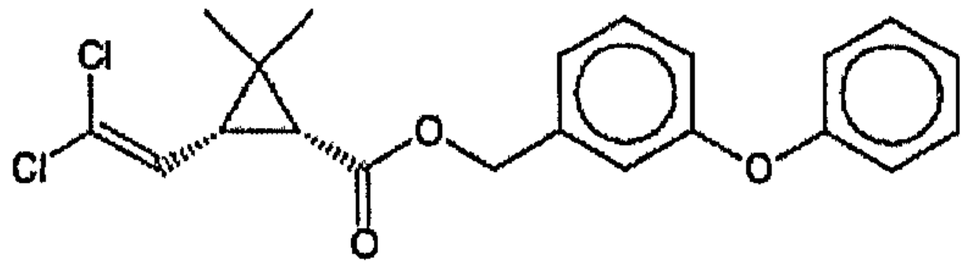
An additional residue found on the IIS5 helix (L925) is also found in close proximity to bound pyrethroids within the O'Reilly model (Davies *et al.* 2008) The mutation L925I may subtly alter the conformation of the binding site within the sodium channel pocket rather than physically interact with pyrethroids (Davies *et al.* 2008). Usherwood *et al.* (2007) showed that the potency of the type-II pyrethroid (deltamethrin) is reduced by the L925I mutation to a greater extent than the type-I pyrethroid, fenfluthrin. Unlike other type-I pyrethroids, fenfluthrin only has one aromatic ring in the alcohol moiety (Figure 4.1).

The extensive use of DDT and pyrethroids to control *B. tabaci* during the 1970s and 1980s inevitably led to widespread resistance across multiple biotypes (Cahill *et al.* 1995; Dittrich *et al.* 1985; Dittrich *et al.* 1990). In many cases, pyrethroid resistance in *B. tabaci* has been attributed to enhanced detoxification through oxidative and hydrolytic pathways. However, two target-site mutations in the Na_V channel, L925I and T929V, have recently been discovered and associated with pyrethroid resistance. The L925I mutation was found in a laboratory strain exhibiting over 100-fold resistance to a mixture of the type-II pyrethroid fenpropathrin plus organophosphate (acephate) as well as in 2000-2001 field collections of *B. tabaci* from Arizona, USA (Morin *et al.* 2002). In the same study, an additional mutation at the super-*kdr* site M918V, was found in a strain derived from field samples collected from the Gila Bend in Arizona (USA) in 1995 but was absent from those whiteflies collected during 2000-2001. L925I and M918V did not occur together on the same allele and M918V has not been detected in whiteflies since. T929V was discovered in *B. tabaci* in two independent studies (Alon *et al.* 2006; Roditakis *et al.* 2006). This mutation was previously found in resistant cat fleas (*Ctenocephalides felis*) (Bass *et al.* 2004). The T929V mutation occurred only in Q biotypes while L925I was present in both B and Q

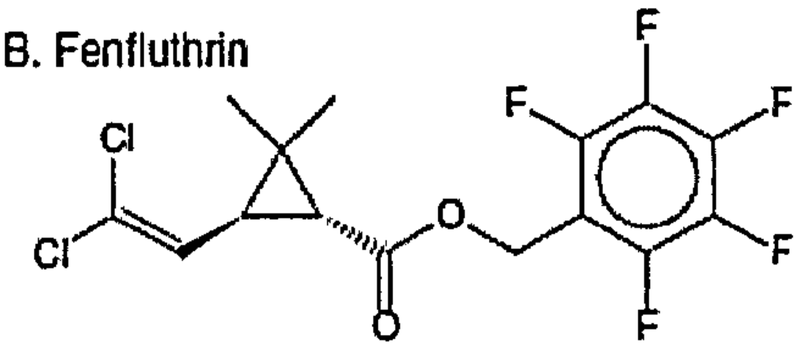
biotypes (Alon *et al.* 2006; Roditakis *et al.* 2006). The two mutations have not been found to occur in the same haplotype but their presence at high frequencies in insect groups from across the world indicates that target-site resistance is an important mechanism in pyrethroid-resistant populations of *B. tabaci.*

Understanding the evolutionary origins of insecticide resistant mutations provides a valuable insight into the spread and emergence of resistance in insect pests. Resistance alleles may arise once in a single population and then subsequently spread via migration or alternatively emerge independently in multiple populations. If resistance alleles from geographically distinct populations exhibit a degree of homology in their gene sequences, this potentially indicates a single origin for the resistance mechanism. Raymond *et al.* (1991) sequenced identical flanking regions of the esterase B2 in *Culex pipiens* from various geographical locations suggesting that organophosphate resistance had spread worldwide from a single event. Identical DNA sequences of FE4 and E4 esterases were described between the peach-potato aphid, *M. persicae*, and the closely related tobacco aphid *Myzus nicotinae* (Field *et al.* 1994). The authors subsequently proposed that the amplified esterases were selected first in *M. persicae* and were then transferred to *M. nicotinae* through sexually produced hybrids between the two species (Field *et al.* 1994). A single global origin was also described for the P450 *CYP6G1* associated with DDT resistance in *Drosophila melanogaster* (Daborn *et al.* 2002). In contrast, Andreev *et al.* (1999) reported independent origins of *rdl* (resistance to dieldrin) alleles in the GABA receptor in the red flour beetle, *Tribolium castaneum*. It has been suggested that the life-history pattern of the insect species greatly influences the distribution pattern of resistance alleles (ffrench-Constant *et al.* 2000). In *B. tabaci, rdl* resistant B biotype alleles were identical and consistent with the passive spread of this biotype throughout the world. Considerable polymorphism was seen however, between non-B biotypes indicating independent origins for resistance within these isolated groups and demonstrating the limited reproductive incompatibility between biotypes (ffrench-Constant *et al.* 2000). The fact that *M. persicae* undergoes several asexual cycles per year compared with only one sexual cycle, means that independent occurrences of resistance mutations are more likely. Multiple origins of *kdr* and super-*kdr* in several different haplotypes of *M. persicae* were identified by Anstead *et al.* (2005).

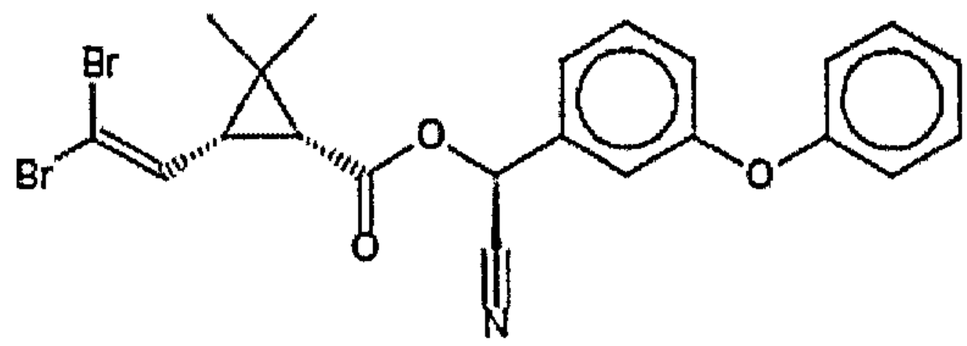
A. Permethrin



B. Fenfluthrin



C. Deltamethrin



D. DDT

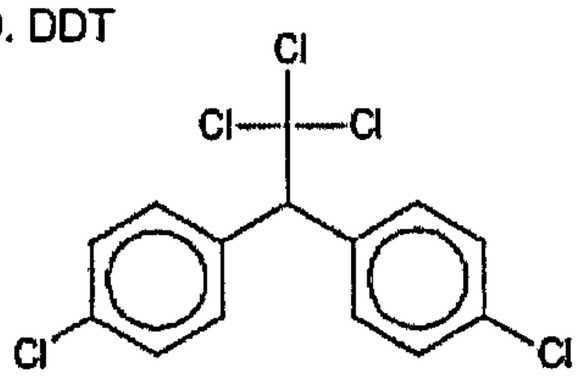


Figure 4.1 The chemical structure of A) permethrin, B) fenfluthrin, C) deltamethrin and D) DDT.

Figure adapted with permission from Usherwood *et al*. (2007).

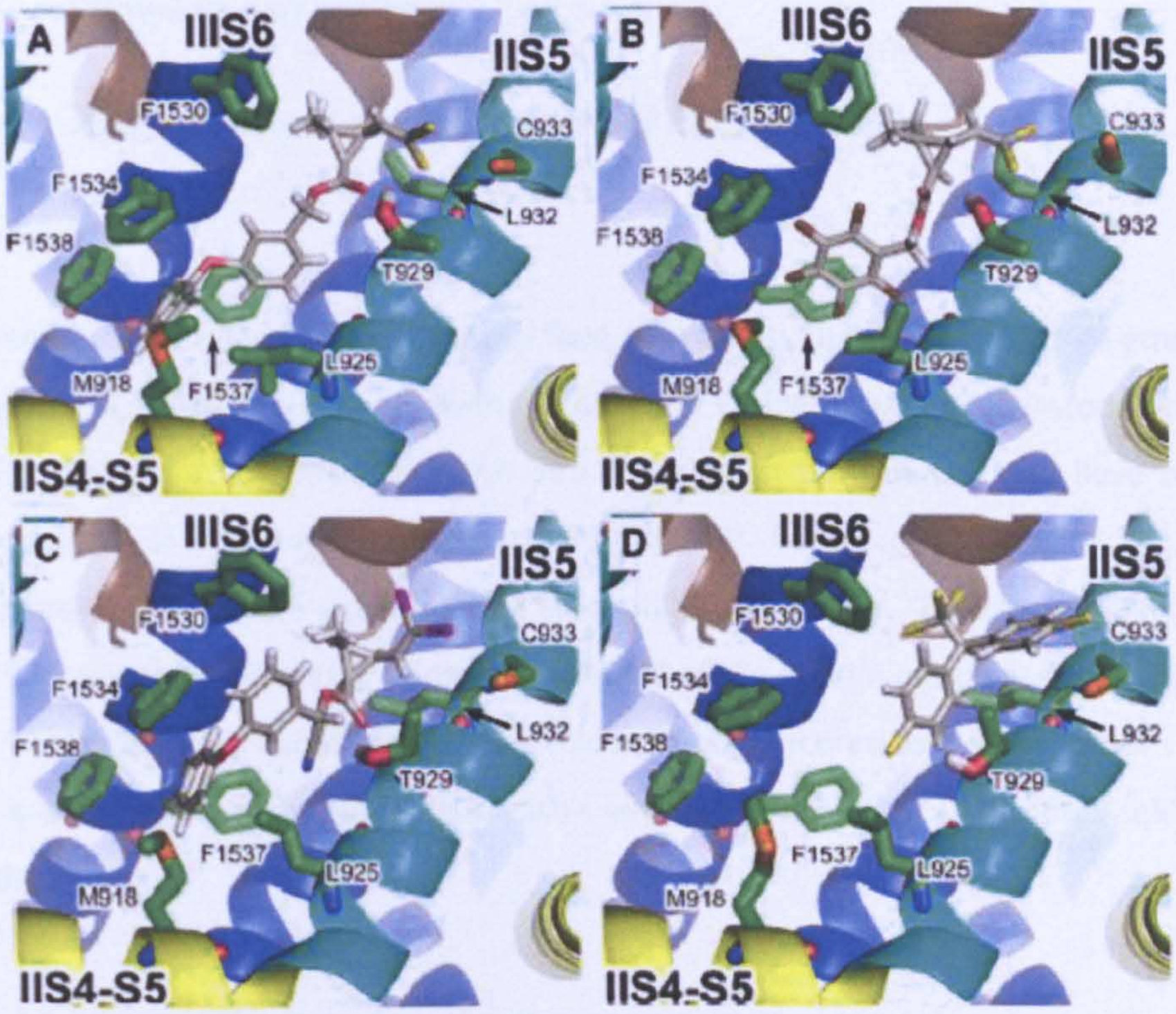


Figure 4.2 The molecular docking of insecticides within the sodium channel binding pocket.

The figure shows the stereochemical interaction between each insecticide (A = permethrin, B = fenfluthrin, C = deltamethrin, D = DDT) and the sodium channel protein. The IIS5 and IIIS6 helices are shown in light and dark blue respectively. Figure adapted from Usherwood *et al.* (2007) with permission.

Knockdown resistance genes are well suited to the evolutionary study of insecticide resistance origins as they have been subject to selection since the 1940s when DDT was first introduced to control insects of agricultural and medical importance (Mellanby 1992; Davies *et al.* 2007). The degree of nucleotide polymorphism in the intron sequence adjacent to the L925 and T929 positions in *B. tabaci* indicates multiple origins for *kdr* alleles in B and Q biotypes (Alon *et al.* 2006).

The purpose of this study was to investigate the occurrence and describe the variation between L925I and T929V alleles from globally-distributed samples of *B. tabaci*. Furthermore, based on our understanding of the function of the L925 and T929 residues from the O'Reilly model and electrophysiology studies, the association of these two mutations with responses to insecticides acting on the Na_v channel in a single polymorphic *B. tabaci* sample was investigated.

4.2 Materials and methods

4.2.1 Distribution and evolutionary origins of L925I and T929V

4.2.1.1 Insects

Whiteflies were collected from various field sites across the world in 80-90% ethanol courtesy of various sources as given in Chapter 3 (Table 3.1). The laboratory strains CRT-1, GUA-MIX and MURCIA were also included in the analysis and have been maintained in long-term culture at Rothamsted Research (Chapter 2.1). The laboratory strain SUD-S was used as a susceptible population. The biotype status of each strain was determined using the TaqMan® allelic discrimination assay described in Chapter 3 and by Jones *et al.* (2008). Biotypes were scored as B, Q or "other"; the latter referring to cases when DNA wasn't amplified by the B or Q-specific TaqMan® probes.

4.2.1.2 PCR amplification of the IIS4-S6 region of the Na_V channel

Genomic DNA (gDNA) was extracted from individual male whiteflies and re-suspended in 15 µl of sterile-distilled water following the protocol described in Chapter 2.3. The final 130bp of the exon containing the L925 and T929 amino acid residues and the complete adjacent intron (730bp) sequence is given in Figure 4.3. The oligonucleotide primers used to amplify this region in two rounds of nested PCR are given in Table 4.1. All PCR reactions were carried out following methods in Chapter 2.5. In the primary PCR, 2-3 µl of gDNA was added to the reaction and subsequently, 1 µl of the primary PCR product was added to the secondary PCR. Temperature cycling conditions were 95°C for 2 minutes followed by 30/25 cycles (primary/secondary PCR) of 95°C for 30 seconds (denaturation), 50°C for 30 seconds (annealing) and 72°C for 60 seconds (extension). Sterile water (1 µl) was used as a blank negative control in each PCR. PCR products (~1kb) were visualised on a 1.4% TAE agarose gel, purified by ethanol precipitation and sequenced following Chapters 2.6, 2.7 and 2.8 respectively.

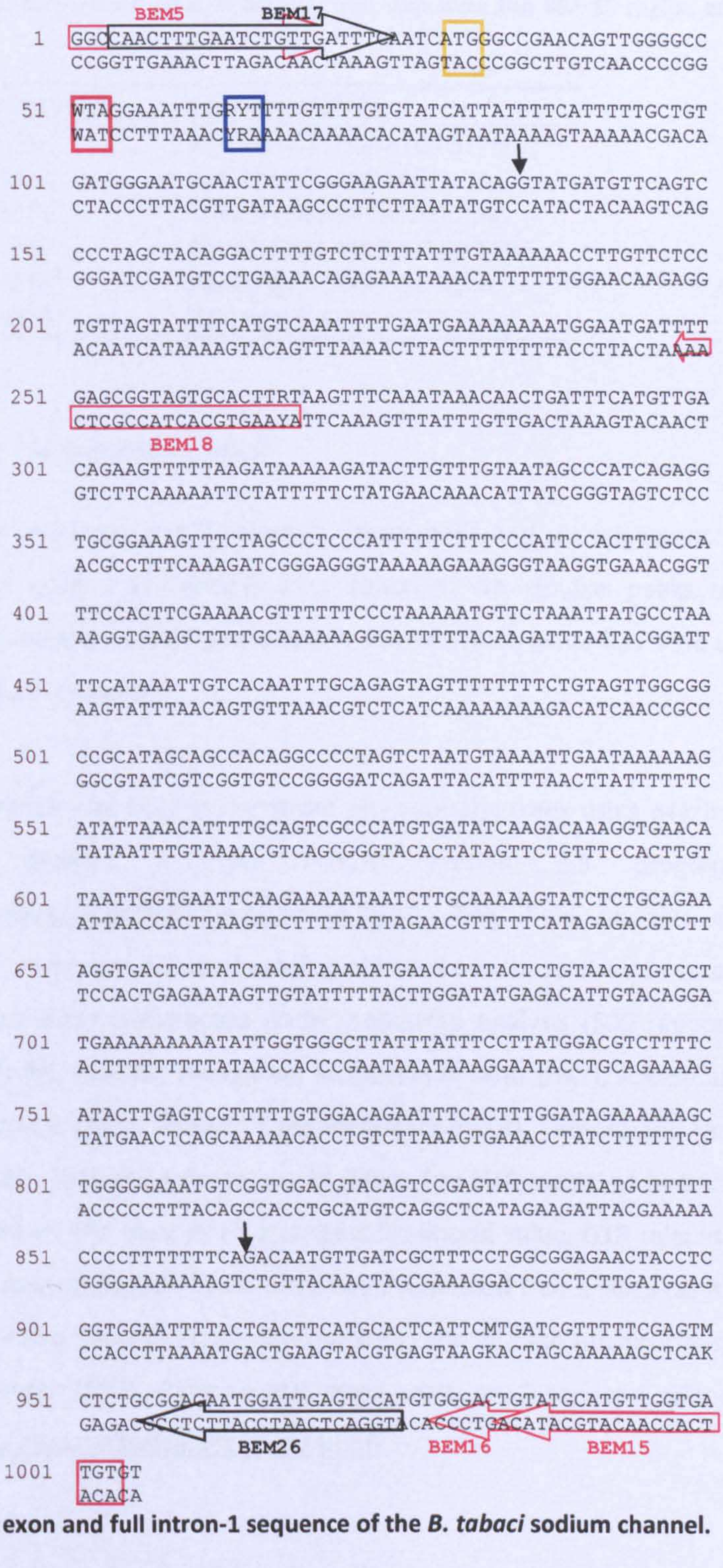


Figure 4.3 The IIS4-S6 exon and full intron-1 sequence of the *B. tabaci* sodium channel.

Oligonucleotide primers used in the nested PCR are shown in red. Sequencing primers are shown in black. Black vertical arrows represent the start and end point of intron-1. The codons of each mutation site are highlighted in the coloured boxes as follows; yellow = M918, red = L925 and blue = T929.

Table 4.1 Oligonucleotide primers used to amplify and sequence the IIS4-S6 region of the sodium channel

Primer name	Sequence (5'-3')
BEM5	GGCCAACTTTGAATCTGTTG
BEM15	CACATCACCAACATGCATAC
BEM16	CAACATGCATACAGTCCCAC
BEM17*	CAACTTTGAATCTGTTGATTTC
BEM18*	YAAGTGCACTACCGCTCAA
BEM26*	TGGACTCAATCCATTCTCCGC

*sequencing primer

4.2.1.3 Sequence and phylogenetic analysis

All sequences were imported into VectorNTI (Invitrogen) and an alignment was created and edited using the ContigExpress function. No double peaks were observed on the sequence chromatograms as only haploid male whiteflies were used to sequence the sodium channel.

The full intron sequence was used to construct phylogenetic trees using Maximum Likelihood (ML) analysis in the PHYLIP version 3.5 programme (http://evolution.genetics.washington.edu/phylip.html). Only field samples were used in the analysis to remove any confounding effects from those strains reared in the laboratory. Trees were constructed under bootstrap analysis (500 replicates) from seven different ML models: Hasegawa, Kishino and Yano (HKY), Jukes-Cantor (JC), Kimura-2-parameter (K2P), General Time Reversible (GTR), Tamura and Nei 93 (TN93), Felsenstein 81 (F81) and Felsenstein 84 (F84). The GTR model of nucleotide substitution was used on the basis of its strongest likelihood value. GTR relaxes the assumption that all four nucleotide bases have equal expected frequencies (as is the case for Jukes-Cantor and Kimura-2-parameter models) and all base substitutions can differ (Waddell & Steel 1997). Phylogenetic trees were produced and edited in MEGA4 (http://www.megasoftware.net/mega.html).

4.2.2 The impact of L925I and T929V on insecticide activity

4.2.2.1 Insects and insecticides

These experiments used seven Chinese *B. tabaci* strains that were analysed for both L925I and T929V and are listed in Table 4.3. All strains were reared at Rothamsted Research (Chapter 2.1). All diagnostic dose bioassays were conducted using technical grade DDT, fenfluthrin (type-I pyrethroid) and deltamethrin (type-II pyrethroid) dissolved in 100% acetone.

4.2.2.2 TaqMan allelic discrimination of kdr mutations

TaqMan® PCR allelic discrimination was used to discriminate *B. tabaci* homozygous and heterozygous for L925I and T929V. The sequence surrounding these residues was analysed using PrimerExpress (Applied Biosystems) and two sets of TaqMan® primers and minor groove binding (MGB) probes were designed by Applied Biosystems to target L925I and T929V (Table 4.2).

Genomic DNA was extracted from individual whiteflies using DNAzol reagent as described in Chapter 2.3. All extracted gDNA samples were placed in sterile 96-well sample plates and mixed with the TaqMan® PCR reagents into reaction plates using the CAS-1200 Robotics system (Corbett) to maximise throughput. Following optimisation, 3 µl of gDNA from each sample was added into two separate TaqMan® PCR reactions; one containing the L925I primer/probe mix and one containing the T929V primer/probe mix. The increase in fluorescence by the VIC and FAM reporter dyes, representing the resistant and wild-type alleles respectively, was monitored in real-time using the Rotor-Gene 6000™ software. The endpoint values of the VIC and FAM fluorescent dyes were corrected for background and plotted against each other in a bi-variate scatter plot to discriminate between heterozygotes and homozygotes.

Individual females whiteflies from five Q biotype and two B biotype Chinese strains (7-10 individuals per strain) were screened for both mutations using the TaqMan® PCR assays. The JZ strain was heterogeneous for both mutations (L925I = 0.2, T929V = 0.8) and chosen to investigate the relationship between the frequency of each *kdr* mutation and insecticide activity.

Table 4.2 TaqMan® PCR primers and probes for allelic discrimination of L925I and T929V in *B. tabaci*.

Assay name	Primer/probe name	Sequence (5'-3')
L925I	BEML925I_F	TCCTGGCCAACTTTGAATCTGTT
	BEML925I_R	GCATTCCCATCACAGCAAAAATGAAAAT
	L925IPROBE-1	CAAATTTCCTATGGCCCC-VIC
	L925IPROBE-2	AAATTTCCTAAGGCCCC-FAM
T929V	BEMT929V_F	TCCTGGCCAACTTTGAATCTGTT
	BEMT929V_R	GTTGCATTCCCATCACAGCAAAA
	T929VPROBE-1	CAAAACAAAAGTCAAATTT-VIC
	T929VPROBE-2	AAAACAAAAACCAAATTT-FAM

Probe sequences are in italics

4.2.2.3 Diagnostic dose leaf-dip bioassay

Three concentrations of DDT, fenfluthrin and deltamethrin (300ppm, 1000ppm & 3000ppm) were used in an adult leaf-dip bioassay (Chapter 2.2) to characterise the response of *kdr* genotypes from JZ males. Only haploid males were tested allowing easy discrimination of hemizygous L925I and T929V genotypes. The laboratory susceptible strain SUD-S was tested with four lower concentrations of DDT and fenfluthrin (10ppm, 50ppm, 100ppm & 200ppm) and three lower concentrations of deltamethrin (3ppm, 30ppm & 300ppm) to provide a baseline response for reference. Dead and alive males were collected at each dose after 48 hours exposure to each insecticide, snap frozen in liquid nitrogen and stored at -80°C prior to gDNA extraction and TaqMan® allelic discrimination.

4.3 Results

4.3.1 Distribution and evolutionary origins of L925I and T929V

4.3.1.1 B. tabaci kdr genotypes

A 1kb fragment of the IIS4-S6 region of the Na_V channel was PCR-amplified and sequenced from gDNA extracted from individual males from 28 strains of *B. tabaci* (Figure 4.4). This encompassed the exon containing the L925 and T929 residues and the complete sequence of the adjacent intron (730bp). The nucleotide and amino acid sequence of some strains at the two *kdr* sites included a single point substitution (T to A) resulting in a L925I mutation or a double substitution (AC to GT) resulting in the T929V mutation (Figure 4.4). No alleles were found that encoded both amino acid substitutions. In total, fourteen strains contained the resistant L925I mutation. The majority of these were B biotypes, three were Q biotypes and two were from strains of an uncharacterised biotype from Pakistan. The T929V mutation was identified from six strains which were all confirmed as Q biotypes. Three Q biotype strains (NZ-1 (New Zealand), CRT-1 (Crete) and Murcia (Spain)) contained individuals with both resistant alleles.

Two further substitutions were found within the IIS4-S6 exon. The USA strain (GRB), collected during the late-1990s, had a single-point substitution (A to G) at the super-*kdr* site conferring the M918V mutation (Figure 4.4). This genotype was previously found by Morin *et al.* (2002) but wasn't identified in any other sample. An additional super-*kdr* mutation, M918L was discovered from two field samples originating from India (Figure 4.4). This is the first time this mutation has been identified in *B. tabaci*. Neither of these Indian strains had L925I or T929V. As expected, the laboratory susceptible strain SUD-S had the wild-type 'susceptible' exon which was also identified from two B biotypes (BRA04 (Brazil) and U'BORG (South Africa)), one Q biotype (ALM-1) and the South African strain BRITS.

B BIOTYPES

ORIGIN	STRAIN	S I M G R T V G A L G N L T F
BRAZIL	BRA04	TCAATCATGGGCCGAACAGTTGGGGCCTTAGGAAATTTGACTTTT
BRAZIL	BRA06	.....A.....
GUATEMALA	GUA-MIX	.....A.....
TAIWAN	EP1	.....A.....
TAIWAN	EP2	.....A.....
TAIWAN	TOM	.....A.....
TAIWAN	CF	.....A.....
SOUTH AFRICA	U'BURG	.....T.....
USA	GRB	.....G.....T.....
EGYPT	EGYPT-1	.....A.....
ISRAEL	YESHA	.....A.....
		V I

Q BIOTYPES

ORIGIN	STRAIN	S I M G R T V G A L G N L T F
SPAIN	ALM-1	TCAATCATGGGCCGAACAGTTGGGGCCTTAGGAAATTTGACTTTT
NEW ZEALAND	NZ-1 (A)	.....A.....AC....
NEW ZEALAND	NZ-1 (B)	.....T.....GT....
NEW ZEALAND	NZ-2	.....A.....AC....
CRETE	CRT-1 (A)	.....A.....AC....
CRETE	CRT-1 (B)	.....T.....GT....
SPAIN	MURCIA (A)	.....A.....AC....
SPAIN	MURCIA (B)	.....T.....GT....
SPAIN	LFNU	.....T.....GT....
JAPAN	TOMATO	.....T.....GT....
JAPAN	MELON	.....T.....GT....
		I V

OTHER BIOTYPES

ORIGIN	STRAIN	S I M G R T V G A L G N L T F
SUDAN	SUD-S	TCAATCATGGGCCGAACAGTTGGGGCCTTAGGAAATTTGACTTTT
SOUTH AFRICA	BRITS	.....T.....
PAKISTAN	PAK-1	.....A.....
PAKISTAN	PAK-9	.....A.....
INDIA	IND-A	.....T.....
INDIA	IND-B	.....T.....
		L I

Figure 4.4 The nucleotide and amino acid sequence of the IIS4-S6 exon of the *B. tabaci* NaV channel

This sequence contains the three sites where mutations have been found in *B. tabaci* and are associated with pyrethroid resistance. The wild-type sequence is shown at the top of each section containing the different biotypes. The mutation sites and nucleotide changes between strains are shown and the dots represent a conserved sequence.

4.3.1.2 Phylogenetic analysis of the IIS4-S6 intron-1

The complete intron sequence (~730bp) adjacent to L925 and T929 was sequenced in all field samples and analysed for nucleotide polymorphism (Appendix 4.1). In total, the intron contained 104 nucleotide substitutions and 18 indels. The high frequency of polymorphism seen was largely due to the inclusion of biotypes other then B and Q in the study (Appendix 4.1). Alon *et al.* (2006) previously reported 36 nucleotide substitutions and four indels from this intron and this was consistent between the B and Q biotypes reported here.

To understand the evolutionary origins of the resistant and susceptible alleles, a maximum likelihood analysis was performed on the intron sequence. The phylogenetic tree revealed two distinct clades (B and Q biotypes) supported by high bootstrap values (>963) (Figure 4.5). Other biotypes formed distinct single branches. The majority of alleles in the B biotype clade contained the L925I mutation apart from a separate 'susceptible group' (bootstrap value = 636), which also included the GRB strain containing the M918V mutation. The Q-biotype clade consisted of alleles with either L925I or T929V. Sequence polymorphism within the intron therefore gave good discrimination between B and Q biotypes.

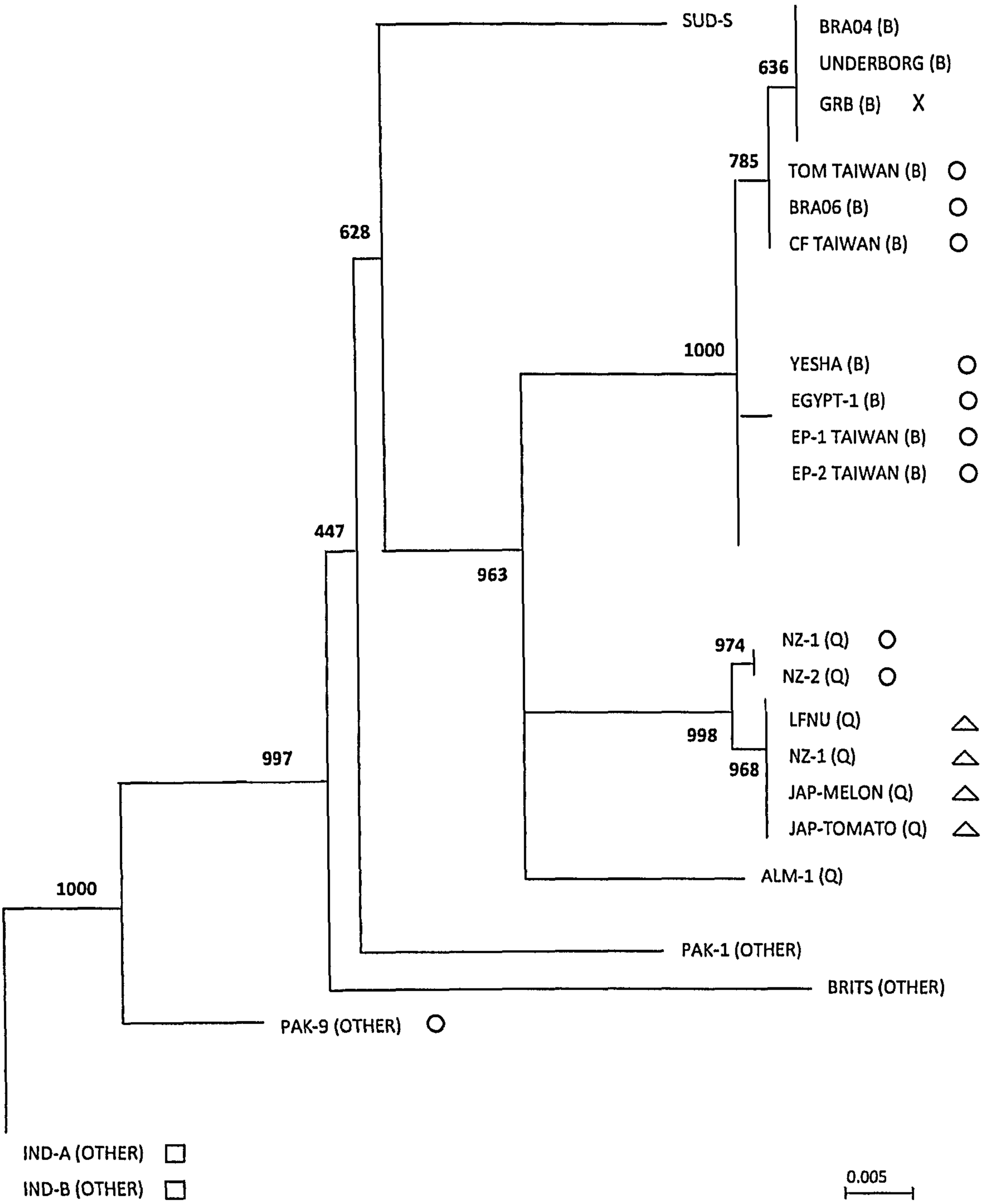


Figure 4.5 Maximum likelihood analysis of the IIS4-S6 intron-1 sequence.

Unrooted maximum likelihood analysis (GTR model) of the intron sequence (~730bp) adjacent to L925 and T929. Bootstrap values were computed over 1000 replications and are shown alongside the branches. The strain name is shown with biotype status in parentheses. The sodium channel genotype is shown next to the strain name with a circle representing L925I, triangle for T929V, square for M918L and a cross for M918V.

4.3.2 The effect of L925I and T929V on insecticide activity

4.3.2.1 TaqMan® PCR allelic discrimination assay for kdr mutations

Two TaqMan® PCR allelic discrimination assays were developed as high-throughput assays to diagnose the *kdr* mutations L925I and T929V in *B. tabaci* individuals. The assays clearly separated those individuals homozygous or heterozygous for either the resistant or susceptible genotypes in real-time fluorescence plots and scatter plot analyses.

4.3.2.2 Screen of Chinese B. tabaci strains for kdr

Female whiteflies from seven Chinese strains (five Q and two B biotype) were screened for L925I and T929V using the TaqMan® assays (Table 4.3). For the two B biotype strains, all *BJ* female whiteflies were L925I homozygotes and this allele was also found at a high frequency in *CHNB* (0.875). T929V was not found in the B biotypes.

The frequency of L925I and T929V varied between the Q biotype strains. No susceptible alleles were detected in any Q biotype individuals. All *WH* individuals were homozygous for L925I while *CHNQ* carried a high frequency of this mutation (0.85). T929V was present at a higher frequency in *JSK* and *JZ* (0.7 and 0.8 respectively) while *GA* carried both mutations at the same frequency.

Table 4.3 The genotype frequency of L925I and T929V in seven strains of *B. tabaci* from China.

Strain	Biotype	N	Genotypes						Genotype frequency		
			L925I/ L925I	L925I/ T929V	T929V/ T929V	L925I/ sus	T929V/ sus	sus/ sus	L925I	T929V	sus
WH	Q	9	9	0	0	0	0	0	1	0	0
JSK	Q	10	1	4	5	0	0	0	0.3	0.7	0
JZ	Q	10	0	4	6	0	0	0	0.2	0.8	0
GA	Q	7	2	3	2	0	0	0	0.5	0.5	0
CHNQ	Q	10	7	3	0	0	0	0	0.85	0.15	0
CHNB	B	8	7	0	0	0	0	1	0.875	0	0.125
BJ	B	7	7	0	0	0	0	0	1	0	0

4.3.2.3 Discriminating dose bioassay

To investigate the effect of L925I and T929V on insecticide activity, the *JZ* strain carrying both mutations (frequency of L925I = 0.2; T929V = 0.8) was exposed to discriminating doses of DDT, fenfluthrin and deltamethrin. The mortality rates from the discriminating dose bioassays are shown in Table 4.4.

SUD-S and JZ exhibited dose response mortality when exposed to DDT however, JZ was far more resistant to this compound with a concentration of 3000ppm required to kill almost 100% of insects. Fenfluthrin was less active than DDT with 200ppm killing only approximately 60% of SUD-S. Concentrations of over 1000ppm were required to kill 90% or more of JZ. Deltamethrin produced an unusual response against both whitefly strains with for example, 300ppm unable to induce 100% mortality against SUD-S. These results indicated good discriminating doses for DDT and fenfluthrin for *kdr* genotype analysis however the ambiguities for deltamethrin suggested that caution should be taken when interpreting results with this insecticide.

Table 4.4 Mortality data for SUD-S and JZ exposed to discriminating doses of DDT, deltamethrin and fenfluthrin.

	SUD-S					*JZ*				
Insecticide	Dose (ppm)	Tested	Dead	Alive	Mortality (%)	Dose (ppm)	Tested	Dead	Alive	Mortality (%)
DDT	10	59	4	55	6.8	300	148	53	95	35.8
	50	40	26	14	65.0	1000	125	98	27	78.4
	100	65	46	19	70.8	3000	131	126	5	96.2
	200	43	42	1	97.7					
Deltamethrin	3	43	11	32	25.6	300	65	26	39	40.0
	30	41	11	30	26.8	1000	111	53	58	47.7
	300	45	37	8	82.2	3000	181	138	43	76.2
Fenfluthrin	10	65	13	52	20.0	300	147	73	74	49.7
	50	114	33	81	28.9	1000	168	155	13	92.3
	100	88	49	39	55.7	3000	156	152	4	97.4
	200	48	30	18	62.5					

4.3.2.4 Frequency of L925I and T929V from individual JZ males

Individual *JZ* males were collected dead and alive from the leaf-dip bioassays and screened for both L925I and T929V using the TaqMan® allelic discrimination assays (Table 4.5). In total, 387 individuals were tested. Sample sizes for some of the survival categories were low due to few survivors at that particular dose. No individual carried L925I and T929V on the same allele and only one individual was susceptible at both residues (L925 and T929).

All male whiteflies carrying the L925I mutation were effectively killed by each concentration of DDT and fenfluthrin. In contrast, 55.6% and 44.4% of L925I hemizygous males survived exposure to 300ppm and 1000ppm of deltamethrin with 90.9% surviving the highest dose (3000ppm).

In general, T929V gave broader resistance to all three compounds. At 300ppm of DDT and fenfluthrin, 60% and 40% of T929V hemizygous males survived exposure while the top dose (3000ppm) gave 17.9% and 11.5% survival. Approximately 50% of all insects carrying T929V survived each dose of deltamethrin.

Table 4.5 Percentage of survivors of L925I and T929V genotypes from discriminating dose bioassays

kdr genotype	Compound	Concentration (ppm)	No. Surviving	No. Dead	n	% survivors
L925I	DDT	300	0	20	20	0.0
		1000	1	4	5	20.0
		3000	0	8	8	0.0
	Fenfluthrin	300	0	16	16	0.0
		1000	0	3	3	0.0
		3000	0	7	7	0.0
	Deltamethrin	300	5	4	9	55.6
		1000	4	5	9	44.4
		3000	10	1	11	90.9
T929V	DDT	300	18	12	30	60.0
		1000	22	20	42	52.4
		3000	5	23	28	17.9
	Fenfluthrin	300	18	27	45	40.0
		1000	12	20	32	37.5
		3000	3	23	26	11.5
	Deltamethrin	300	17	16	33	51.5
		1000	15	14	29	51.7
		3000	10	14	24	41.7

4.4 Discussion

The unprecedented increase in the frequency and severity of whitefly outbreaks during the 1980s and worldwide spread of invasive biotypes led to an increasing reliance on chemical control, based largely on the pyrethroid insecticides. As a result, pyrethroid resistance is now widespread in *B. tabaci* (Cahill *et al.* 1995; Denholm *et al.* 1996; Dennehy & Williams 1997; Luo *et al.* 2010; Roditakis *et al.* 2009). In many instances, resistance has been attributed to an increase in P450 or esterase enzymes (Byrne *et al.* 2000; Byrne & Devonshire 1993; Dittrich *et al.* 1990; Moores *et al.* 2005). More recently however, two target-site mutations (L925I and T929V) in the Na_V channel have been found in *B. tabaci* exhibiting resistance to a range of pyrethroids (Alon *et al.* 2006; Morin *et al.* 2002; Roditakis *et al.* 2006).

The distribution and evolutionary origins of L925I and T929V was investigated from 28 strains of globally collected field samples of *B. tabaci*. L925I was found in B, Q and unknown biotypes whereas T929V was found only in the Q biotype. This is consistent with previous findings (Alon *et al.* 2006) although L925I has not been previously documented from a biotype other than B and Q (PAK-1, Pakistan). The global status of L925I and T929V is consistent with the worldwide distribution of pyrethroid resistance.

The presence of these mutations in strains maintained in the laboratory without selection pressure (CRT-1, GUA-MIX and MURCIA) suggests that any associated fitness cost is low. Furthermore, both mutations were not found on the same allele in any individual in this survey. This possibly reflects one of two scenarios, either; a) the substantial conformational change required in the sodium channel membrane protein to accommodate both mutations results in a severe fitness cost or b) the chance of recombination occurring is very low.

The Q biotype specific nature of T929V is interesting as it is likely that both B and Q biotypes have been subject to similar selection pressure from pyrethroids. T929V requires a double base pair substitution in the nucleotide sequence (AC to GT). T929V has also been reported from the cat flea, *Ctenocephalides felis* (Bass *et al.* 2004). The T929I variant has been reported to occur in combination with the

mutations L1014F and L932F from resistant diamondback moth (*Plutella xylostella* L; Schuler *et al.* 1998) and headlice (*Pediculus capitis;* Lee *et al.* 2003) respectively. T929I has been described as a super-*kdr* mutation resulting in high resistance levels similar to those exerted by other super-*kdr* mutations and T929V (Alon *et al.* 2006). The two resistant genotypes at position 929, valine and isoleucine, are both hydrophobic amino acids indicating that molecular interactions at this site with an insecticide would be similar. T929 is a critical position for pyrethroid and DDT binding (Atkinson 2002; O'Reilly *et al.* 2006; Usherwood *et al.* 2007). The presence of T929V in Q biotype whiteflies may offer a selective advantage over B biotypes when subjected to pyrethroids.

A novel mutation at the super-*kdr* position (M918L) was identified in field samples originating from the South Indian state of Tamil Nadu. This mutation was reported from the cotton-melon aphid, *Aphis gossypii* (Yang, X. & Williamson, M.S. 2001 *GenBank Accession No. AF412815*) but no subsequent studies investigated the consequences for resistance phenotype. The type-II pyrethroid, lambda-cyhalothrin is sprayed in the Coimbatore area (Tamil Nadu, India) where this mutation was discovered (N. Pazhanisamy, Syngenta, pers. comm.) and could feasibly have selected for M918L. Super-*kdr* mutations are usually found in association with a basal kdr mutation (e.g. L1014F) and therefore an investigation downstream of the Na_V IIS6 region is required. Furthermore, characterisation of this mutation and its role in pyrethroid resistance warrants further investigation. The super-*kdr* mutation M918V was identified from Gila River Bend in Arizona (USA) shortly after a switch to synergised pyrethroids to control B biotype whiteflies resistant to all other available insecticides (Morin *et al.* 2002; Dennehy & Williams 1997). However, it has not been reported in another strain collected in the USA or elsewhere. It is possible that this mutation has been lost from subsequent generations as a result of associated fitness costs, and supplanted by L925I.

To gain an insight into the evolutionary origins of L925I and T929V, an intron adjacent to these mutations in the IIS4-S6 region was sequenced to analyse the extent of polymorphism between resistant and susceptible alleles. Sequencing revealed a very high level of polymorphism that was biased by the inclusion of biotypes other than B and Q in the study. The intron contained thirty-six

substitutions and five indels in a re-analysis with only the B and Q-biotypes and this supports results found previously (Alon *et al.* 2006). Inspection of the intron sequence shows no evidence of gene flow between the B and Q-biotypes, strengthening the argument that these biotypes are both genetically and reproductively isolated and this is supported by the two distinct clades (B and Q-biotype) from the phylogenetic analysis (Figure 4.5).

The intron sequence data suggest that L925I has multiple origins. This mutation is present in both B and Q biotypes. Considering the lack of gene flow and reproductive isolation between these biotypes (Dinsdale *et al.* 2010; Liu *et al.* 2007), L925I must have arisen independently in each biotype. Analysis of the surrounding exon and intron sequence suggests that there is no evidence that resistant alleles carrying L925I appear in the different B and Q-biotype clades as a result of recombination (J. Brookfield pers. comm.) and recombination over such a short sequence is unlikely. It is worth noting that little or no intron variation exists between some B biotype resistant alleles from geographically distinct regions implying a single origin for at least one of the L925I alleles followed by migration. This is likely considering the rapid spread of the B biotype throughout the world that is likely to have been caused by long-distance traffic in infested plant material. Similarly, resistant alleles containing T929V from various localities have an identical intron background. This suggests a single origin for this mutation and is consistent with the recent and rapid spread of the Q-biotype. Few samples carried the susceptible wild-type allele demonstrating how widespread these resistance alleles have extended following the spread of invasive biotypes and selection pressure from pyrethroids, and more historically DDT.

Recent electrophysiological and modelling studies have demonstrated that mutations at the L925 and T929 sites within the Na_V channel have a significant effect on insecticide activity and binding. L925I reduces the activity of type-II to a greater extent than the type-I fenfluthrin but not the type-I permethrin (Usherwood *et al.* 2007). T929V appears to be a crucial site for both pyrethroid and DDT binding. The presence of both these mutations in *B. tabaci* offers an interesting opportunity to study the relative contribution of L925I and T929V to resistance. Tsagkarakou *et al.* (2009) reported the frequency of these mutations from various sites in Crete with

varying degrees of pyrethroid resistance however no strong association between resistance and genotype was shown.

The effect of L925I and T929V upon the activity of DDT, fenfluthrin and deltamethrin was investigated in the *JZ* strain from China. Clear associations between the frequency of the *kdr* genotype and dead/alive status could be made by using solely haploid males in the analysis. Unlike the type-I permethrin, fenfluthrin contains only one aromatic ring in the alcohol moiety of the pyrethroid (Figure 4.1). The chemical structure of fenfluthrin is therefore more closely related to DDT than the type-II deltamethrin. Both deltamethrin and permethrin extend further into the binding pocket than DDT and fenfluthrin (O'Reilly *et al.* 2006; Figure 4.2) and therefore, interact with the super-*kdr* site on the IIS4-S5 linker. As a result, mutations at M918 give stronger resistance to deltamethrin and permethrin than either DDT or fenfluthrin (Usherwood *et al.* 2007). DDT and fenfluthrin killed virtually all whiteflies carrying L925I indicating a lack of protection offered by this mutation against these compounds. The L925 residue is positioned on the IIS5 helix upstream and further into the sodium channel binding site than T929V (O'Reilly *et al.* 2006; Figure 4.2) and consequently has a much greater effect on compounds which extend into the sodium channel 'pocket' such as deltamethrin and permethrin. The greater proportion of L925I-carrying whiteflies surviving deltamethrin than DDT or fenfluthrin reflects this hypothesis. On the other hand, T929V offered a broad degree of protection against all insecticides tested. The hydrophilic T929 is the main point of contact for DDT which has a restricted binding area on IIS5 helix while the threonine residue is believed to form tight hydrogen bonds with the ester linkage of type-II pyrethroids (e.g. fenvalerate) increasing neurotoxicity (Davies *et al.* 2008). The data presented here, together with recent modelling studies, suggest that Q biotype *B. tabaci* carrying T929V, unlike other biotype counterparts, have a degree of protection against all compounds targeting the sodium channel.

DDT is an environmentally persistent compound with applications now largely restricted to indoor-habiting public health pests (Hemmingway *et al.* 2004). Fenfluthrin is active against a number of insects and in particular, pests of hygiene (e.g. mosquitoes) and stored products (Behrenz *et al.* 1985). There is little information in the literature describing the use of this compound to combat

whiteflies. The data presented here suggest that fenfluthrin could play an active role in controlling *B. tabaci* and more specifically be used in a resistant management strategy to combat those biotypes carrying the L925I. Regardless of the practical opportunities, the data provide a clear demonstration on how modelling the insecticide target protein and genotyping can help to unravel the effect of target-site mutations upon the activity of insecticides.

A PCR-RFLP molecular diagnostic has been developed to detect both the L925I and T929V mutations (Tsagkarakou *et al.* 2009). The TaqMan® PCR assays described in this chapter offer a high-throughput and sensitive diagnostic for these mutations with the capacity to screen large numbers of field samples and as outlined previously, improve the power of laboratory experiments.

Chapter 5 Molecular characterisation of imidacloprid resistance in *Bemisia tabaci*

5.1 Introduction

The development of the neonicotinoid class of insecticides represents a significant milestone in crop protection. Prior to the introduction of the forerunner neonicotinoid imidacloprid (Bayer CropScience) in 1991, a lack of chemical diversity offering new modes of action placed existing groups of insecticides under increasing threat of resistance. Neonicotinoids act agonistically against post-synaptic acetylcholine receptors (AChR) in the insect nervous system (Tomizawa & Casida 2003). This unique mode of action, coupled with their versatility and selectivity, is largely responsible for their rapid growth. Neonicotinoids target a range of sucking insect pests including whiteflies, aphids, and planthoppers (order Hemiptera) as well as economically-important members of the orders Coleoptera (beetles), Thysanoptera (thrips) and Lepidoptera (moths) (Elbert *et al.* 2008). They exhibit both plant systemicity and translaminar activity and therefore can be applied as seed, soil or foliar treatments (Elbert *et al.* 2008). Today, neonicotinoids are one of the leading insecticide classes on the market and imidacloprid is currently the top-selling insecticide in the world (Nauen *et al.* 2008b). The chemical structure and interaction with nAChRs of the seven commercialised neonicotinoids are reviewed by Jeschke & Nauen (2008).

Despite their success and widespread use, neonicotinoid resistance has been surprisingly, and somewhat fortunately, slow to develop compared with other insecticide groups (Nauen & Denholm 2005). Significant levels of neonicotinoid resistance have been documented in field populations of the cotton whitefly, (*Bemisia tabaci*) (Horowitz *et al.* 2007; Nauen & Denholm 2005), the glasshouse whitefly (*Trialeurodes vaporarium*) (Gorman *et al.* 2007), the Colorado potato beetle (*Leptinotarsa decemlineata* Say) (Zhao *et al.* 2000), the brown planthopper (*Nilaparvata lugens* Stål) (Gorman *et al.* 2008; Wang *et al.* 2009) and more recently the peach potato aphid (*Myzus persicae* Sulzer) (Puinean *et al.* 2010). Laboratory selection of resistance has been achieved in *Drosophila melanogaster* Meigen

(Daborn *et al.* 2001) as well as in houseflies (*Musca domestica*) and German cockroaches (*Blatella germanica*) (Wen & Scott 1997).

B. tabaci was the first species to develop resistance to a neonicotinoid, highlighting its status as a primary crop pest and demonstrating the challenges associated with managing insecticide resistance in this species (Denholm *et al.* 1998; Nauen & Denholm 2005). The first report of imidacloprid resistance came from Q biotype *B. tabaci* originating from the Almeria region of southern Spain in 1994 (Cahill *et al.* 1996). Resistance in this area of intensive horticultural production increased gradually to over 100-fold by the year 2000 following continuous systemic and foliar applications (Nauen *et al.* 2002). The first documented case of imidacloprid resistance in field collected B biotypes was made from Guatemala (Byrne *et al.* 2003). Continued reliance on neonicotinoids for *B. tabaci* control has led to widespread reports of resistance (Horowitz *et al.* 2004; Luo *et al.* 2010; Nauen & Denholm 2005; Roditakis *et al.* 2009; Vassillou *et al.* 2009). Resistance management tactics have been implemented in some countries as part of integrated pest management (IPM) programmes in order to preserve the efficacy of neonicotinoids (Horowitz *et al.* 1994; Palumbo *et al.* 2001). For example, *B. tabaci* infestations on Arizona cotton, melon and vegetable crops have been successfully controlled since the early 1990s by rotating neonicotinoids with the insect growth regulators (IGR) buprofezin and pyriproxyfen (Dennehy & Williams 1997; Ellsworth & Martinez-Carrillo 2001; Palumbo *et al.* 2001).

Much of our understanding on the mechanisms underlying imidacloprid resistance comes from studies on *B. tabaci*. Susceptibility was recovered in imidacloprid-resistant *B. tabaci* populations following treatment with the synergist piperonyl butoxide, indicating the involvement of microsomal monooxygenase activity (Nauen *et al.* 2002). Rauch & Nauen (2003) demonstrated an increase in O-deethylation of 7-ethoxycoumarin, a measure of cytochrome P450-dependent monooxygenase activity, in resistant whitefly strains. The authors also showed the presence of the 5-hydroxy imidacloprid metabolite (IMI-5-OH) in resistant strains following exposure to radiolabelled [^{14}C] imidacloprid whereas IMI-5-OH was not found at detectable levels in the susceptible strain.

The discovery of a mutation (Y151S) present in two nAChRs subunits (Nla1 and Nla3) of a strain of *N. lugens* selected in the laboratory for resistance to imidacloprid indicated the presence of target-site resistance (Liu *et al.* 2006). Despite an extensive screen of field populations this mutation has not been detected since (Gorman *et al.* 2008). Target-site resistance to neonicotinoids has also been proposed, though not confirmed, in *D. melanogaster* (Perry et al. 2008) and *L. decemlineata* (Tan *et al.* 2008). In ligand-binding experiments using [^{3}H]imidacloprid, the lack of any difference between resistant Q biotypes and a susceptible strain of *B. tabaci* suggested an absence of any altered nAChRs (Rauch & Nauen 2003).

Several specific *CYP* genes encoding P450-dependent monooxygenases have been implicated in enhanced detoxification leading to insecticide resistance. For example, over-transcription of *CYP6G1* in *D. melanogaster* is necessary for DDT resistance and causes broad cross-resistance to an insect growth regulator, organophosphates, carbamates and neonicotinoids (Daborn *et al.* 2001; Daborn *et al.* 2002; Le Goff *et al.* 2003; Pyke *et al.* 2004). Le Goff *et al.* (2003) identified two more genes, *CYP12D1* and *CYP6A8*, upregulated in DDT-selected *D. melanogaster* strains. A number of P450s from the *CYP6* family have been associated with pyrethroid resistance in mosquitoes (Amenya *et al.* 2008; David *et al.* 2005; Muller *et al.* 2007; Muller *et al.* 2008; Nikou *et al.* 2003) and *CYP6P3* has been expressed *in vitro* and shown to metabolise type-I and type-II pyrethroids (Muller *et al.* 2008).

Puinean *et al.* (2010) recently conducted a thorough characterisation of the mechanisms conferring resistance to four neonicotinoids in *M. persicae*. Microarray and subsequent quantitative PCR analysis revealed the over expression (via gene amplification) of the P450 *CYP6CY3* and several genes encoding cuticular proteins. No evidence of target-site resistance was found confirming previous findings. This study strongly indicates that neonicotinoid resistance in *M. persicae* is due primarily to P450-induced detoxification and other mechanisms, based on reduced cuticular penetration, may play a significant role.

Karunker *et al.* (2008) isolated several P450 cDNAs from *B. tabaci* representing the *CYP4* and *CYP6* families and characterised their expression in imidacloprid-resistant and -susceptible insects. The authors demonstrated a strong correlation between

mRNA levels of one of these genes, *CYP6CM1*, and the LC_{50} values of imidacloprid in B and Q biotypes. Moreover, three SNPs in the first intron of the Q biotype *CYP6CM1* allele (*CYP6CM1-Q*) were associated with reduced susceptibility to imidacloprid. Following this association between *CYP6CM1* and imidacloprid resistance, the Q biotype *CYP6CM1* allele (*CYP6CM1-Q*) was heterologously expressed *in vitro* and catalysed imidacloprid into IMI-5-OH (Karunker *et al.* 2009). The authors also identified the key amino acids and hydroxylation site within *CYP6CM1-Q*.

It is widely assumed that resistance to insecticides is consistent throughout the life cycle of the insect. Recently, however, Nauen *et al.* (2008a) demonstrated in leaf-dip bioassays that nymphs of laboratory susceptible *B. tabaci* were 4-10 times less sensitive to imidacloprid compared with their adult counterparts. In the same study, the LC_{50} of adults from resistant field strains was significantly reduced in nymphs (N2/N3), to levels close to or not significantly different from the susceptible adult population (Nauen *et al.* 2008a). Few other examples of age-specific resistance exist (Daly *et al.* 1988). The recent association of *CYP6CM1* with imidacloprid resistance (Karunker *et al.* 2008; Karunker *et al.* 2009) makes this enzyme the most likely candidate underlying this age-specific resistance.

Imidacloprid residues remain largely on the surface of the leaf following a foliar spray whereas the insecticide is metabolised completely in a soil or seed treatment (Nauen *et al.* 1999). Metabolites of imidacloprid and other neonicotinoids have been characterised from various biological systems (Ford & Casida 2006; Ford & Casida 2008; Nishiwaki *et al.* 2004). A summary of imidacloprid metabolites reported in the literature is shown in Figure 5.1.

Metabolites of imidacloprid show activity against *B. tabaci* and the peach-potato aphid, *M. persicae*, in bioassays (Nauen *et al.* 1998; Nauen *et al.* 1999). For example, the 4-hydroxy (IMI-4-OH) metabolite is as active as the parent compound (imidacloprid) whereas the 5-hydroxy (IMI-5-OH) metabolite is less active against resistant and susceptible *B. tabaci* strains (Nauen *et al.* 1999; Rauch & Nauen 2003). The olefin metabolite (IMI-ole) is at least 10-fold more potent than imidacloprid against *B. tabaci* although this compound is found at much lower levels. Thin layer chromatography revealed that IMI-5-OH is the major metabolite present in resistant

strains and is at undetectable levels in susceptible populations (Rauch & Nauen 2003).

The aim of the study presented here was to characterise and improve our understanding of the mechanisms underlying imidacloprid resistance in *B. tabaci*. The gene expression of *CYP6CM1* was analysed in adults, pupae and larvae from four *B. tabaci* strains to assess whether this P450 is responsible for age-specific imidacloprid resistance. The same four strains were subject to imidacloprid treatment and the imidacloprid metabolite, IMI-5-OH, was detected and quantified using liquid chromatography/mass spectrometry (LC/MS). Finally, a TaqMan® allelic discrimination approach was used to assess the previously documented association of a SNP within the intron of *CYP6CM1* and imidacloprid resistance in Q biotype *B. tabaci*.

Figure 5.1 Imidacloprid and associated metabolites reported from the literature.

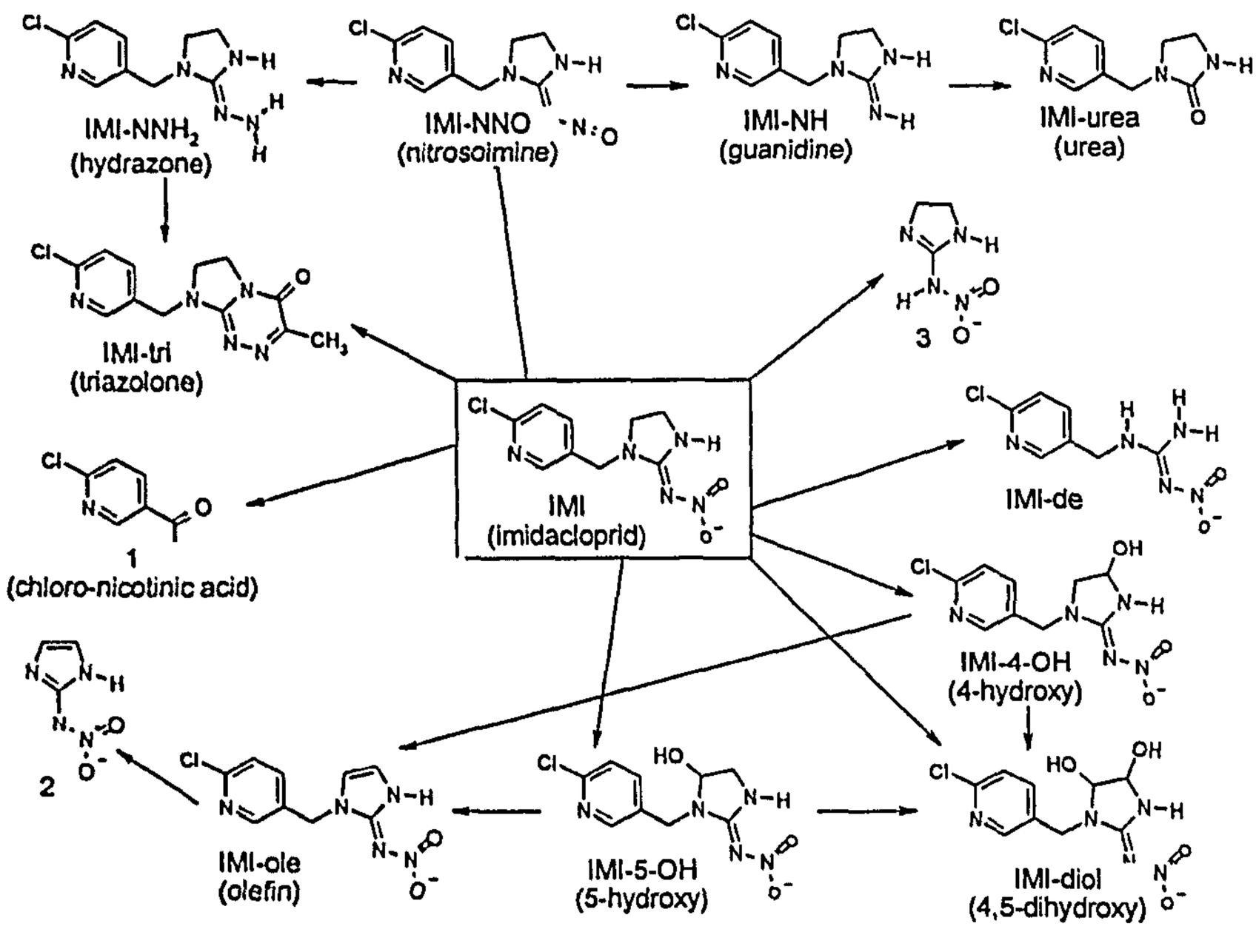


Figure courtesy of M. Daniels (Jealot's Hill, Syngenta)

5.2 Materials and methods

5.2.1 CYP6CM1 expression

5.2.1.1 Insect strains

All insects were collected from *B. tabaci* strains reared at Rothamsted Research on cotton plants (*Gossypium hirsutum* cv. Linda) at 26 ± 2°C under a 16 hour photoperiod. The Q biotype strains used throughout the experiments were ALM07 (Spain 2007) and CRT-1 (Crete 2006) and the B biotype strains were GRB/MEX (USA/Mexico 2006) and PIRGOS (Cyprus 2003). The biotype status of each strain was determined using the B/Q TaqMan allelic discrimination assay described in Chapter 3 and by Jones *et al.* (2008).

5.2.1.2 Insecticides

The formulated neonicotinoids used in leaf-dip bioassays were imidacloprid ('Confidor', 25% SL, Bayer Crop Science, Monheim) and thiamethoxam ('Actara', 20% WG, Syngenta). All serial dilutions were made in 0.01% of the non-ionic wetter Agral® (Zeneca Agrochemicals).

5.2.1.3 Leaf-dip bioassays

The resistance levels of adult *B. tabaci* to both imidacloprid and thiamethoxam were assessed using the adult leaf-dip bioassay described in Chapter 2.2. For any assessment of insect mortality following exposure to an insecticide, it is important to account for any potential experimental or biological variation. All insects must be from a fit and healthy population and be of a consistent age, sex and size. Only 10-15 day-old diploid female whiteflies were used and all concentrations were assayed in triplicate with approximately 20 adults placed in each dish. Mortality was recorded after 72 hours as this end time point has provided the most consistent data following neonicotinoid treatment (Kevin Gorman *pers. comm.*).

Resistance in *B. tabaci* nymphs was characterised using a modified leaf-dip bioassay described by Nauen *et al.* (2008a). A dose-response was generated only for those nymphs treated with imidacloprid due to a lack of N1/N2 numbers on the leaves

treated with thiamethoxam. Leaves on 15-day old cotton plants were cut into rectangles approximately 40 x 50mm and 30-50 female *B. tabaci* per leaf were allowed to lay eggs on these leaves for 24 hours in a cage. The adults were removed and the eggs left to hatch and develop into the N1 or N2 stage for 9 days. Leaves harbouring the nymphs were then dipped into insecticide solutions diluted in Agral®. Five concentrations of insecticide were used to generate a dose-response curve and for controls the leaves were dipped solely in (0.01%) Agral®. The number of nymphs per leaf varied (20-200) due to a difference in fecundity between strains and the initial number of egg-laying females. Each bioassay was therefore conducted in triplicate. Bioassays were incubated at 28°C and mortality scored 20 days following egg lay with those individuals which did not emerge as adults scored as 'dead'.

5.2.1.4 RNA extraction

Total RNA for gene expression was extracted from separate collections of one-hundred and fifty adult (mixed sex), pupae and N1/N2 nymphs from each strain using TRIzol® reagent (Molecular Research Inc.) as described in Chapter 2.4. Immature *B. tabaci* were collected from lower cotton leaves from standardised whitefly rearing cages. The insects were confirmed as either N1 or N2 under a light microscope and were placed in a 1.5 ml sterile plastic tube and snap-frozen in liquid nitrogen. RNA samples were re-suspended in 15 µl of DEPC-treated water and treated with 1 µl of DNase I (Promega) to remove any contaminating gDNA. All RNA samples were quantified using the ND-1000 spectrophotometer (NanoDrop Technologies Inc.) and the integrity of the RNA was checked on an agarose gel (Chapter 2.6).

5.2.1.5 cDNA synthesis

First strand cDNA was synthesised from total RNA using Superscript II Reverse Transcriptase (Invitrogen) following the methods in Chapter 2.8. For amplification of the *CYP6CM1* and *actin* genes, 1 µl of oligo(dT)$_{20}$ (500 µl/ml) was added to the reaction whereas for *18S rRNA*, 1 µl of a gene specific primer (18SR2; 5'-GTTGAGTCCCGCATCGGTATT-3') was used. To ensure homogeneity between different biological samples for relative gene expression the amount of input RNA should be approximately the same between samples (Pfaffl 2006), although it cannot

be guaranteed that each cDNA synthesis reaction will be equally efficient across all reactions. An approximate final total of cDNA synthesised was calculated using:

$$\text{cDNA concentration} = \frac{\text{sample RNA concentration } \left(\frac{\text{ng}}{\mu\text{l}}\right) \times \text{total RNA input}}{\text{total volume cDNA synthesis reaction (20 } \mu\text{l)}}$$

The cDNA was stored at -20°C and diluted to the required concentration for gene expression in nuclease-free sterile distilled water.

5.2.1.6 Relative gene expression

Reverse transcription (RT) followed by PCR amplification is a powerful method to quantify gene expression (Pfaffl 2006). The use of real-time quantitative RT-PCR (qRT-PCR) is highly specific, sensitive and reproducible, covering a wide dynamic range of gene expression even at low levels. Relative quantification measures the change in steady-state mRNA levels of a target gene relative to a reference group such as an untreated control (Bustin *et al.* 2005). In relative gene expression studies, constantly expressed endogenous control genes are required to normalise target quantities and as a means of correcting data that may be affected by differences in cDNA input. Suitable reference genes for relative gene expression include *GAPDH*, *ß-actin* and *rRNA*.

To measure gene expression in samples relative to an untreated control, the comparative C_t method (also known as the $\Delta\Delta C_t$ or $2^{-\Delta\Delta Ct}$ method) is a widely used tool (Livak & Schmittgen 2001). $\Delta\Delta C_t$ uses an arithmetic formula to calculate the relative changes in gene expression based on the threshold cycle (C_t) of the real-time PCR reactions. The derivation of this formula is discussed in Livak & Schmittgen (2001). The $\Delta\Delta C_t$ method may only be used if the PCR amplification efficiencies between the target and endogenous control gene are approximately equal. This is determined by checking how C_t values vary with template dilution using:

$$(\Delta Ct = Ct\ \text{Target} - Ct\ \text{Endogenous Control})$$

The ΔC_t values are plotted against the log of RNA input. If the value of the slope of the semi-log regression line is <0.1 then this is a general criterion for passing the validation successfully (Appendix 8.3).

It is important to assess the accuracy and reproducibility of results with different endogenous control genes and therefore, in this study, *18S rRNA* and *actin* were tested as reference genes for quantification of *CYP6CM1*. Standard curves were made for each gene by testing the assay on serial dilutions of cDNA from 150 whiteflies from the ALM07 strain. The R value, gradient of the slope (M value) and the PCR efficiency of each curve is presented in Appendix 5.1. The C_t values of each gene were assessed for continuity across different strains of *B. tabaci* and a validation assay, as described above, was performed against *CYP6CM1* to ensure the reaction efficiencies were similar (Appendix 5.1).

5.2.1.7 Design of TaqMan® relative gene expression assays

The target and endogenous control genes were amplified using TaqMan® chemistry. Each gene-specific probe was labelled with the dye 6-FAM. A comprehensive description of TaqMan® chemistry is given in Chapter 2.10.

Gene sequences were available from the National Centre for Biotechnology Information (NCBI) database (GenBank) for B and Q biotype *CYP6CM1* (EU642555 and EU344879 respectively) and for *B. tabaci 18S rRNA* (Z15051). The *B. tabaci actin* gene sequence was kindly provided by Shai Morin (Hebrew University, Jerusalem, Israel). The suitability of using TaqMan® PCR to quantify each gene was checked using the Primer Express Software version 2.0 (Applied Biosystems, Foster City, CA, USA). A 100-300bp region of the *CYP6CM1, 18S rRNA* and *actin* sequences were sent to Applied Biosystems for assay design and manufacture. Any SNP between B and Q biotypes and intron sequences were avoided in the design. The design of each TaqMan® gene expression assay including the oligonucleotide primer and 6-FAM probe sequences are shown in Table 5.1, Figure 5.2, Figure 5.3 and Figure 5.4.

Table 5.1 TaqMan® relative gene expression oligonucleotide probes and primers.

Assay Name	Primer/Probe	Sequence (5'-3')
CYP6CM1-cm1c	CYP6CM1-F	CACCCGAAACTTCCTGTTCTTCTTA
	CYP6CM1-R	CCTCTTTAATGGCCTCCCTGAA
	CYP6CM1-probe	*TCGCACAGCTTTCC-6FAM*
18SrRNA-18Sb	18SrRNA-F	TTTCGACGGTCGGGTATTGG
	18SrRNA-R	CGAACCCTGATTCCCCGTTA
	18SrRNA-probe	*CCCGTCGCAACCAC-6FAM*
Actin	Actin-F	GTGTAATGGTCGGTATGGGTCAAAA
	Actin-R	ATGATACCGTGCTCGATGGG
	Actin-probe	*ACGAAGCCCAGTCCAG-6FAM*

All primers and probes for each assay were designed by Applied Biosystems from a submitted sequence. The three probes each contain a 6-FAM reporter dye and are highlighted in italics.

```
GATGACGCTC CCCGTGCTGT CTTCCCCTCC ATCGTCGGTC GGCCAAGACA 50
TCAGGGTGTA ATGGTCGGTA TGGGTCAAAA AGACTCGTAC GTAGGTGACG 100
AAGCCCAGTC CAACAGAGGT ATCCTCACCC TGAAATACCC CATCGAGCAC 150
GGTATCATCA CAAACTGGGA TGACATGGAA AAGATCTGGC ATCACACCTT 200
CTACAACGAG CTCCGAGTTG CCCCAGAAGA GCACCCCATC CTCCTGACCG 250
AAGCCCCCCT CAACCCCAAG GCCAACCGTG AAAAGATGAC TCAGATCATG 300
TTTGAAACCT TCAACACACC CGCCATGTAC GTCGCCATCC AGGCTGTGCT 350
CTCCCTGTAC GCCTCCGGTC GTACCACCGG TATCGTCTTG GACTCCGGAG 400
ATGGTGTCTC CCACACCGTC CCCATCTACG AAGGTTACGC CCTCCCCACG 450
CCATCCTNCG TTTGGACTTG GCTGGTCGTG ACTTGACCGA CTACCTCATG 500
AAAATCTACT GAACGTGGTT ACAGCTTCAC CACCACAGCT GAGAGAGAAA 550
TTGTCCGTGA CATCAAGGAG AAATTATGTT ACGTCGCCTT ANACTTCGAG 600
CAGGAAATGG CCACCGGCGC TGCCTTCACC TNATTAAAAA AGTNCTACGA 650
ATTGGCCGAC GGACAGGTCA TNCAATTCGG AAAACGAAAG GTTCCGTTGT 700
CC                                                      702
```

Figure 5.2 *Bemisia tabaci* actin sequence

TaqMan® primers (blue) and the FAM MGB probe (green) used in expression of *actin* are highlighted.

CTGGTTGATCCTGCCAGTAGTCATATGCTCGTCTCAAAGACTAAGGCATG 50

CATGTCTCAGTGCAAGCCGAACTAAGGTGGAACCGCGAAAGGCTCATTAA 100

ATCAGTTGTCGTTCACAGGATCGTGCCACACCCTACATGGATAACTGTGG 150

TAATTCTAGAGCTAATACATGCCGATAGAGTCCCGGCAACTCCCCGGGCT 200

CGCCCGGGGCAGGGACGCTTTTATTAGATCAAAACCGACACCGCGGGCCT 250

CCGGGTCCGCGGTTTCTCCAGCACTTCGCGTGCACACTGGACGATTCCGA 300

ATAACTCCGGCCGATCGCATCGGACCCTCGCGGTCCGGGCGACGCATCCT 350

TCAAATGTCTGCCTTATCAACTTTCGACGGTCGGGTATTGGCCTACCGTG 400

GTTGCGACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCCTG 450

AGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAC 500

TCCCGGCACGGGGAGGTAGTGACGGAAAATACCGATGCGGGACTCAACCG 550

AGGCCCCGTGATCGGAATGAGCGCCCCGTAAACAAGGCGACGAGGACCCA 600

TTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGG 632

Figure 5.3 *Bemisia tabaci* 18S rRNA sequence

TaqMan® primers (blue) and the FAM MGB probe (green) used in expression of 18S rRNA are highlighted.

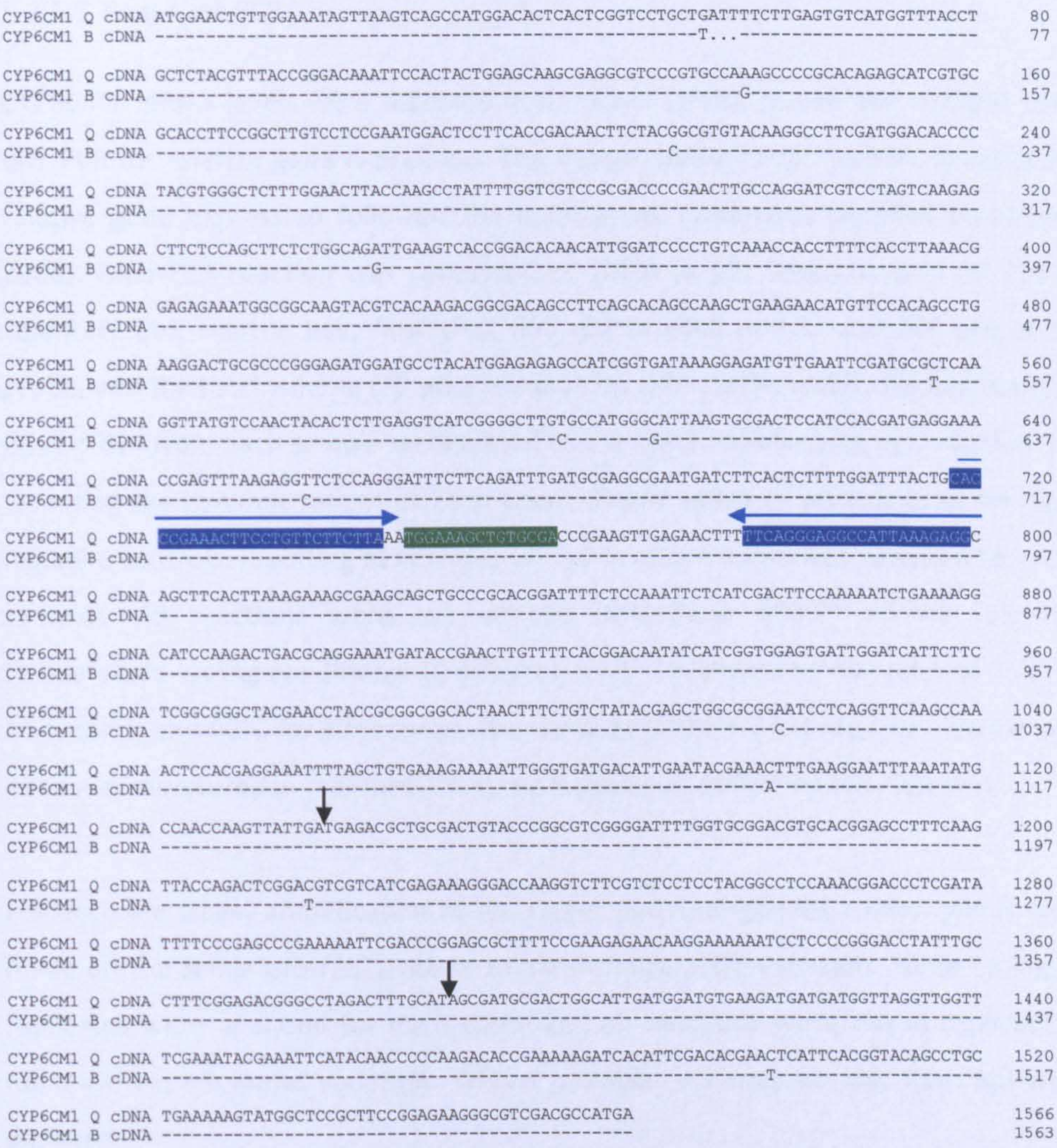


Figure 5.4 Alignment of *Bemisia tabaci* B and Q biotype full length CYP6CM1 cDNA sequences.

TaqMan gene expression primers (blue) and the FAM MGB probe (green) for gene expression are highlighted. SNPs between biotypes are shown and the conserved sequence is represented by a dash (-). Black arrows indicate the position of the intron.

5.2.1.8 Real-time PCR

CYP6CM1 mRNA levels were assessed in *B. tabaci* adults, pupae and nymphs using qRT-PCR for relative gene expression. The design, optimisation and performance of relative gene expression followed the appropriate guidelines (Applied Biosystems 2008). Each PCR reaction was composed of cDNA (5 µl), SensiMix DNA kit (10 µl; Quantace Ltd, Neutral Bay, Australia), 900 nM of each primer and 200 nM of the probe, and the total volume (20 µl) was made up with sterile water. Approximately 5 ng of cDNA from each sample were added to the reaction following optimisation and assessing the dynamic ranges of each assay. Sterile water (5 µl) was used for blank negative controls. Pipetting at volumes of 5 µl or above minimises variation between samples. All reactions were run on the RotorGene 6000™ at the following temperature cycling conditions: 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The increase in the 6-FAM reporter dye for each gene was monitored in real-time using the RotorGene 6000™ system software.

For each life stage, amplification of the target and endogenous control genes were made on the same 100-well plate to minimise intra-plate variation. Three biological replicates were analysed for each strain and all reactions were run in triplicate to minimise experimental variation. Where possible, volumes no less than 5µl were pipetted.

5.2.1.9 Data Analysis

Adult and nymphal dose-response data were subject to probit analysis using PoloPlus© statistical software (LeOra Software, Berkeley, CA) (Chapter 2.1). The LC_{50} of ALM07, CRT-1 and GRB/MEX were divided by the LC_{50} of the most susceptible strain PIRGOS (LC_{50} equivalent to the standard susceptible strain SUD-S) to generate resistance ratios.

The C_t values from the RotorGene 6000™ software were imported directly into Microsoft Excel. The fold change in *CYP6CM1* expression, normalised to *actin* and relative to PIRGOS, was calculated using the $2^{-\Delta\Delta Ct}$ formula within each life stage. The standard deviation and 95% confidence limits of$2^{-\Delta\Delta Ct}$ were determined from

the triplicate samples. Significance between strains was assumed if the 95% confidence limits of the $2^{\wedge -\Delta\Delta Ct}$ values did not overlap.

5.2.2 CYP6CM1 intron SNP analysis

5.2.2.1 Insect strains and insecticides

The Q biotype *B. tabaci* strains used were CRT-1 (Crete 2006) and MURCIA (Spain 2007) reared at Rothamsted Research under standard conditions (Chapter 2.1). Formulated imidacloprid ('Confidor', 25% SL, Bayer Crop Science, Monheim) was used in all bioassays.

5.2.2.2 Discriminating dose bioassay

Prior to the discriminating dose bioassay, an imidacloprid dose-response curve was generated for the MURCIA strain. MURCIA exhibited high resistance to imidacloprid with an LC_{50} of 408ppm and a resistance ratio of 136 compared with the susceptible PIRGOS strain. Consequently, discriminating concentrations in leaf-dip bioassays of 128 ppm for MURCIA and 32 ppm for CRT-1 were used, intended to kill half of the population. Approximately 20 female whiteflies were assayed at each concentration in triplicate. Whiteflies killed by imidacloprid at 24, 48 and 72 hours as well as those individuals still alive after 72 hours were collected for DNA extraction for SNP genotyping.

5.2.2.3 DNA extraction

All genomic DNA extractions were performed on individuals following the protocol described in Chapter 2.3. Haploid males were used for sequencing the *CYP6CM1* intron whereas diploid females were used for genotyping. DNA was re-suspended in 15 µl of sterile distilled water and quantified using the ND-1000 spectrophotometer (NanoDrop Technologies Inc.).

5.2.2.4 Sequencing the CYP6CM1 intron

Forward and reverse primers were designed to amplify a 325bp fragment of the *CYP6CM1* intron containing the SNPs reported by Karunker *et al.* (2009) (Table 5.2).

gDNA (1 µl) samples from two Q biotype strains maintained at Rothamsted Research (CRT-1 and CHLORAKA) were PCR amplified following the conditions outlined in Chapter 2.5. The PCR products were run on an agarose gel (Chapter 2.6) and then sequenced following the protocols outlined in Chapter 2.7 and 2.8.

5.2.2.5 TaqMan allelic discrimination assay

An alignment of the *CYP6CM1* phase-1 intron from Q-biotype resistant and susceptible alleles is shown in Figure 5.5. Karunker *et al.* (2009) identified three SNPs associated with imidacloprid resistance named here as SNP1 (C/A at intron position 195), SNP2 (C/G at position 230) and SNP3 (A/G at position 242). Following sequence analysis with Primer Express, SNP1 was chosen as the target SNP for allelic discrimination. TaqMan® primers and MGB probes were designed by Applied Biosystems to target SNP1 (Figure 5.5).

Table 5.2 Oligonucleotide primers and TaqMan® primer and probes used in CYP6CM1 intron experiments.

Primer name	Sequence (5'-3')	Reporter dye
BEMINTRON-F1	ATATGCCAACCAAGTTATTGATG	-
BEMINTRON-F2	CAACCAAGTTATTGATGGTGAGT	-
BEMINTRON-R1	AACGATAACTCCGCAAGTCTGA	-
BEMINTRON-R2	CTCCGCAAGTCTGAATTATCG	-
SNP1-F	GCCTTTGATTTACAGGATTTTTATTTTTATTTACTATAGGT	-
SNP1-R	GAAATCAATAGATAACTCCTCCTACAATAGCA	-
SNP1-VIC	ATGCAGACACACATC	VIC
SNP1-FAM	ATGCAAACACACATC	FAM

Primers named *BEMINTRON* were used to PCR amplify the *CYP6CM1vQ* intron and those named *SNP1* were used in the TaqMan® allelic discrimination assay.

5.2.2.6 TaqMan PCR

Allelic discrimination was performed following the protocol outlined in Chapter 2.10. TaqMan® Genotyping Master Mix was used to ensure a clear discrimination between homozygous and heterozygous genotypes. The increase of VIC and FAM reporter dyes, representing the SNP1-SUS and SNP1-RES alleles respectively, was monitored in real-time using the Rotor-Gene 6000™ software.

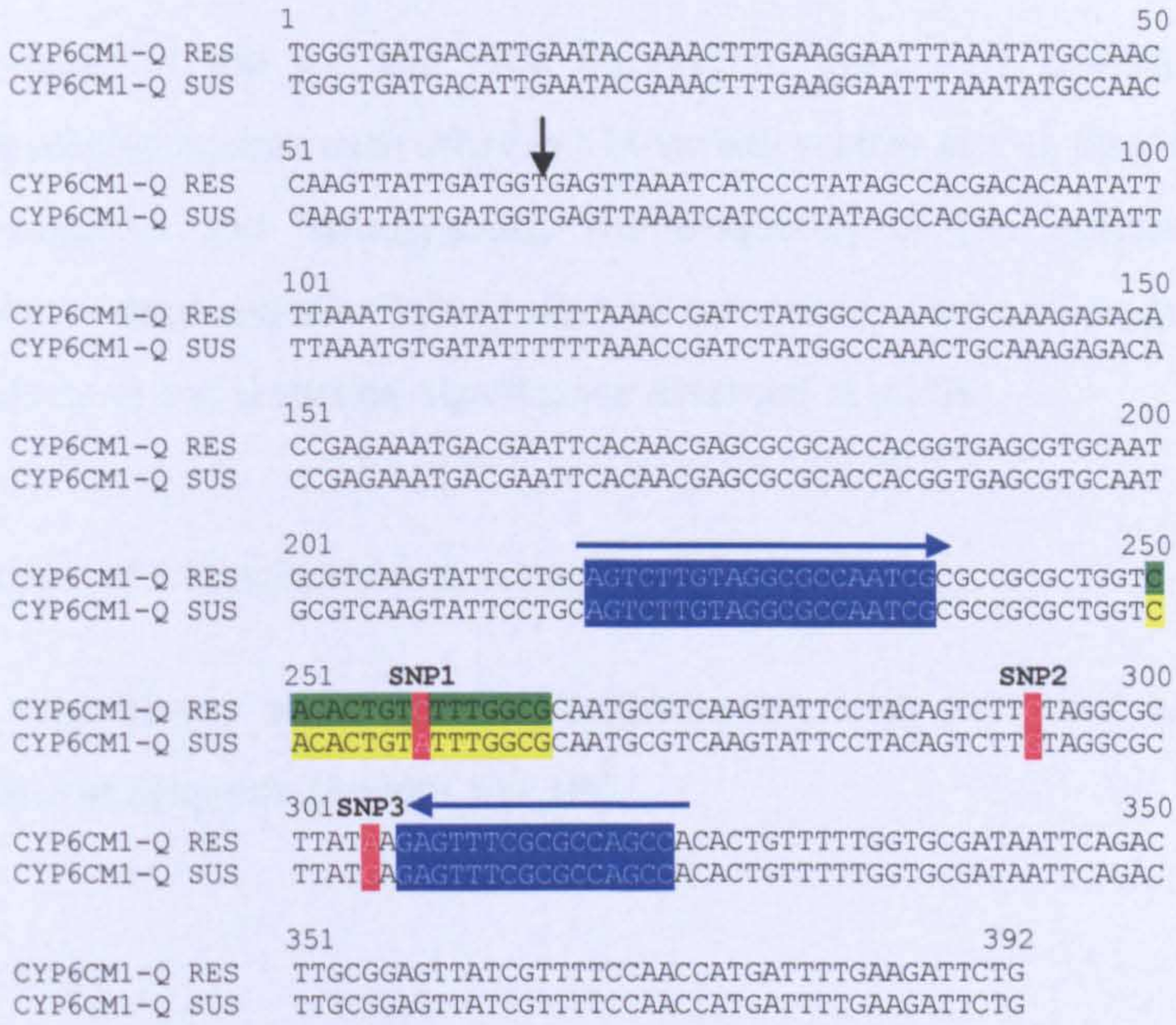


Figure 5.5 Alignment of the resistant and susceptible alleles of the CYP6CM1-Q intron.

Imidacloprid resistance associated SNPs are highlighted in red. The black arrow represents the intron start. TaqMan® oligonucleotide primers (blue) and the fluorescent dye-labelled probes (VIC (yellow); FAM (green)) are shown.

5.2.2.7 Data analysis

The endpoint values of the VIC and FAM fluorescent dyes were corrected for background and plotted against each other in a bi-variate scatter plot to discriminate between heterozygotes and homozygotes. The frequency of the resistant and susceptible alleles in dead and alive individuals was compared using a chi-square test (χ^2) (GenStat Software) and statistical significance assumed at ≤0.05.

5.2.3 Metabolism of imidacloprid in B. tabaci

The following experiments were done in collaboration with Rob Lind and the Biochemistry team at Syngenta (Jealot's Hill, UK).

5.2.3.1 Insect strains

The *B. tabaci* strains used in the metabolism experiments were the same as those used in the analysis of *CYP6CM1* expression and are described in Chapter 2.2.1.1.

5.2.3.2 Imidacloprid treatment

Technical grade imidacloprid was dissolved in 60% acetone to make a concentration of 1ppm. This is the dose required to kill 50% of a susceptible insect population. Individual whiteflies (mixed sex) were placed on cotton leaf discs (40 mm) maintained on agar in a plastic Petri dish and treated with 0.25 µl of imidacloprid using a micro-applicator. Insects and leaf discs were chilled on ice during application. Care was taken to ensure that no physical damage to the insect was caused from the application needle. Approximately four-hundred insects from each strain were treated and left for 5 hours as the optimum time for recovery of the IMI-5-OH metabolite. Collections of 400-500 insects were made from the rearing cage for matrix matched standards and all samples were frozen at -80°C prior to preparation for LC/MS.

5.2.3.3 Sample preparation

The fresh weight of untreated and treated whitefly samples was determined to account for any differences between strains. Whitefly samples were placed in

extraction tubes (Precellys 24, 0.5 ml, lysing matrix VK05; Bertin Technologies; France) and 100% acetonitrile (500 µl, HPLC-grade; Merck, Germany) was added to each sample. Samples were homogenised for 5 x 20s using a Fast Prep® FP120 (Qbiogene, UK). Extraction tubes were centrifuged at 10,000 rpm for 20 minutes and the supernatant was transferred into HPLC vials. Parent compound and major metabolites present in the samples were determined using LC-MS.

5.2.3.4 Liquid chromatography-mass spectrometry (LC/MS)

The LC/MS detection of imidacloprid metabolites was developed and performed under the supervision of Miriam Daniels (Syngenta, UK).

5.2.3.5 High performance liquid chromatography (HPLC)

Whitefly samples were analysed on a Waters ACQUITY UPLC. Gradient elution of the analyte was achieved using a Phenomenex Luna (C18, 5 µm, 4.60 x 250 mm) analytical column. The mobile phase consisted of a gradient of water (+0.2% formic acid) and acetonitrile with a flow rate of 0.8 ml/min. Summaries of the conditions used are outlined in Appendix 8.4.

5.2.3.6 Mass spectrometry

The mass spectrometer used was a Finnigan TSQ Quantum Discovery (Thermo Electron Corporation, USA) equipped with an Ion Max source operating in positive ion mode. Operating parameters for the electrospray ionisation are outlined in Appendix 8.4. Compounds of interest were detected using selected-reaction-monitoring (SRM).

5.2.3.7 Sample analysis, calibration and quantitation

Integration of peaks was performed using Xcalibur™ 2.0 (Thermo Electron Corporation). Standard curves were constructed using reference calibration solutions prepared in 100% acetonitrile. The parent compound (IMI) and 6 metabolites (IMI-urea, IMI-ole, IMI-5-OH, IMI-de, IMI-1 and IMI-3) were available as authentic standards for quantification (Table 5.3). For all other compounds (IMI-4, IMI-NNH2,

IMI-NH, IMI-tri, IMI-diol, IMI-NNO), the transitions were predicted by staff at Syngenta. Matrix effects were taken into account by spiking control samples containing whitefly samples with the reference compounds. Total compound in samples was determined from the standard curve.

Table 5.3 Molecular weight of imidacloprid metabolites and availability of authentic standards for quantification using LC/MS.

Compound	Molecular weight	Standards available
IMI	255.67	Yes
IMI-NH	210.67	No
IMI-NNO	239.67	No
IMI-NNH_2	255.68	No
IMI-tri	277.72	No
IMI-ole	253.65	Yes
IMI-5-OH	271.66	Yes
IMI-4-OH	271.66	No
IMI-diol	287.66	No
IMI-urea	211.65	Yes
IMI-de	229.63	Yes
IMI-1	157.56	Yes
IMI-2	128.00	No
IMI-3	130.10	Yes

5.3 Results

5.3.1 CYP6CM1 expression

5.3.1.1 Adult response to imidacloprid and thiamethoxam

The LC_{50}s, probit line slopes, resistance ratios and probit lines from adult leaf dip bioassays are given in Table 5.4. The LC_{50} and dose-response of the B biotype strain PIRGOS (LC_{50} = 3.0ppm) was comparable to a laboratory susceptible strain (SUD-S). PIRGOS was therefore was considered fully susceptible and all strains were compared to PIRGOS to calculate resistance ratios. ALM07 was highly resistant to both imidacloprid (LC_{50} = 548ppm) and thiamethoxam (LC_{50}=239). CRT-1 and GRB/MEX responded similarly to both compounds displaying low-moderate resistance (Table 5.4).

Table 5.4 LC_{50} values, 95% confidence limits, probit line slopes and resistance ratios for responses of adult *B. tabaci* to imidacloprid and thiamethoxam.

Compound	Strain	LC_{50} (ppm)	95% cl	Slope	95% cl	RR	95% cl
Imidacloprid	PIRGOS	3.0	0.8-7.1	0.7	0.6-0.8	-	-
	GRB/MEX	74.9	21.9-163	0.9	0.9-1.0	24.9	9.0-68.7
	CRT-1	55.0	26.9-152	1.1	0.9-1.3	18.3	7.0-47.8
	ALM07	548	346-903	1.6	1.2-1.9	182	71.3-466
Thiamethoxam	PIRGOS	7.5	2.4-18.6	1.9	1.7-2.1	-	-
	GRB/MEX	34.4	14.4-72.8	1.0	0.8-1.1	4.8	2.7-8.6
	CRT-1	80.5	32.5-142	1.7	1.4-2.0	11.4	6.5-19.8
	ALM07	239	164-328	1.8	1.6-2.1	31.9	20.2-50.3

5.3.1.2 Response of nymphs to imidacloprid

Results of the probit analysis for N1/N2s subject to imidacloprid in nymphal leaf dip bioassays are given in Table 5.5. The LC_{50} of PIRGOS (0.48ppm) was similar to the LC_{50} of the laboratory susceptible strain reported by Nauen *et al.* (2008a). This represents a 6-fold increase in sensitivity to imidacloprid compared to adults of the same strain. Similarly, the LC_{50} for the other strains in response to imidacloprid dropped significantly in nymphs from that of their adult counterparts. The magnitude of the drop in imidacloprid resistance was approximately 90-fold in ALM07 compared with

25-30-fold in CRT-1 and GRB/MEX. Furthermore, the LC_{50} of ALM07 nymphs (LC_{50} = 6.0 ppm) was only double that of PIRGOS adults (LC_{50} = 3.0ppm). Using PIRGOS as the baseline strain, the change in resistance ratios is shown in Figure 5.6. Each strain exhibited positive resistance and the order of the most resistant strains remained the same suggesting a quantitative change in resistance. The results here suggest an age-specific quantitative change in imidacloprid resistance comparable with that reported by Nauen *et al.* (2008a).

Table 5.5 LC_{50} values, 95% confidence limits, probit line slopes and resistance ratios for responses of *B. tabaci* nymphs to imidacloprid.

Compound	Strain	LC_{50} (ppm)	95% cl	Slope	95% cl	RR	95% cl
Imidacloprid	PIRGOS	0.48	0.13-1.07	1.3	1.0-1.5	1	1
	GRB/MEX	2.55	0.82-41.8	0.9	0.7-1.1	5.4	1.5-19.0
	CRT-1	2.12	0.85-6.2	1.2	1.0-1.4	4.5	1.8-11.1
	ALM07	6.0	1.98-34.3	1.1	0.9-1.2	12.6	5.4-29.3

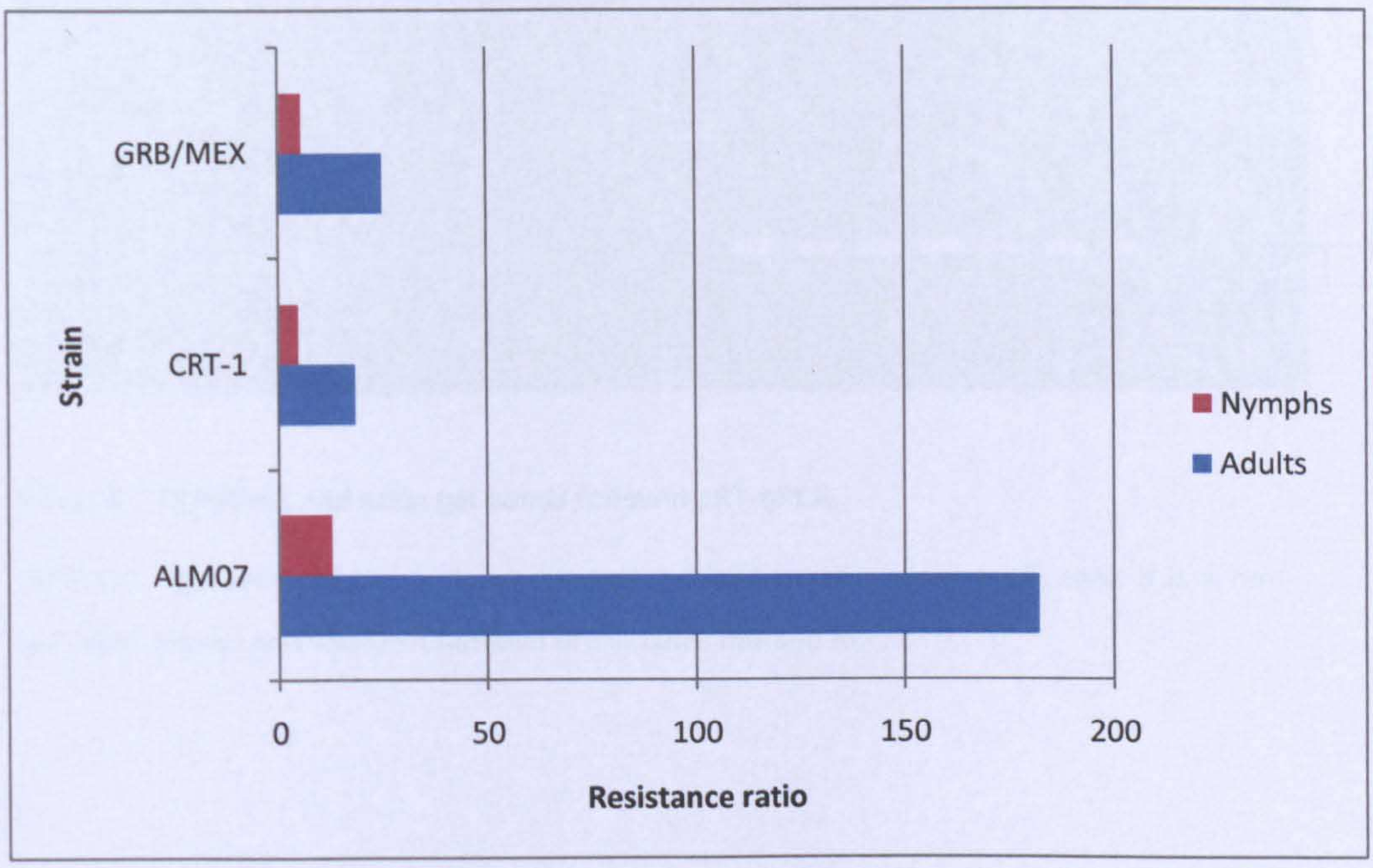


Figure 5.6 *B. tabaci* adult and nymph resistance ratios in response to imidacloprid.

5.3.1.3 CYP6CM1 gene expression assay

TaqMan® gene expression assays were designed for the target gene, *CYP6CM1*, as well as two endogenous control genes, *actin* and *18S rRNA*. Relative gene expression using the ΔΔCt method requires the PCR amplification efficiencies of the target and the endogenous control gene to be equivalent (Pfaffl 2006). The *actin* gene was chosen as the appropriate endogenous control for *CYP6CM1* expression following validation and optimisation (Appendix 5.1). Furthermore, to ensure the correct amplification of the PCR product, endpoint *CYP6CM1* and *actin* PCR samples were run on an agarose gel (Figure 5.7). The gel shows two different bands at 82bp and 102bp representing the correct target fragment sizes of *CYP6CM1* and *actin* respectively.

Figure 5.7 CYP6CM1 and actin gel bands following RT-qPCR.

CYP6CM1 PCR products are in lanes marked 1-8 and *actin* in lanes 9-16. Lane B is a non-template control and 1kb DNA markers are in lanes marked M.

5.3.1.4 Age-specific gene expression

Once optimised, the relative gene expression assay was tested on adults, pupae and nymphs (N1/N2) of *B. tabaci*. 150 insects were collected for each life stage with three biological replicates for each strain. The $2^{\wedge-\Delta\Delta Ct}$ values were calculated according to Livak & Schmittgen (2001) along with 95% confidence limits (Figure 5.8). Following the bioassay results presented in Chapter 5.3.1.1 and Chapter 5.3.1.2, and as the most imidacloprid-susceptible strain, PIRGOS was chosen as the control sample from which to compare all strains.

In adults and in comparison to PIRGOS, the *CYP6CM1* mRNA levels were highest in ALM07 ($2^{\wedge-\Delta\Delta Ct}$ = 24.2) followed by CRT-1 (17.3) and then GRB/MEX (6.7). This is consistent with ALM07 being the most imidacloprid-resistant strain although the 95% C.L. of $2^{\wedge-\Delta\Delta Ct}$ overlap slightly with CRT-1. In pupae, ALM07 showed an extremely high level of expression (>200) compared with PIRGOS. Although the exact $2^{\wedge-\Delta\Delta Ct}$ values were higher in the pupal stage in CRT-1 and GRB/MEX, the 95% C.L. overlap with PIRGOS. The levels of *CYP6CM1* expression were dramatically reduced in N1/N2 nymphs across all strains. The $2^{\wedge-\Delta\Delta Ct}$ values were comparable and did not significantly differ from those for PIRGOS. These results suggest that *CYP6CM1* expression is a possible cause of age-specific resistance in *B. tabaci*.

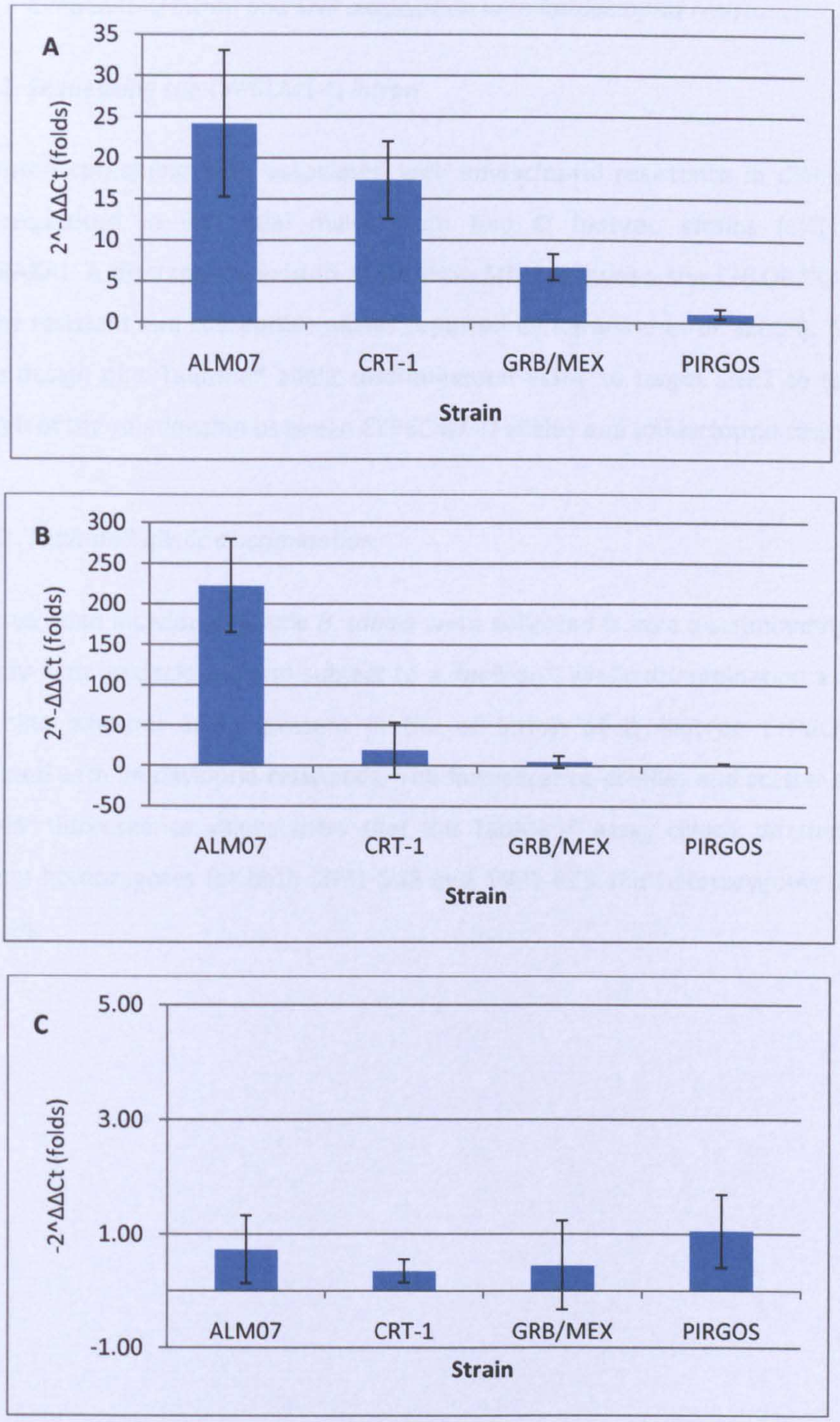


Figure 5.8 Quantitative PCR analysis of CYP6CM1 expression from different *B. tabaci* life stages.

The $2^{-\Delta\Delta Ct}$ values for each strain are shown along with 95% confidence limits for A) adults, B) pupae and C) nymphs.

5.3.2 CYP6CM1-Q intron and SNP association with imidacloprid resistance

5.3.2.1 Sequencing the CYP6CM1-Q intron

The intron containing SNPs associated with imidacloprid resistance in *CYP6CM1-Q* was sequenced in individual males from two Q biotype strains (CRT-1 and CHLORAKA). A discrepancy existed at position SNP2 between the CHLORAKA allele and the resistant and susceptible alleles reported by Karunker *et al.* (2008). This led to the design of a TaqMan® allelic discrimination assay to target SNP1 to test the strength of the relationship between *CYP6CM1-Q* alleles and imidacloprid resistance.

5.3.2.2 TaqMan® allelic discrimination

Alive and dead individual female *B. tabaci* were collected from a discriminating dose bioassay with imidacloprid and subject to a TaqMan® allelic discrimination assay to determine whether SNP1, present in the of intron of Q biotype *CYP6CM1*, is correlated with imidacloprid resistance. The fluorescence profiles and scatter plot of endpoint fluorescence values show that the TaqMan® assay clearly discriminated between homozygotes for both SNP1-SUS and SNP1-RES and heterozygotes (Figure 5.9-5.10).

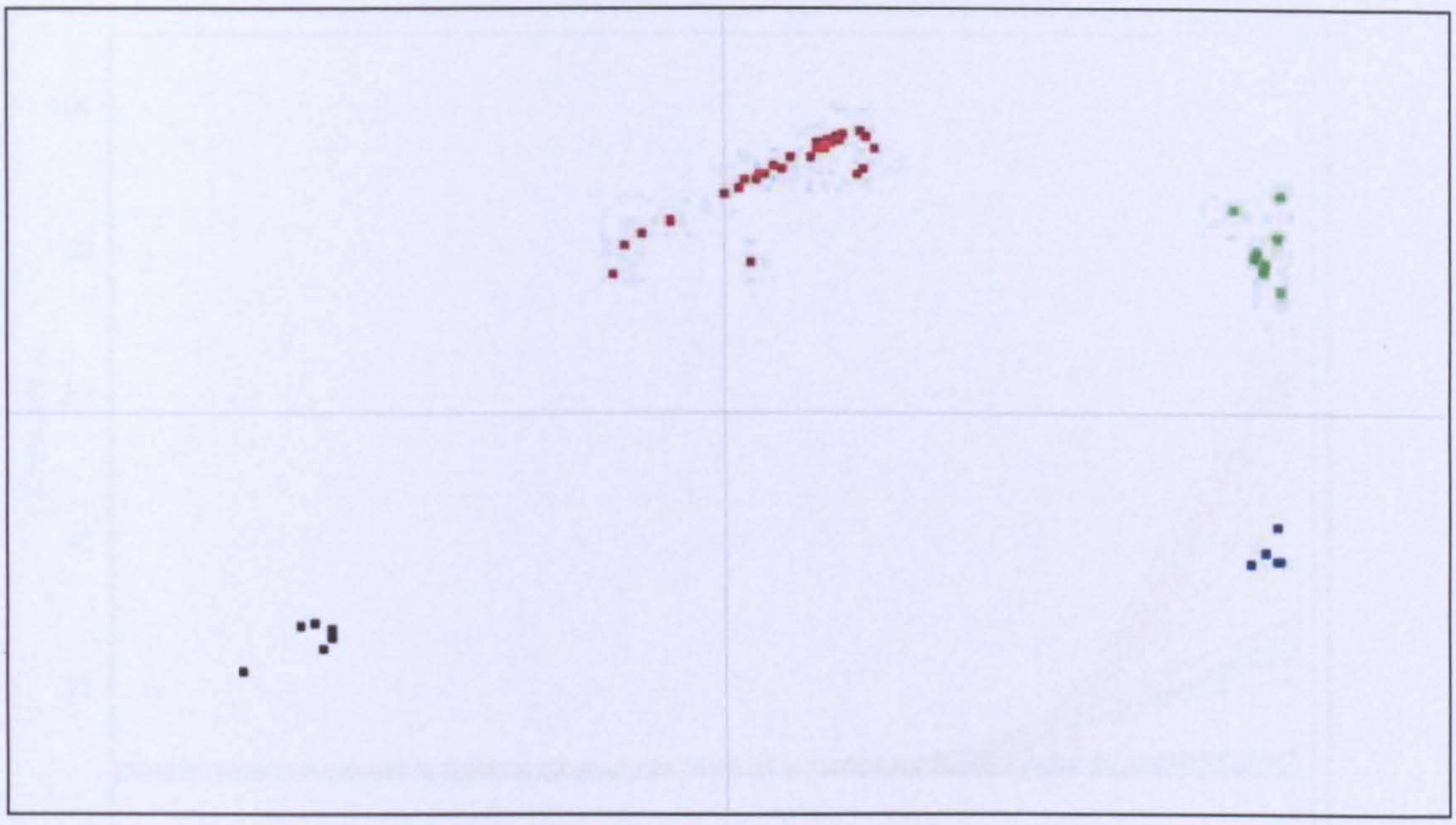


Figure 5.9 An example scatter plot analysis of CYP6CM1-Q intron SNP1 detection.

Endpoint fluorescence values for each allele specific probe are corrected for background and plotted against each other. Individuals of each genotype form clear clusters and are represented by the different coloured points. SNP1-RES homozygous (red); SNP1-SUS homozygous (blue); heterozygotes (green); blank negative controls (black).

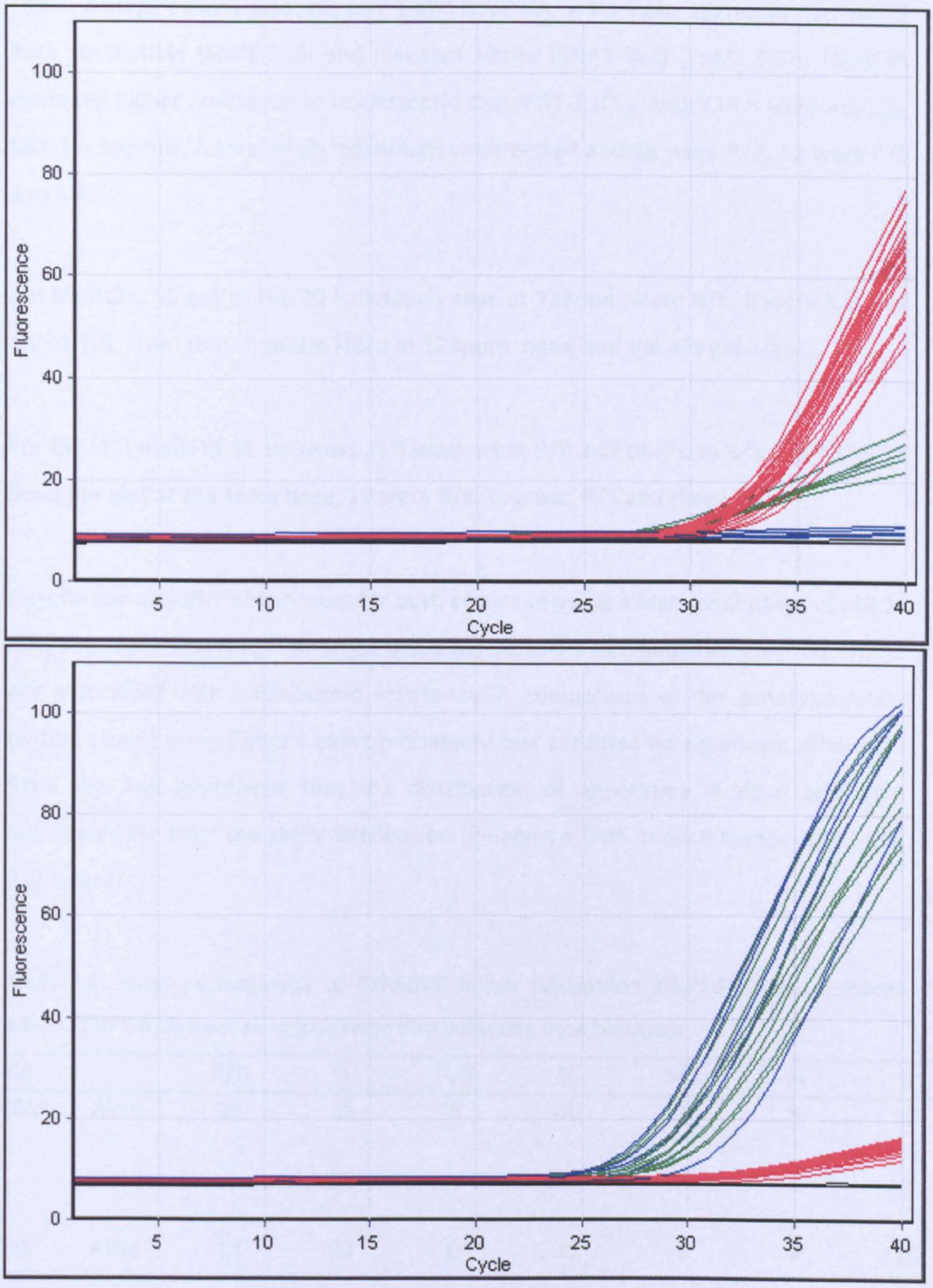


Figure 5.10 Fluorescence plots of CYP6CM1 intron SNP genotypes.

In this diagram, fluorescence plots for each probe are shown over 40 PCR cycles. **A** Cycling of FAM; specific for SNP1-RES. **B** Cycling of VIC; specific for SNP1-SUS. Red lines represent individuals homozygous for SNP1-RES, blue lines represent SNP1-SUS and green lines represent heterozygotes.

The Q biotype strains assayed, CRT-1 and MURCIA, were heterogeneous containing both susceptible (SNP1-SUS) and resistant alleles (SNP1-RES) (Table 5.6) . MURCIA exhibited higher resistance to imidacloprid than CRT-1 (LC_{50} MURCIA = 408ppm; LC_{50} CRT-1 = 55ppm). A total of 65 individuals were tested and 48 were R/R, 12 were R/S and 5 S/S.

For MURCIA, 11 out of the 20 individuals alive at 128ppm were R/R, 8 were R/S and only 1 S/S. From the 7 females killed at 128ppm, none had the S/S genotype.

For CRT-1, 14 out of 15 survivors at 32ppm were R/R and only one S/S. From the 23 dead females at the same dose, 19 were R/R, one was R/S and three S/S.

Despite the majority of survivors for both strains carrying a high percentage of SNP1-RES, the high percentage amongst those individuals dead, suggests that this SNP is not associated with imidacloprid resistance. A comparison of the genotype ratios (within strain) using Fisher's exact probability test confirms no significant difference from the null hypothesis that the distribution of genotypes in dead and alive individuals are from the same distribution (P-values > 0.05 in each comparison using 2x2 tables).

Table 5.6 Genotype frequency of *CYP6CM1* intron susceptible (SNP1-SUS) and resistant alleles (SNP1-RES) from an imidacloprid discriminating dose bioassay.

Strain		R/R	%	R/S	%	S/S	%	n
MURCIA	Alive	11	55	8	40	1	5	20
	Dead	4	57	3	43	0	0	7
CRT-1	Alive	14	93	0	0	1	7	15
	Dead	19	83	1	4	3	13	23
	Total	48		12		5		65

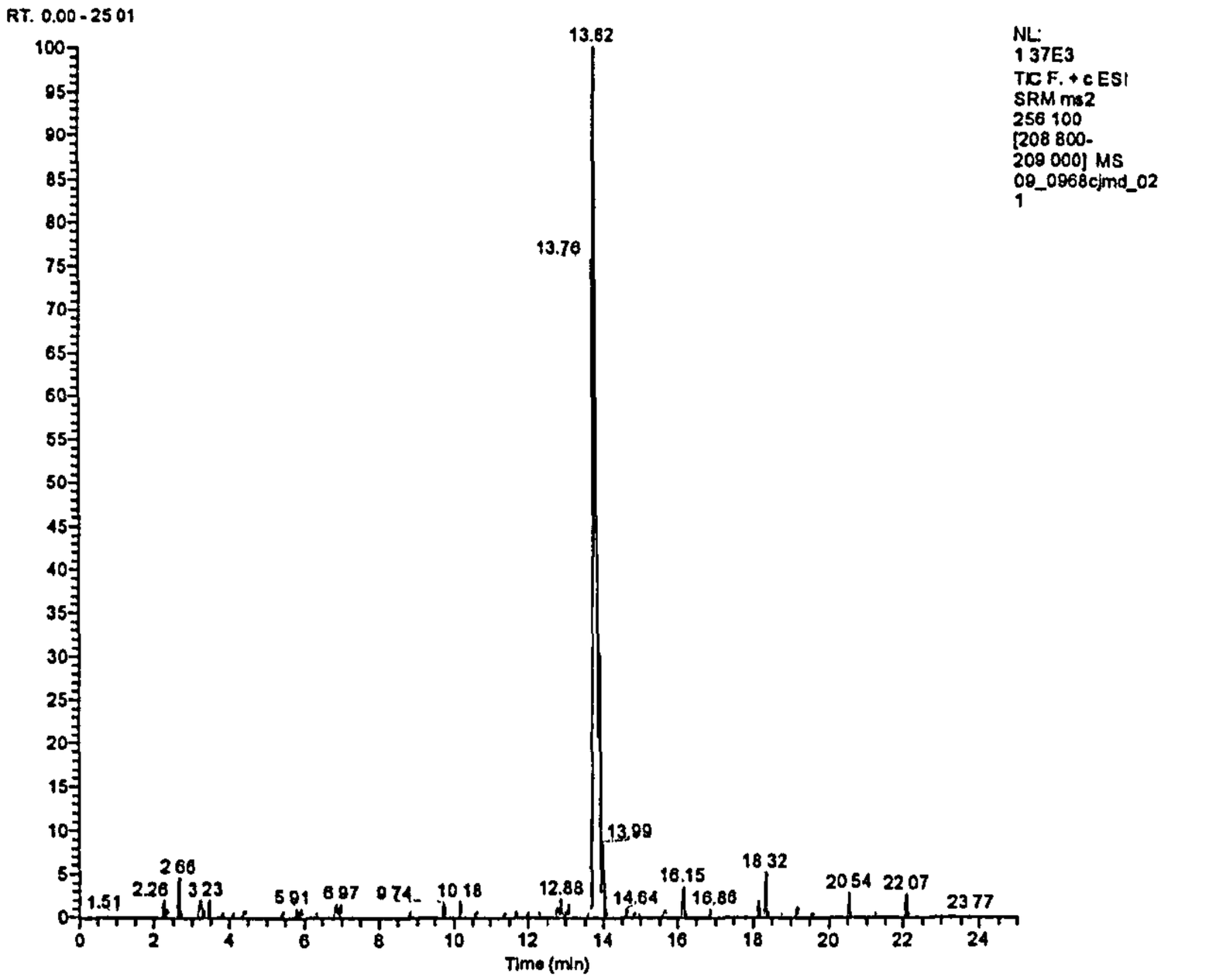
5.3.3 Characterisation of imidacloprid metabolism

5.3.3.1 LC/MS detection of imidacloprid metabolites

Following exposure to 1ppm of imidacloprid, 400-500 individuals from the four *B. tabaci* strains ALM07, CRT-1, GRB/MEX and PIRGOS were analysed for eleven known metabolites of imidacloprid using LC/MS. Imidacloprid and IMI-5-OH were the only compounds found at the levels of detection (LOD) and quantification (LOQ) in the insecticide-treated whiteflies. The LC/MS chromatograms of IMI and IMI-5-OH are shown in Figure 5.11 . Trace amounts of IMI-4-OH and IMI-diol were detected in the treated samples but below the threshold levels for LOD and LOQ. An anomalous detection of IMI-4-OH was seen in untreated whitefly samples. It is possible that in the whitefly matrix there is a compound with the same retention time and transition as IMI-4-OH resulting in detection (M. Daniels pers. comm.).

5.3.3.2 Recovery of imidacloprid and IMI-5-OH

The total amount of imidacloprid and IMI-5-OH recovered (µg/mg of whitefly sample) per strain is given in Figure 5.12. The calculated total of imidacloprid present in each strain varies. This is a consequence of experimental variation and in particular, the difficulty in treating individual whiteflies with such a small volume of insecticide. The extraction efficiencies of imidacloprid are, however, similar across different *B. tabaci* strains following the validation of LC/MS for metabolite recovery, discounting any strain-specific effects on the total amount of compound. The proportion of IMI-5-OH relative to imidacloprid was determined as a measure of metabolism in each strain (Figure 5.13). The ratio of IMI-5-OH to imidacloprid was highest in ALM07 (0.54) compared with 0.32 and 0.16 in CRT-1 and GRB/MEX respectively whereas PIRGOS had the lowest ratio (0.04). The rate of metabolism shown in Figure 5.13 follows a similar pattern to the levels of imidacloprid resistance and *CYP6CM1* expression reported in these four strains and presented in Table 5.4 and Figure 5.8 respectively.



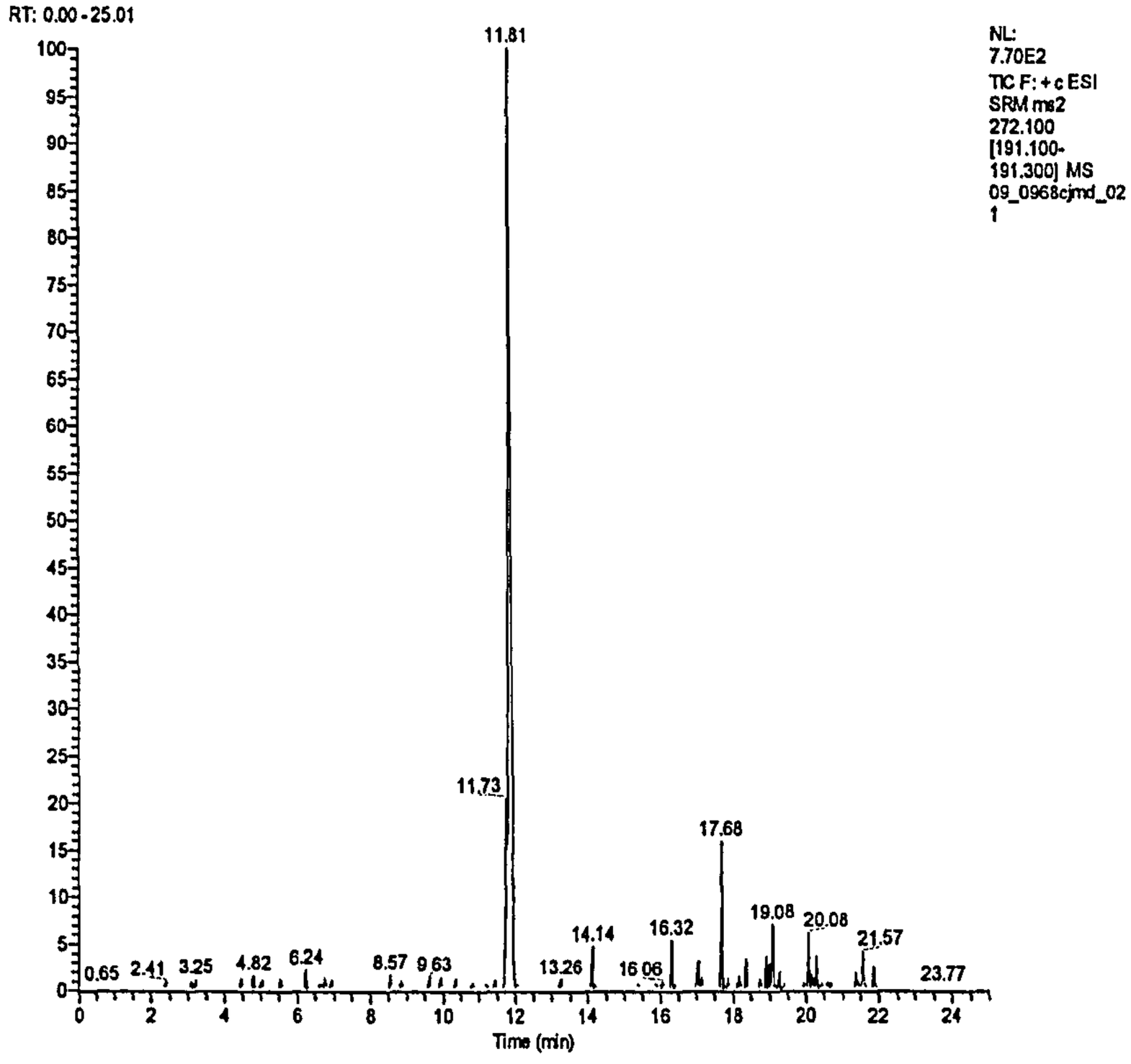


Figure 5.11 LC/MS detection of imidacloprid and the 5-hydroxy metabolite.

LC/MS chromatograms of A) imidacloprid and B) IMI-5-OH from a standard solution (0.00156ppm) of the whitefly matrix.

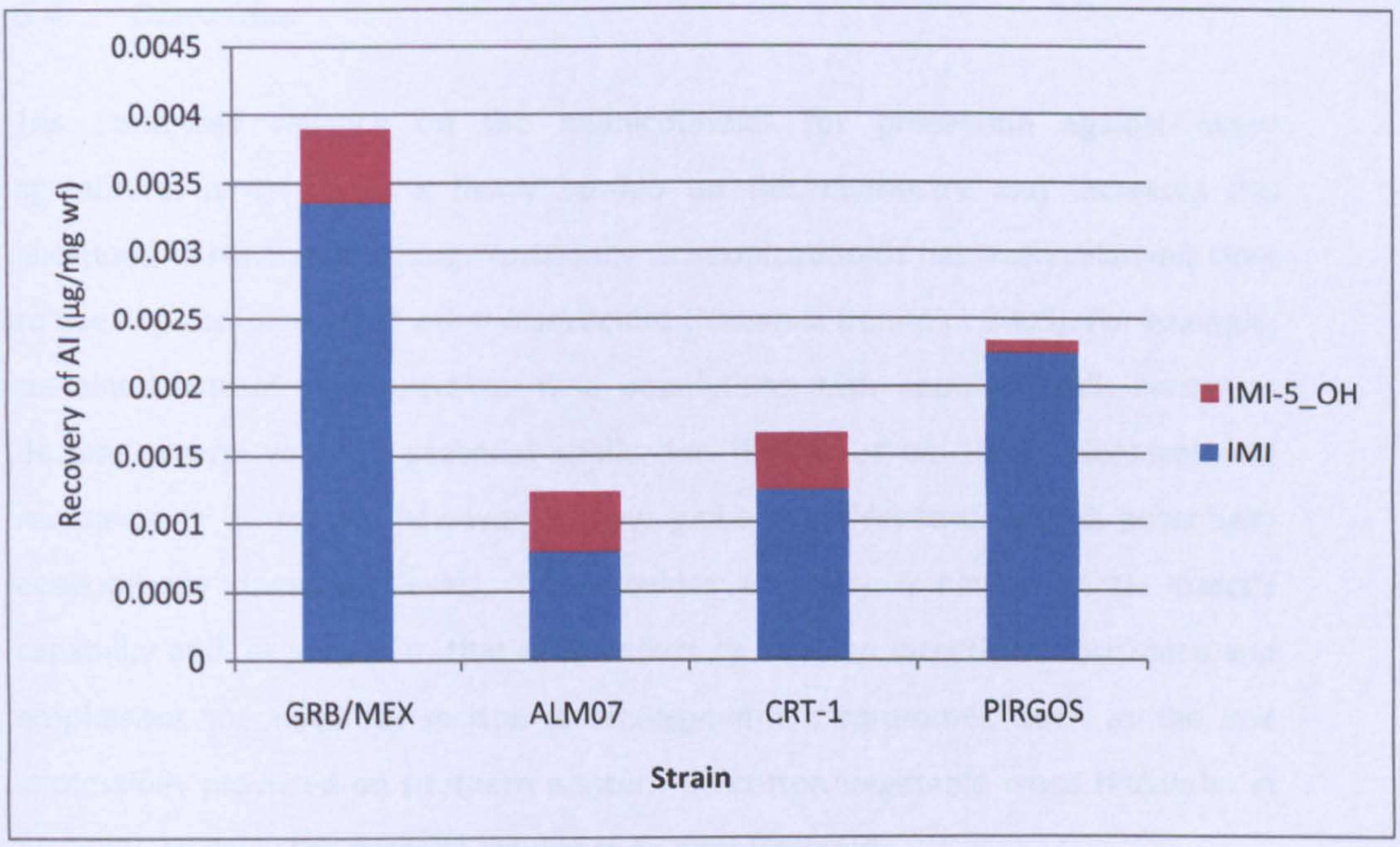


Figure 5.12 Recovery of the metabolites IMI and IMI-5-OH from each *B. tabaci* strain.

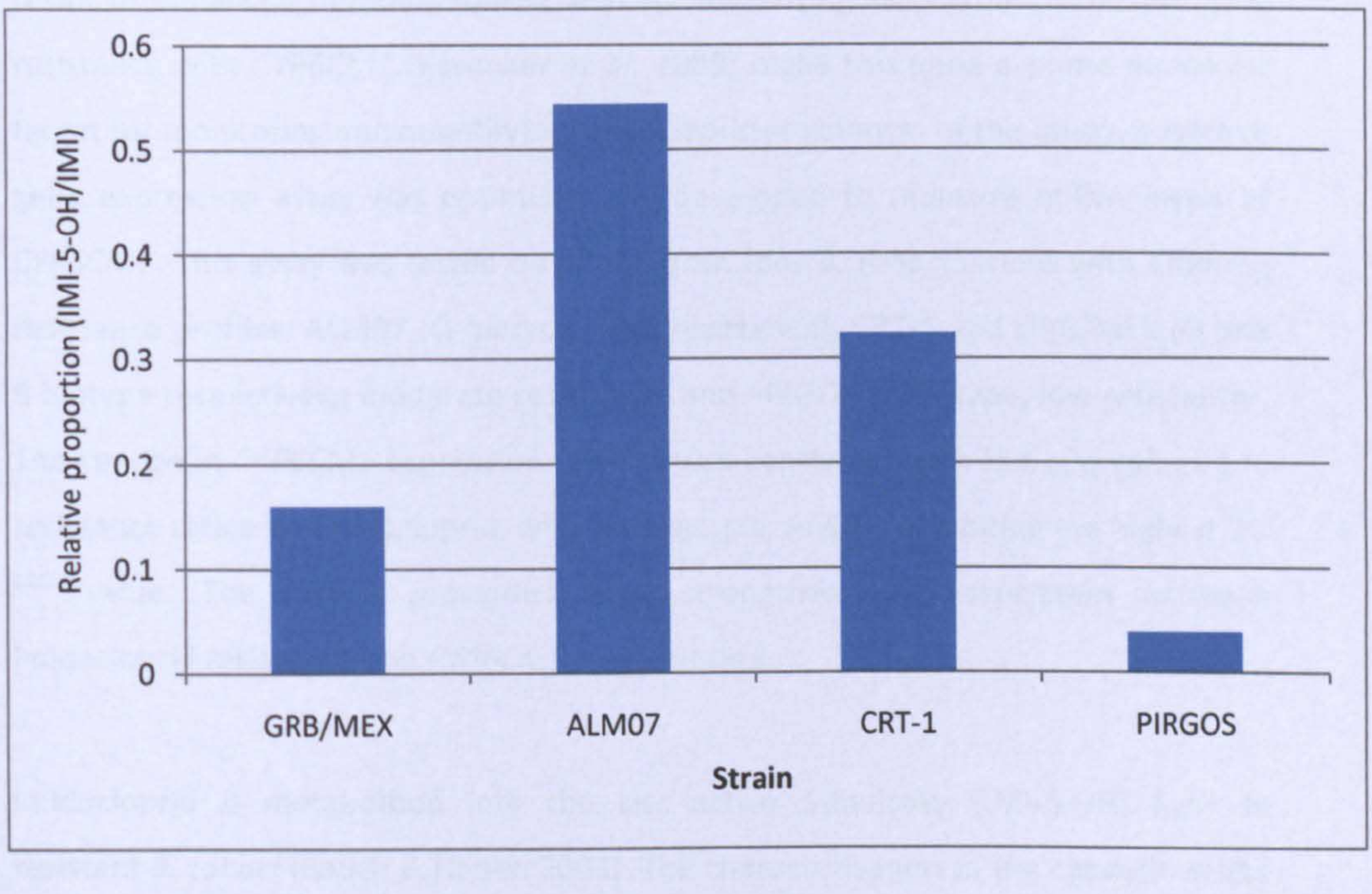


Figure 5.13 Proportion of IMI-5-OH relative to imidacloprid recovered from adult whiteflies.

5.4 Discussion

The continued reliance on the neonicotinoids for protection against many agricultural pests places a heavy burden on this chemistry and increases the likelihood of resistance arising. Resistance to neonicotinoids has been relatively slow to develop compared with other insecticides (Nauen & Denholm 2005). For example, sustained control of *M. persicae* field populations with neonicotinoids continues despite several years of seasonal application (Foster *et al.* 2008). Neonicotinoid resistance in *B. tabaci*, however, is now globally widespread and at potentially economically damaging levels. This provides a timely reminder of an insect's capability and, in particular, that of *B. tabaci*, to develop insecticide resistance and emphasises the need for resistance management programmes, such as the one successfully practiced on southern western US cotton/vegetable crops (Palumbo *et al.* 2001), to delay the onset of resistance to neonicotinoids.

Recent evidence suggests that resistance to imidacloprid in *B. tabaci* is largely a result of enhanced monooxygenase activity. The strong association of imidacloprid resistance with *CYP6CM1* (Karunker *et al.* 2008) make this gene a prime molecular target for monitoring and quantifying imidacloprid resistance. In this study, a relative gene expression assay was optimised and developed to measure mRNA levels of *CYP6CM1*. This assay was tested on adults from four *B. tabaci* strains with differing resistance profiles; ALM07 (Q biotype; high resistance), CRT-1 and GRB/MEX (Q and B biotype respectively; moderate resistance) and PIRGOS (B biotype; low resistance). The change in *CYP6CM1* expression ($2^{\wedge-\Delta\Delta Ct}$) was consistent with the LC_{50} values and resistance ratios for imidacloprid, with for example, ALM07 exhibiting the highest $2^{\wedge-\Delta\Delta Ct}$ value. The results presented here strengthen the association between imidacloprid resistance and *CYP6CM1* transcription.

Imidacloprid is metabolised into the less active 5-hydroxy (IMI-5-OH) form in resistant *B. tabaci* (Rauch & Nauen 2003). The characterisation of the catalytic ability of *CYP6CM1* to hydroxylate imidacloprid into IMI-5-OH provided the first functional link between genotype and imidacloprid resistance in *B. tabaci* (Karunker *et al.* 2009). To investigate the quantitative relationship between *CYP6CM1* and imidacloprid resistance LC/MS was used to detect and quantify a range of

metabolites from the four whitefly strains. Only the parent compound and IMI-5-OH could be detected but the proportion of IMI-5-OH recovered from imidacloprid (Figure 5.13) suggests a quantitative link between imidacloprid resistance, *CYP6CM1* expression and metabolite production.

Although resistance to thiamethoxam in the strains examined here was slightly lower than to imidacloprid, the pattern of resistance was similar and therefore consistent with *CYP6CM1* expression. Any relationship between thiamethoxam and *CYP6CM1* expression however, must be treated as correlative rather than causal, until the enzyme is shown to catalyse this compound. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants (Jeschke & Nauen 2008) and in this case, metabolism by *CYP6CM1* may not be directly on the parent compound. A correlation between *CYP6CM1* levels and various neonicotinoids was recently found in Chinese Q biotypes (Qiong *et al.* unpublished data). Many P450 enzymes are capable of metabolising several substrates (Feyereisen 1999). Functional expression of the *D. melanogaster CYP6A2* enzyme metabolised the organophosphate diazinon (Dunkov *et al.* 1997). Broad cross resistance to a range of insecticides was associated with increased transcripts of *CYP6G1* suggesting that this enzyme can metabolise a range of compounds (Daborn *et al.* 2002). The ability of P450 enzymes to catalyse the breakdown of more than one insecticide class is particularly worrying for resistance management. The human CYP3A4 is capable of detoxifying a broad spectrum of drugs and can metabolise imidacloprid into IMI-5-OH (Schulz-Jander & Casida 2002). This enzyme shares a conserved region with *CYP6CM1* which has been implicated in breaking down several substrates (Karunker *et al.* 2009). It is conceivable that the up-regulation of *CYP6CM1* in *B. tabaci*, as a consequence of imidacloprid selection, could cause reduced susceptibility to other neonicotinoids or even other classes of insecticide. Monitoring levels of *CYP6CM1* is therefore a crucial part of resistance management in *B. tabaci* and further characterisation of the enzyme's ability to metabolise other insecticides is required.

Gene expression via qRT-PCR is a powerful and sensitive tool to measure changes in mRNA transcripts (Pfaffl 2006) and once the target gene is known, screening of an insect population for a gene associated with insecticide resistance can be regularly performed. It must be noted however, that relative gene expression is not high-

throughput and, to be robust and reliable, needs significant optimisation. Furthermore, the technique requires the extraction of RNA, necessitating the need for live insects or insect material stored at -80°C. The assay described here would make a useful practical contribution to resistance management by measuring levels of *CYP6CM1* associated with imidacloprid resistance in live populations collected from the field. The expression of *CYP6CM1* from globally distributed and heterogeneously resistant populations would enhance our understanding of the relationship between genotype and phenotype.

The design of any molecular diagnostic for insecticide resistance would be ideally based on a DNA marker. This would allow easy storage and transportation of insect material as well as offering more opportunities for high-throughput techniques. In this regard, the association of three SNPs in the first intron of *CYP6CM1-Q* alleles with imidacloprid insensitivity was promising (Karunker *et al.* 2008). Following a discriminating dose bioassay, dead and alive Q biotype individuals were screened using a TaqMan® allelic discrimination PCR assay to detect resistance or susceptible alleles. The results from this screen suggested that no such link between genotype and phenotype exist. Furthermore, sequencing of this intron from the CRT-1 population revealed a discrepancy between the SNPs in the gene sequence and those reported by Karunker *et al.* (2008). The lack of a relationship between the SNP and resistance is unfortunate as this would greatly enhance our ability to monitor resistance across multiple samples and/or groups. The genetic basis underlying the increase in *CYP6CM1* transcription in resistant *B. tabaci* remains unknown. Gene insertions/deletions or mutations in the promoter region may increase transcription. The insertion of the *Accord* transposable element 300bp upstream of *CYP6G1* in globally widespread alleles of *D. melanogaster* has been connected with resistance to imidacloprid and DDT (Daborn *et al.* 2002). This mechanism of *CYP6G1* up-regulation is conserved in the sister species *Drosophila simulans* (Schlenke & Begun 2004). Over-expression of *CYP6A1* in *M. domestica* has been linked to a loss-of-function in a *trans*-acting regulatory gene (Sabourault *et al.* 2001). Identifying the molecular basis of *CYP6CM1* up-regulation in resistant *B. tabaci* would potentially provide an opportunity to develop a rapid and reliable molecular diagnostic to study the global prevalence of imidacloprid resistance.

The concept of age-specific resistance is an intriguing prospect; for insect control, the selection of resistant genotypes and resistance management strategies. Nauen *et al.* (2008b), from three independent studies, proposed that imidacloprid-resistant *B. tabaci* adults (B and Q biotypes) gain a selective advantage over immature stages. The resistance data presented here on nymphs exposed to imidacloprid in leaf-dip bioassays support this theory. The most resistant strain ALM07 remained the most resistant in nymphs, albeit with a significantly reduced LC_{50}. The data suggest that the mechanism causing this phenomenon is potentially qualitative. Following the development of the *CYP6CM1* gene expression assay and report of age-specific resistance in *B. tabaci* (Nauen *et al.* 2008b), *CYP6CM1* mRNA levels were analysed across three different life stages; adults, pupae and nymphs (N1/N2). The $2^{\wedge\Delta\Delta Ct}$ values dropped remarkably in nymphs of the resistant strains (ALM07, CRT-1 and GRB/MEX) to a similar level as the susceptible PIRGOS strain. The drop in *CYP6CM1* expression in nymphs is consistent with the dramatic fall in imidacloprid resistance levels. Interestingly, *CYP6CM1* mRNA levels in the pupal stage of ALM07 increased dramatically compared with PIRGOS. The high levels of *CYP6CM1* in pupae may not necessarily be due an increased insensitivity to an insecticide, but suggest the involvement of this gene in another developmental process as the insect undergoes morphological change from nymph to pupa. Similar gene expression patterns have been found elsewhere between resistant and susceptible *B. tabaci* (pers. comm. Mel Andrews, Syngenta). The correlation of age-specific expression of *CYP6CM1* with imidacloprid resistance provides further experimental evidence of the involvement of this enzyme in imidacloprid detoxification.

The low control mortality among N1/N2 nymphs indicates that the methods used here were appropriate; however, an investigation to ensure no discrepancy exists between adult and nymphal leaf-dip bioassays would be welcomed. Adult whiteflies are able to move freely within the Petri-dish and encounter the insecticide either through tarsal contact or ingestion following feeding whereas the whole nymph body is encapsulated completely within solution. The latter potentially results in a greater exposure to the insecticide and may explain why nymphs are killed at reduced doses.

Age-specific resistance to neonicotinoids in *B. tabaci* offers some potential alternative tactics to manage this pest. Targeting immatures with an early-season

systemic treatment and by strategic applications to lower leaves where the nymphs reside has been proposed (Nauen *et al.* 2008b). The possibility of selecting for resistance in other life-stages would require laboratory investigation prior to employing such tactics combining bioassay data with biochemical and genotype information.

Chapter 6 General Discussion

Insecticide resistance continues to pose a threat to effective insect control and this is compounded by the limited spectrum of insecticide chemistry available. Insecticide resistance management (IRM) strategies play a key role in pre-empting, preventing and circumventing the onset of resistance and many IRM strategies are enforced as part of whitefly control programmes (Dennehy *et al.* 2008; Horowitz *et al.* 2008). Molecular biology has advanced remarkably over the past 20 years and this has vastly improved our ability to detect, incriminate, manipulate and quantify genes associated with insecticide resistance. The advent of microarray technology and next-generation sequencing are beginning to offer some real insights behind the mechanisms underpinning insecticide resistance in *B. tabaci* as well as in other insect pests of agriculture and human disease (Colvin *et al.* 2009; David *et al.* 2005; David *et al.* 2010; Ghanim & Kontsedalov 2007; Saldivar *et al.* 2008).

The extent and speed with which *Bemisia tabaci* develops resistance to insecticides makes this species an ideal organism to study the dynamics of heritable resistance traits. Resistance has now been reported from *B. tabaci* field populations to the majority of insecticides registered for whitefly control (Reviewed by Horowitz *et al.* (2007). The haplodiploidy system of *B. tabaci* greatly influences the evolution of insecticide resistance in this pest (Crowder *et al.* 2009; Denholm *et al.* 1998). The work presented here describes three separate studies where molecular tools were used to monitor resistant groups of *B. tabaci* and improve our understanding of the mechanisms responsible for insecticide resistance.

The taxonomy of *Bemisia tabaci* has undergone several changes since first described as *Aleyrodes tabaci* by Gennadius in 1889 (Perring 2001). Indeed, during the course of this study there has been a significant shift from the concept of a 'complex species' comprising of several biotypes, towards the notion that *B. tabaci* is in fact composed of a host of reproductively isolated sibling species. The classification of biotypes and the many different techniques used to distinguish them has caused much confusion and debate amongst researchers. Adhering to the classical 'biological species concept' (Mayr 1942), current evidence suggests that 'biotypes' of *B. tabaci* do not freely interbreed to produce viable fertile offspring and therefore

must be elevated to species status (Crowder *et al.* 2010; Xu *et al.* 2010). The challenge is to now build on these recent studies and gather as much information on mating behaviour, life-history patterns and resistance status between as many extant biotypes as possible. Competitive displacement studies in population cage experiments are beginning to provide an insight into the invasiveness of various biotypes (Crowder *et al.* 2010) and larger, collaborative experiments are planned.

Regardless of taxonomic status, the TaqMan® assay successfully distinguished between the economically impacting and often co-existing B and Q biotypes from a worldwide collection of field samples. The assay was recently used to identify biotypes from China showing various levels of pyrethroid and neonicotinoid resistance (Luo *et al.* 2009). Identifying the biotype status of a whitefly infestation must remain an important component of control programmes and resistance management strategies as biotypes/species of *B. tabaci* continue to demonstrate differing levels of resistance to various insecticides (Fernandez *et al.* 2009; Horowitz *et al.* 2008; Luo *et al.* 2009; Nauen & Denholm 2005; Roditakis *et al.* 2009).

The main advantage of the TaqMan® assay compared to previous biotype-diagnostic methods is its ability to process large numbers of insects. The power of the assay was demonstrated in a study investigating the interaction between Chinese strains of B and Q biotypes treated with or without imidacloprid in population cages (Luo *et al. in prep*). In this experiment, the Q biotype proliferated in those cages sprayed with imidacloprid whereas the B biotype dominated in those cages untreated providing evidence of insecticide resistance driving biotype displacement in China. The planned biotype/species interaction experiments described above could benefit from a high-throughput assay such as the TaqMan® diagnostic.

Insecticide resistance not only significantly impacts crop protection but is perhaps the best known example of human activity artificially driving the selection of genes in the environment. Several questions are often raised regarding this phenomenon and the implications for insecticide resistance, such as:

a) How often and where do mutations spread as a consequence of insecticide use?

b) Why are insecticide resistance mutations so conserved between different insect species?
c) Why do individual resistance mutations often not occur on the same allele?

The answers to some of these questions were explored by examining the intron sequence adjacent to two pyrethroid resistant mutations (L925I and T929V) across global field collections of individual *B. tabaci*. The data presented in Chapter 4 showed that such insecticide resistant mutations have the ability to arise multiple times and are conserved between reproductively isolated groups (L925I present in B and Q biotypes). Whether the absence of L925I and T929V on the same allele is evidence of reduced fitness cost requires further study but is an indication that the conformational changes required within the sodium channel would be too deleterious to exist.

The similar selection pressure exerted from the same class of insecticide presumably pre-disposes certain amino acid residues to mutation given the conservative nature of some of these genetic changes between different insect species. However, several environmental factors including variation in insecticide application, the reproductive nature of the insect and host plant-insect interaction will undoubtedly play a large role in which mutations arise in space and in time. Through target-site modelling, electrophysiology and phenotypic data, we are beginning to understand why certain mutations are more prone to selection and in particular those found within the sodium channel. Indeed, these studies form a basis for similar studies investigating the interaction between other classes of insecticide and target-site.

The arsenal of insecticides available to combat insect pests remains limited. Furthermore, the number of new compounds coming on to the market is waning as a result of high development costs and increasingly complex legislation (Bielza *et al.* 2008). It is feared that this will lead to increasingly widespread reports of resistance with novel approaches required to develop new insecticides and manage resistance. Chapters 4 and 5 describe two pieces of research which may help in this regard; a) the design of new insecticides based on target-site protein modelling b) exploiting age-specific imidacloprid resistance to target the 'susceptible' immature stage of the insect compared with their 'resistant' adult counterparts.

The design of a new insecticide based on existing chemistry rather than on a novel mode of action is an interesting and challenging prospect. The premise is based on altering the chemical structure of the insecticide in order to avoid the conformational changes induced within the target-site protein by a particular genetic mutation. For example, this idea has generated much interest in the control of malaria-carrying mosquitoes, as pyrethroids remain the sole compound registered for use on widely distributed bednets (Hemingway *et al.* 2006). In Chapter 4, the effect of L925I and T929V on the insecticidal activity of DDT, fenfluthrin and deltamethrin was investigated. Insects carrying L925I were clearly at a disadvantage compared with those individuals carrying T929V when treated with either DDT or fenfluthrin. This suggests that either compound could effectively control L925I carrying whiteflies. The genotype data is supported by the stereochemical interactions between the insecticide and residues within the sodium channel (O'Reilly *et al.* 2006). Controlling infestations of *B. tabaci* with these compounds on the basis of whether either mutation is present or absent is not a practical option for whitefly control. However, the work does demonstrate how our knowledge of the target-site (or the metabolic enzyme) through modelling approaches could be used to custom-design an insecticide to overcome the resistance mechanism.

One of the most intriguing aspects of the project is the description of age-specific resistance to imidacloprid, confirming the original discovery by Nauen *et al.* (2008b). In Chapter 5, some of the possibilities of targeting immature *B. tabaci* as an effective resistance management strategy for whitefly control with imidacloprid were explored. Further studies are required to demonstrate whether this strategy would be as successful as other IRM tactics such as the rotation, mixture or mosaic application of insecticides. Preliminary attempts were made in laboratory simulators to demonstrate whether 'susceptible' larvae could be targeted in such a strategy. An initial systemic treatment of imidacloprid was applied to cages as a means of controlling the emerging immature stages, whereas adults were intermittently sprayed with imidacloprid over a pre-defined time period. A pilot simulation such as this is necessary before serious attempts could be made to execute this resistance management strategy in the field.

The recent association of *CYP6CM1* and imidacloprid metabolism is one of the most comprehensive associations between a single P450 enzyme and insecticide resistance. In Chapter 5, *CYP6CM1* levels correlated with imidacloprid resistance in adults and larvae and this was supported by a similar correlation between resistance and metabolism rate in adults using LC-MS. The link between this *CYP6CM1* and imidacloprid resistance in this species is now very strong. As further proof of association, an additional study was conceived to knockdown *CYP6CM1* mRNA levels in *B. tabaci* adults using RNA interference (RNAi). Double-stranded RNA was synthesised and several attempts were made to feed dsRNA through an artificial feeding system. Having reduced *CYP6CM1* mRNA levels, insects fed on dsRNA would be compared with those unfed in imidacloprid leaf-dip bioassays to test the phenotype. Insects were fed rather than injected with dsRNA as it was believed that injection would induce high levels of mortality and as a result mask the phenotype when treated with an insecticide. Unfortunately, using the *CYP6CM1* relative gene expression described in Chapter 5, *CYP6CM1* expression did not differ in those whiteflies fed dsRNA compared with those unfed. This experiment would add further credence to *CYP6CM1* up regulation as a main cause of imidacloprid resistance.

The work presented here encompasses three very different areas of insecticide resistance in *B. tabaci* at the molecular level. Molecular biology must never substitute standard toxicological data but be used practically to complement studies monitoring fluxes in insecticide resistance. In the near future, *B. tabaci* will continue to hamper control programmes worldwide and therefore it would be remiss not to make the most of the significant advancements made in molecular biology to improve our understanding of insecticide resistance and the biology underlying this species.

Chapter 7 References

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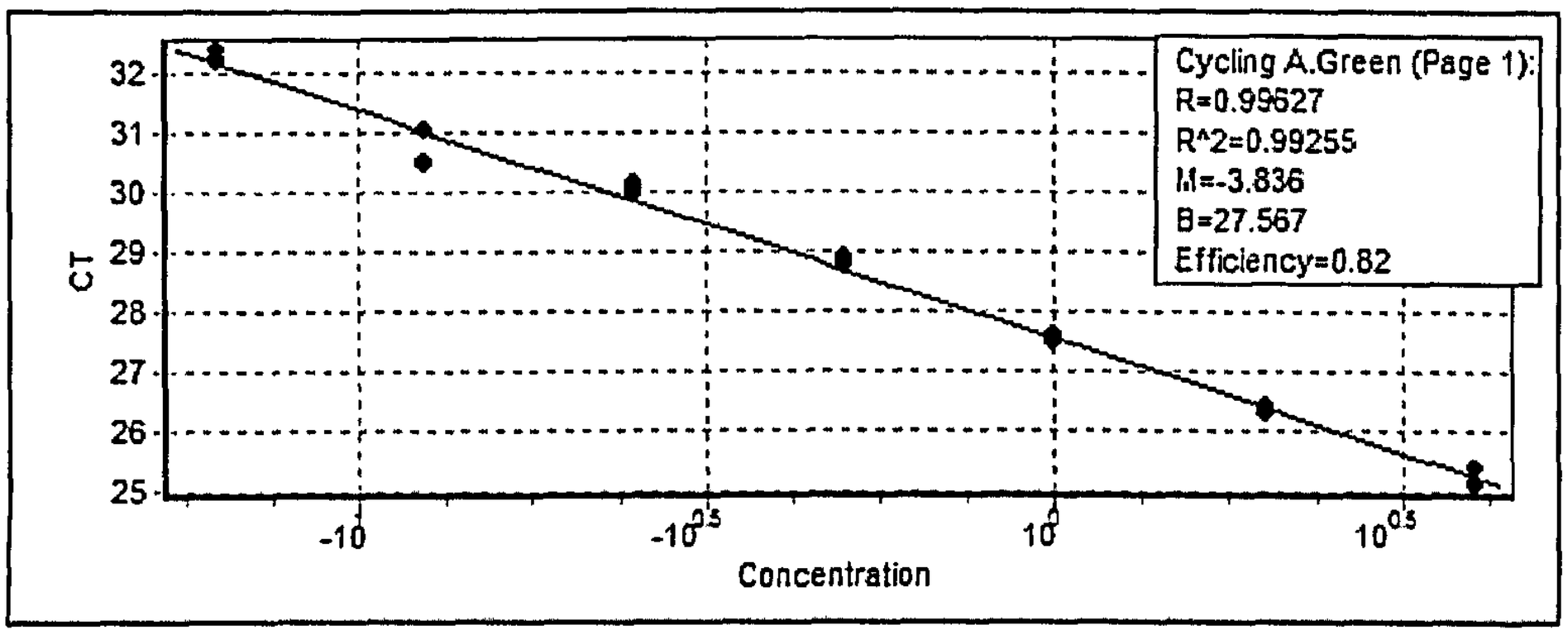
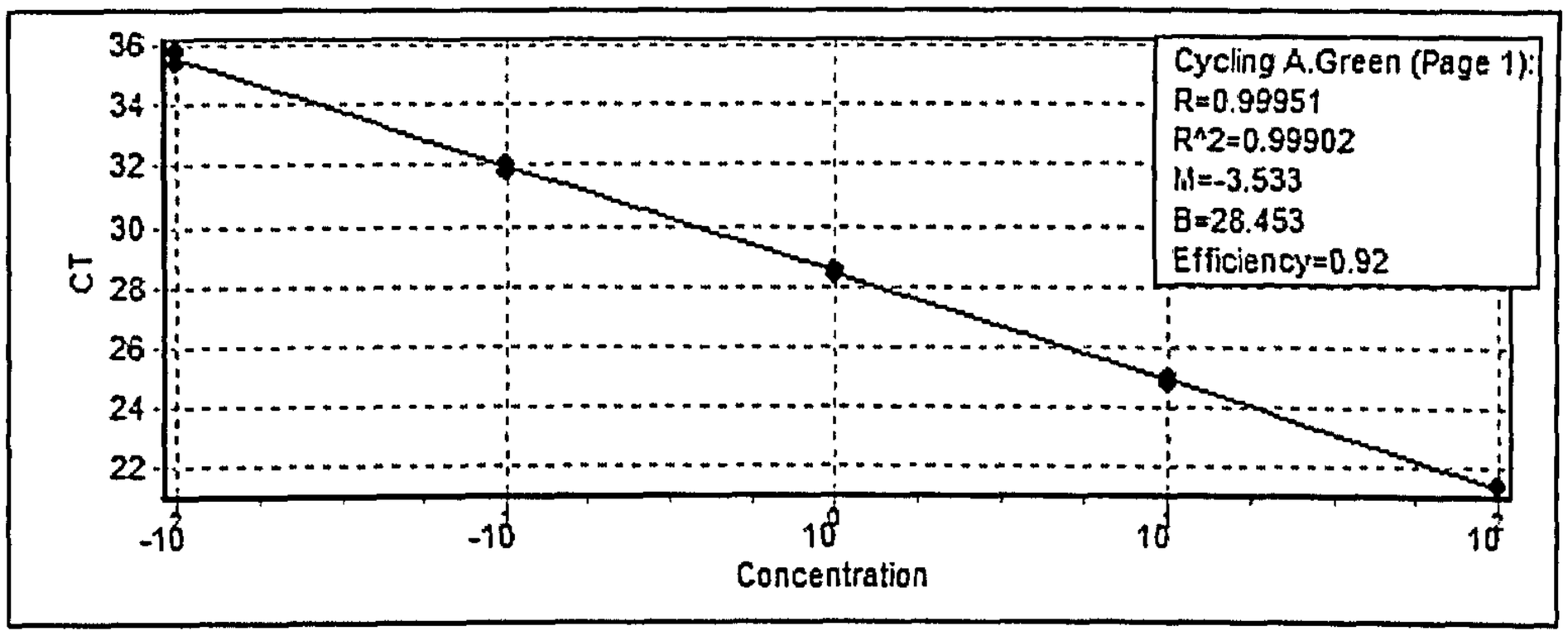
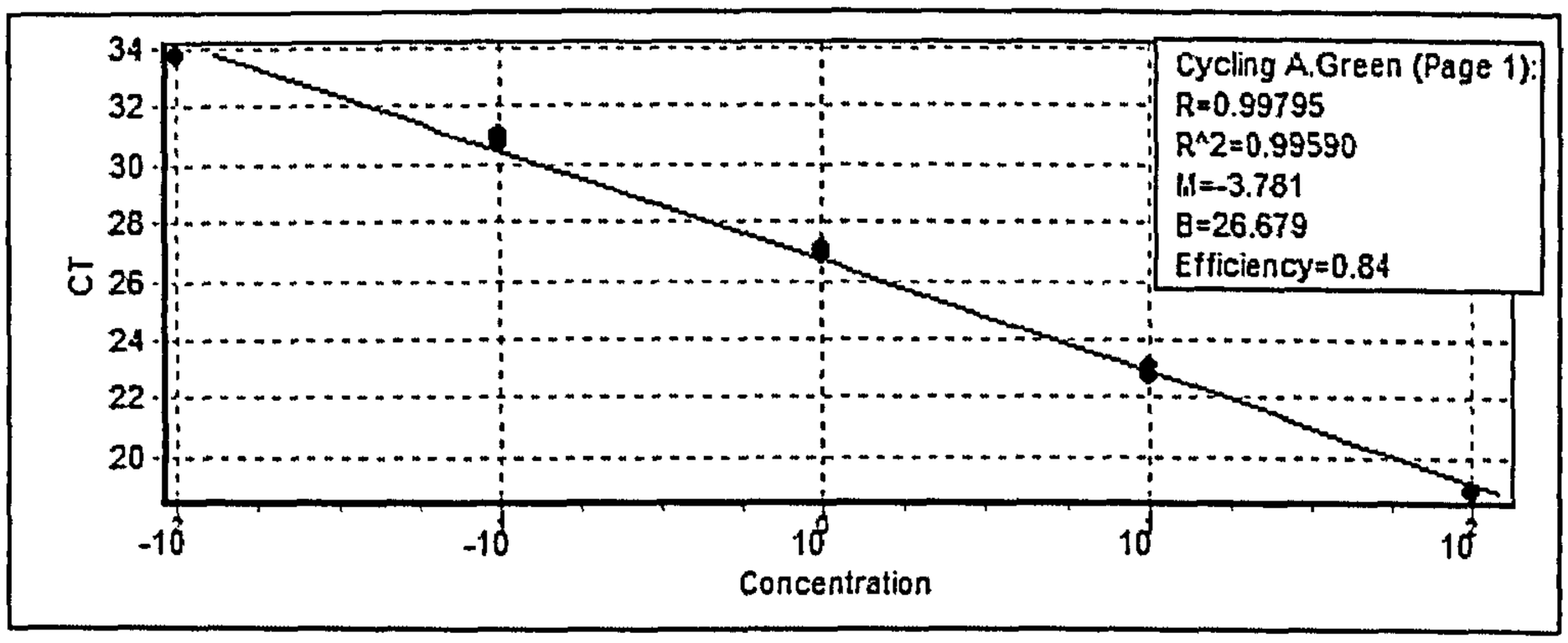
Chapter 8 Appendix





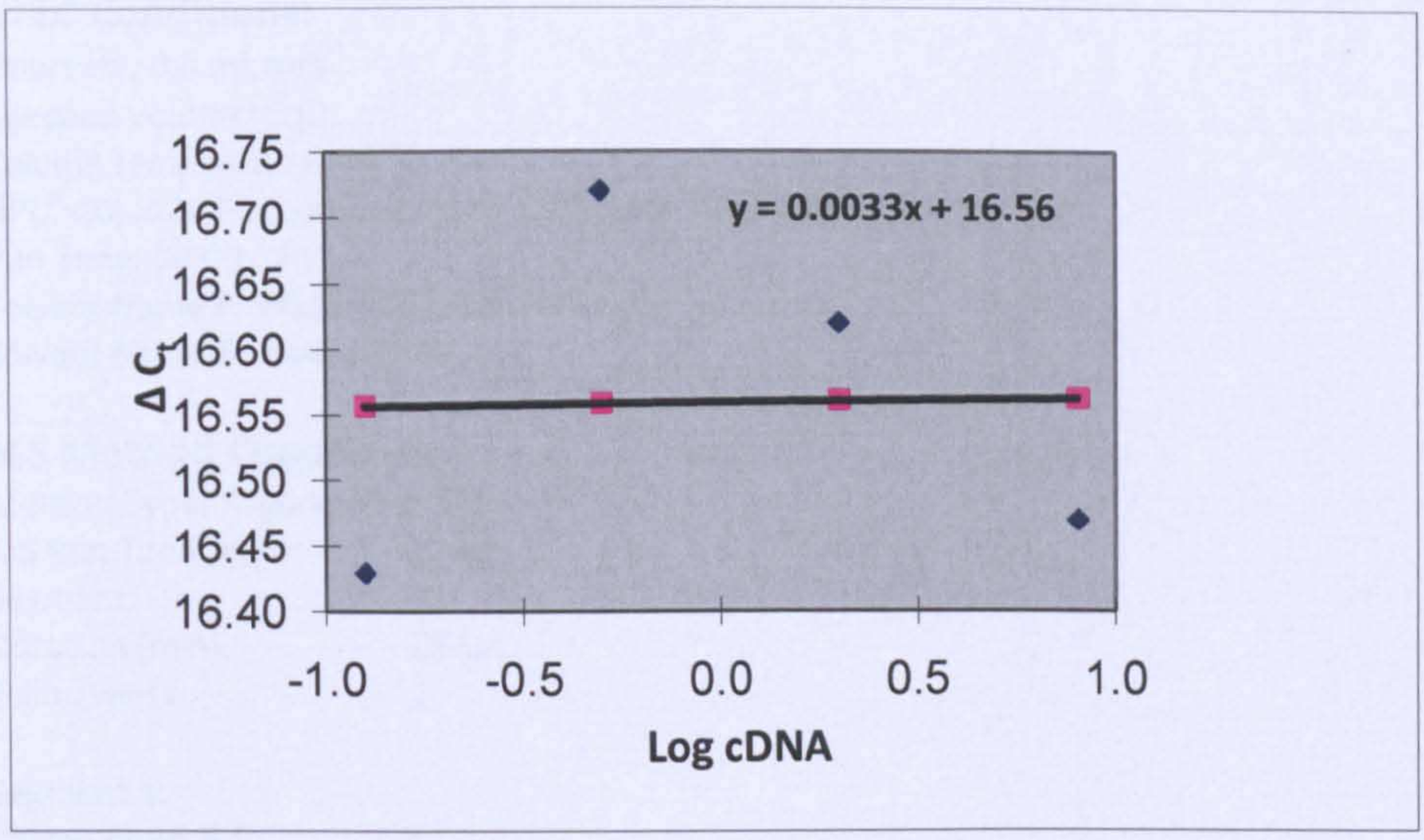


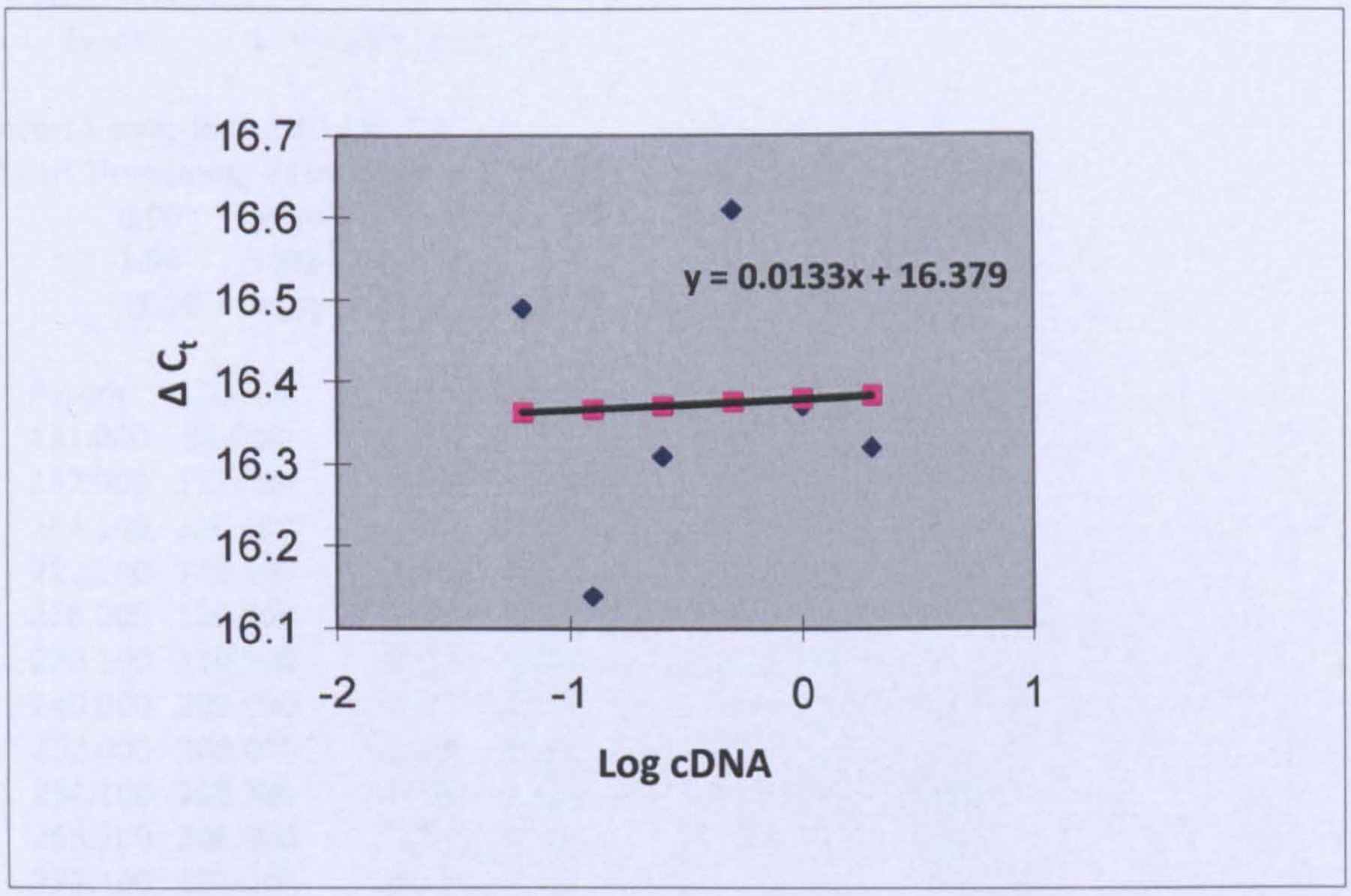
Appendix 8.1 Quantitative PCR standard curves for A) *CYP6CM1*, B) *actin* and C) *18S rRNA* genes used in relative gene expression in Chapter 5.

The standard curves made using *B. tabaci* cDNA serially diluted over 5 fold-logs with each dilution assayed in triplicate.

Standard curve	R	R^2	M	PCR Efficiency
CYP6CM1	0.996	0.993	-3.863	0.82
18S rRNA	0.998	0.996	-3.781	0.84
Actin	0.999	0.999	-3.533	0.92

Appendix 8.2 A summary of R values, slope gradients and PCR efficiencies of the standard curves made for each gene used in relative gene expression.





Appendix 8.3 Validation of the endogenous control genes for *CYP6CM1* expression.

Validation curves were made from serial dilutions of cDNA made from RNA extracted from 150 homogenised whiteflies (ALM07). The target gene, *CYP6CM1*, and the endogenous controls, A) *actin* and B) *18S rRNA*, were amplified by TaqMan® PCR for each serial dilution in triplicate. The ΔC_t ($C_{t,\ CYP6CM1} - C_{t,\ 18S\ rRNA}$) was calculated and equation of each plot is shown on the graph. A gradient of 0.01 or less is considered a suitable criterion for the use of an endogenous control gene.

HPLC Conditions:

Flow rate: 0.8 mL min^{-1}
Injection volume: 5 µL
Column temperature: 40 °C
HPLC Column: Phenomenex Luna C18, 5 µm, 4.60 x 250 mm
Run Time: 30.00 min
Solvent Name A: Water (0.2% formic acid)
Solvent Name B: Acetonitrile

MS Method Conditions:

Method Type: Regular Method

MS Run Time (min):	25.00
Segment:	1
Duration (min)	25.00
Scan Events	1

Segment 1:
Chrom filter: 6.0
Q2 Gas Pressure: 1.4
Syringe Pump: Off
Scan Events: 1: + c SRM Micro Scans 1

Divert Valve: in use during run

Divert Time (min)	Valve State
0.00	Inject \ Waste
1.94	Load \ Detector
21.99	Inject \ Waste

Parent	Center	Width	Time	CE	Q1PW	Q3PW	Compound
131.000	84.000	0.200	0.100	22	0.70	0.70	IMI-3
157.900	122.000	0.200	0.100	24	0.70	0.70	IMI-1
211.100	126.100	0.200	0.100	17	0.70	0.70	IMI-NH
212.200	128.100	0.200	0.100	24	0.70	0.70	IMI-urea
226.000	126.000	0.200	0.100	22	0.70	0.70	IMI-NNH_2
230.100	110.900	0.200	0.100	22	0.70	0.70	IMI-de
240.000	209.000	0.200	0.100	22	0.70	0.70	IMI-NNO
252.000	209.000	0.200	0.100	22	0.70	0.70	IMI-?
254.100	205.700	0.200	0.100	17	0.70	0.70	IMI-ole
256.100	208.900	0.200	0.100	17	0.70	0.70	IMI
272.100	191.200	0.200	0.100	22	0.70	0.70	IMI-5-OH
288.000	207.000	0.200	0.100	22	0.70	0.70	IMI-diol

Appendix 8.4 Summary of conditions used for HPLC and mass spectrometry of imidacloprid and metabolites.

Pest Management Science *Pest Manag Sci* 64:12–15 (2008)

Rapid Report

High-throughput allelic discrimination of B and Q biotypes of the whitefly, *Bemisia tabaci*, using TaqMan allele-selective PCR

Christopher M Jones,[1,2] Kevin Gorman,[1] Ian Denholm[1] and Martin S Williamson[2]*

[1]*Department of Plant and Invertebrate Ecology, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK*
[2]*Department of Biological Chemistry, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK*

Abstract

BACKGROUND: B and Q biotypes of the whitefly, *Bemisia tabaci* (Gennadius), are generally regarded as the most significant given their global distribution and strong resistance to insecticides. Since these biotypes can coexist and differ markedly in their insecticide resistance profiles, a rapid but reliable means of discriminating between them would be a valuable complement to resistance monitoring and management programmes. Recently, PCR-based methods have been developed to determine the biotype status of *B. tabaci* populations. However, these require post-amplification procedures, which increase time and labour.

RESULTS: The authors have developed an allelic discrimination real-time PCR assay using fluorescent dye-labelled probes to distinguish the B and Q biotypes. The assay targets a single nucleotide polymorphism (SNP) in the mitochondrial cytochrome oxidase I (mtCOI) gene. To evaluate the assay, DNA was extracted from individual whiteflies of six known biotype strains, and all scored correctly as either a B or Q biotype. As further validation, 72 individuals from field samples collected in different parts of the world were also tested by the assay. No failed reactions were observed, with all 72 samples scoring clearly as either the B or Q biotype.

CONCLUSION: The development of this rapid and high-throughput assay has important potential for routine monitoring of B and Q biotypes on ornamental plants and for the screening of *B. tabaci* populations in countries where these biotypes are not yet established.

Keywords: *Bemisia tabaci*; B and Q biotypes; allele-selective PCR; diagnostics; routine monitoring

1 INTRODUCTION

The whitefly, *Bemisia tabaci* (Gennadius), is an increasingly important pest of agricultural and horticultural crops worldwide.[1] Damage is caused directly by phloem feeding, by the transmission of several associated plant-pathogenic viruses[2] and through the excretion of honeydew which serves as a substrate for fungal infections.[1] *B. tabaci* exists as a complex of morphologically indistinguishable biotypes that possess distinct biological characteristics[3,4] and were originally characterised by their differing non-specific esterase profiles.[5,6] Over the last 20 years the B biotype (also described as *B. argentifolii* Bellows & Perring[7]) has proven to be the most widely problematical as a result of its relatively high fecundity, broad host range and strong resistance to insecticides.[4,8] Since the mid-1990s the Q biotype, which was first reported in Southern Europe, has superseded the B biotype in many areas around the Mediterranean and in the Middle East.[9–12] It is also now present in Japan and China[13,14] and was reported for the first time from the USA in 2004.[15] Transportation of the Q biotype around the world has occurred as a consequence of the global trade in infected plant material. This is of particular concern because the Q biotype characteristically shows strong resistance to novel insecticides (including neonicotinoids and insect growth regulators) that have in many areas provided successful control against other biotypes.[16,17]

A number of DNA-based techniques have been designed to complement or improve upon traditional biochemical methods for diagnosing biotypes.[9,18–21] These techniques can, however, be time consuming and labour intensive. Mitochondrial (cytochrome oxidase I or COI) and nuclear gene sequences (ribosomal intergenic transcribed spacer 1 or ITS1) have also been used as markers to study genetic

* Correspondence to: Martin S Williamson, Department of Biological Chemistry, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK
E-mail: martin.williamson@bbsrc.ac.uk
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variation in *B. tabaci*, and an alternative classification into genetic races based on phylogenetic clades has been proposed.[22]

Polymerase chain reaction (PCR)-based allelic discrimination assays using fluorescent dye-labelled probes provide a fast and reliable way for detecting single nucleotide polymorphisms (SNPs) and have been used, for example, to diagnose mutations causing insecticide resistance.[23,24] In the present paper, an analogous allelic discrimination assay based on mtCOI sequence polymorphism to determine the biotype status of *B. tabaci* individuals is reported.

2 MATERIALS AND METHODS

2.1 Insect strains and DNA extraction

Strains of *B. tabaci* were reared at Rothamsted Research on cotton plants (*Gossypium hirsutum* L. cv. Linda) at 26 ± 2 °C under a 16:8 h light:dark photoperiod. The biotype status of each strain was predetermined by polyacrylamide gel electrophoresis of non-specific esterases. B biotype strains were PIRGOS (Cyprus, 2003), GUA-MIX (Guatemala, 2004), GRB (USA, 1997) and MEX-2 (Mexico, 2004). Q biotype strains were CHLORAKA (Cyprus, 2003) and CRT-1 (Crete, 2006). For further validation of the assay, 72 adults were tested from ten field samples of *B. tabaci* that were likely to contain either the B or Q biotypes from locations in Japan, Taiwan, Brazil and Egypt. The field samples were collected and transported in 90–100% ethanol.

Genomic DNA for genotyping was extracted from individual whiteflies homogenised in 200 µL of DNAZOL® (Invitrogen) at a fifth scale of the supplier's recommended protocol. The DNA was resuspended in 20 µL of Tris-EDTA buffer (10 mM Tris-HCl, pH 8 + 0.1 mM EDTA).

2.2 TaqMan allelic discrimination assay

A nucleotide alignment of 95 mtCO1 gene sequences from a range of *B. tabaci* B and Q biotypes available in the National Centre for Biotechnology Information (NCBI) database (GenBank) revealed a number of SNPs that discriminate between these biotypes (Fig. 1). The target SNP chosen for this assay was within a conserved region of the mtCOI gene sequence to facilitate the design of TaqMan probes and primers.

Two primers and two fluorescent dye-labelled probes were designed and manufactured by Applied Biosystems (Foster City, CA, USA). The forward primer BEMBQ-SNP1F (5′-GCCTTTGATTTAC-AGGATTTTTATTTTTATTTACTATAGGT-3′) and the reverse primer BEMBQ-SNP1R (5′-GAAATCAATAGATAACTCCTCCTACAATA-GCA-3′) were unmodified PCR primers. The probe SNP1V2 (5′-ATGCAGACACACATC-3′) was labelled with the reporter dye VIC at the 5′ end for detection of B biotype individuals, and the probe SNP1M2 (5′-ATGCAAACACACATC-3′) was labelled with 6-FAM at the 5′ end specific for the Q biotype. Both probes contained a non-fluorescent quencher dye (NFQ) and minor groove binding (MGB) groups at their 3′ ends. The NFQ dye suppresses VIC or FAM fluorescence until the probe is broken down during PCR, while the MGB groups increase the stability of matched duplexes, thereby resulting in a more accurate allelic discrimination.[25]

Each PCR reaction composed of gDNA (1 µL), SensiMix DNA kit (12.5 µL; Quantace Ltd, Neutral Bay, Australia), 900 nM of each primer and 200 nM of each probe, and the total volume (25 µL) was made up with sterile water. Sterile water (1 µL) was used for blank negative controls. Real-time PCR was performed on a Rotor-Gene 6000™ (Corbett Research) using temperature cycling conditions of 10 min at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. The increase in VIC and FAM reporter dyes, representing B and Q biotypes respectively, was monitored in real time using the RotorGene software. For each assay, fluorescence values of the negative controls were averaged and subtracted from the raw data to correct for background fluorescence. The endpoint values of fluorescence for each dye were then plotted against each other in bivariate scatter plots that gave a clear clustering of

Figure 1. Nucleotide alignment of a 100 bp region of the mitochondrial cytochrome oxidase I sequence from a sample of B, Q and other biotypes of *Bemisia tabaci* obtained from GenBank (accession numbers are shown in the figure). The vertical arrow marks the SNP at position 46 targeted in the TaqMan allelic discrimination assay.

the samples and enabled easy scoring of the B and Q biotypes.

3 RESULTS AND DISCUSSION

The TaqMan assay is an allelic discrimination PCR that uses fluorescent dye-labelled probes to determine which allele is present.[26] A single SNP within a conserved region of the *B. tabaci* mtCOI gene was targeted to develop the B/Q assay (Fig. 1). When DNA from a B biotype adult is tested, the VIC-labelled B-selective probe anneals to its target sequence in the mtCOI gene and is then cleaved in the PCR by the 5′ nuclease activity of Taq DNA polymerase, thereby releasing the VIC dye away from its quencher and resulting in an increase in VIC fluorescence. Conversely, when DNA from a Q biotype adult is tested, only the FAM-labelled Q-selective probe anneals, resulting in cleavage of this probe and an increase in FAM fluorescence. The relative fluorescence of these two dyes is measured in real time during the PCR, with the endpoint values corrected for background and plotted against each other in a bivariate scatter plot.

An initial analysis of individual adult whiteflies from six laboratory strains of known biotype status (two Q and four B biotypes) is given in Fig. 2A and shows that the assay works extremely well, with the B and Q biotypes grouping into two distinct clusters. To validate further and demonstrate the high-throughput nature of the assay, 72 adults from ten field samples of unknown biotype were tested and also found to group into two clear clusters that enabled unambiguous scoring of all samples as either B or Q biotype (Fig. 2B). No failed reactions were observed, with all individuals within a particular field sample conforming to the same biotype. Thus, it is concluded that the assay is fast and accurate and can be carried out on ethanol-preserved samples that have been transported over long distances.

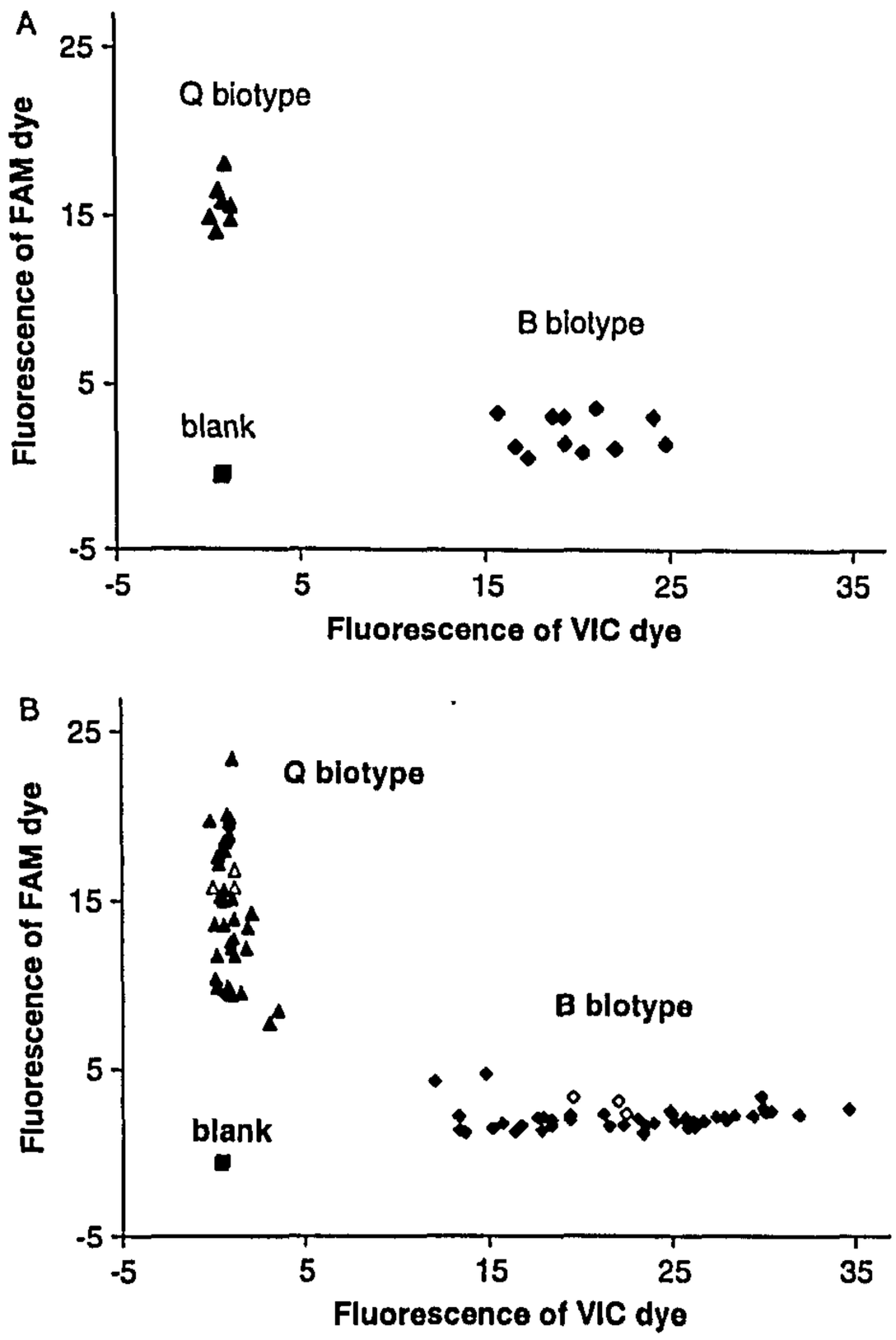

Figure 2. Scatter plot analysis of fluorescence data from the TaqMan assay facilitates scoring of B and Q biotypes. **A**, adults of known biotype from the laboratory strains show tight clustering according to their respective increases in VIC (B biotype) or FAM (Q biotype) fluorescence. Negative controls (water) are represented in the 'blank' cluster. **B**, analysis of 72 individuals from field samples of unknown biotype. All samples group clearly into the B- or Q-type clusters. Three each of water controls (blank), known B types (open diamonds) and known Q types (open triangles) were also included.

The B and Q biotypes represent the most damaging biotypes of *B. tabaci*, and the recent global spread of multiresistant insects of the Q biotype has heightened the need for a rapid means of distinguishing between them. For example, in the USA, a national whitefly Q biotype task force has been formed to monitor and combat the spread of this biotype (http://www.cottoninc.com/Entomology/WhiteflyQBiotypeTaskForce).

Current PCR-based methods to discriminate biotypes are not high throughput and require post-amplification procedures which increase both time and labour.[21] The allelic-discriminating PCR assay outlined in this paper required minimum optimisation and is designed to run in a 96-well plate format from which results can be obtained in less than 90 min.

This assay was specifically designed to discriminate between B and Q biotypes given their agricultural and economical importance. As it stands, the assay is not applicable to other biotypes of *B. tabaci* owing to variation in the mtCOI sequence (Fig. 1), which would result in either a failure of the flanking primers to anneal or a mismatch of the fluorescent probes. TaqMan assays could be designed to target alternative SNPs, thereby enabling discrimination between other biotype combinations.

Biotypes are defined according to differing biological characteristics; any single SNP present within the nuclear or mitochondrial genome is therefore most likely to correlate with, rather than determine, biotype status. Although the SNP chosen in this assay consistently separated B and Q biotypes in 95 sequences from the available database, verification of this assay by occasional sequencing of the target gene may be beneficial in cases where the assay fails to score either biotype.

One potential drawback of using a mitochondrial rather than nuclear marker is its inability to detect potential genetic introgression between B and Q biotypes. However, successful mating between these biotypes is known to be extremely rare, if it occurs at

all (Rothamsted Research, unpublished data). Given the consistency of the SNP used here, the authors believe that the assay is not only highly reliable but can significantly reduce the time and expense associated with existing molecular approaches for the determination of *B. tabaci* biotypes.

Given the falling costs of fluorescent dye-labelled probes, the costs of running this assay are estimated to be less than one dollar per insect. In addition, the assay only requires one-tenth of a whitefly, offering the opportunity of combining the biotype diagnostic work with finding other traits associated with SNPs, such as insecticide resistance mutations. Furthermore, recent work by the present authors has shown that these assays can be performed on single, homogenised insects without the need for prior DNA extraction, which further enhances throughput and offers the potential for a single operator to test several hundred samples per day.

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