Evolutionary Genetics of Insecticide Resistance in *Culex quinquefasciatus*

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

By

Walter Fabricio Silva Martins

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Abstract

Culex quinquefasciatus mosquitoes play an important role in the transmission of vector-borne diseases of public health importance including lymphatic filariasis (LF) as well as many arboviruses. Insecticide-based approaches are one of the most important interventions to mitigate disease burden; nevertheless increased resistance of vectors to insecticides imposes a challenge for the sustainability and effectiveness of both current and future vector control interventions. Hence, understanding the dynamics and likely mechanisms underlying the evolution of resistance will be critical to effective decision-making in insecticide resistance management strategies. The present study was set out to investigate the genetic basis of insecticide resistance in *C. quinquefasciatus* from Uganda. Two objectives were developed, 1) to investigate patterns of insecticide resistance across the south of the country and how this might reflect local selection and genetic structure and 2) to investigate the basis of the molecular mechanisms underlying resistance to all four classes of insecticides recommended for vector control.

The population genetic study compared and contrasted microsatellite markers and two resistance-associated loci (*Vgsc*-1014F and *Ace1*-119S). While no significant difference in genetic diversity across populations were detected by microsatellites, higher frequency of *Vgsc*-1014F compared to the *Ace1*-119S mutations was observed in all populations suggesting that the Ugandan Eastern – Southwest populations are under a heterogeneous selection pressure, which created a pattern of local adaptation in these populations. Additionally, the copy number (CN) assay developed in this study indicated the presence of CN variation in the voltage gated sodium channel (*Vgsc*) gene in about 10% of the individuals assayed from these populations. Genotypic/phenotypic association tests conducted on bendiocarb resistant-individuals suggested that this resistant phenotype was not underlying solely by the 119S target-site mutation in the *Ace-1* gene. Indeed, synergist bioassays show an increase of mortality of around 25% in mosquitoes pre-exposed to either TTP or PBO, indicating a possible resistance mediated by detoxification enzymes. Using a novel whole-transcriptome microarray we profiled the bendiocarb-resistant phenotype and implicated two P450s (*Cyp-Cx1* and *Cyp6n23*) with the highest up-regulation expression compared to a susceptible strain. Remarkably, the predicted *Cyp-Cx1* is closely related to two P450s from the family *Cyp6*, which were already validated *in vitro* as insecticide metabolizers in *A. gambiae* and *A. aegypti*, which corroborates a likely association of metabolic resistance in the investigated bendiocarb-resistant phenotype.

Taken together the results yielded by genomic and transcriptomic experiments provide evidence that Ugandan *C. quinquefasciatus* mosquitoes are under heterogeneous selection pressure imposed by insecticides from distinct classes, and that the evolution of insecticide resistance is mediated by at least two main genetic mechanisms; target-site mutations (*Vgsc*-1014F and *Ace1*-119S) as well as over-expression of detoxification enzymes.

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List of Abbreviations

Ace	Acetylcholinesterase
AWDI	Alternative Wet and Dry Irrigation
BIC	Bayesian Information Criterion
CNP	Copy Number Polymorphism
CNV	Copy Number Variation
DAPC	Discriminant Analysis of Principal Components
ddPCR	Digital Droplet PCR
DEC	Diethylcarbamazine
DEM	Diethyl Maleate
F_{IS}	Inbreeding Coefficient
GABA	Gamma-Aminobutyric Acid
GO	Gene Ontology
GPELF	Global Program to Eliminate Lymphatic Filariasis
GSTs	Glutathione S-transferases
H_E	Expected Heterozygosity
H_O	Observed Heterozygosity
HWE	Hardy-Weinberg Equilibrium
IBD	Isolation-By-Distance
IRS	Indoor Residual Spraying
ITNs	Insecticide-Treated Bed Nets
JEV	Japanese Encephalitis Virus
kdr	Knockdown resistance
LLITNs	Long Lasting Insecticide-Treated Bednets
LF	Lymphatic Filariasis
MDA	Mass Drug Administration
MF	Microfilaria
Ne	Effective Population size
NTDs	Neglected Tropical Diseases
P450	Cytochrome P450
PBO	Piperonyl Butoxide
PIC	Polymorphism Information Content
qPCR	Quantitative PCR
Rdl	Resistance to Dieldrin
RQPS	Reference Query Pyrosequencing
*R*s	Allelic Richness
RT-qPCR	Reverse transcription Quantitative PCR
RVF	Rift Valley Fever

SLEV	Saint Louis Encephalitis Virus
SNPs	Single Nucleotide Polymorphisms
TFBS	Transcription Factor Binding Sites
TPP	Triphenyl Phosphate
TPRI	Tropical Pesticides Research Institute
ULV	Ultra Low Volume
Vgsc	Voltage-Gated Sodium Channel
WNV	West Nile virus

CHAPTER I

Background and Study Design

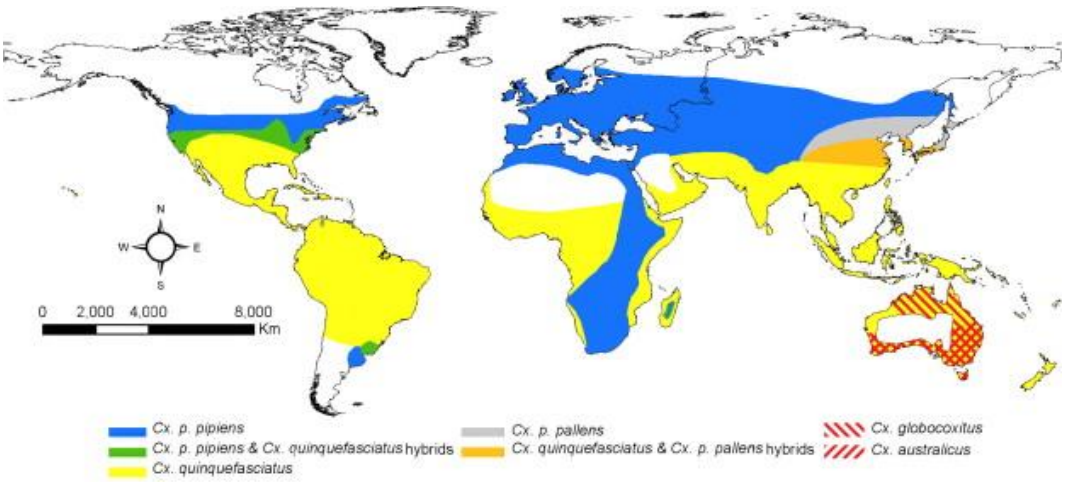
1.1. Ecology and medical importance of *Culex* complex mosquitoes

1.1.1 Ecology of *Culex* mosquitoes

With more than 700 species, the genus *Culex* is the largest within the family Culicidae, containing a vast number of species which have the capacity to transmit lymphatic filariasis (LF), as well as arboviruses such as West Nile virus (WNV), Japanese Encephalitis Virus (JEV), Rift Valley Fever (RVF) and Saint Louis Encephalitis Virus (SLEV) (Farajollahi et al. 2011, Harbach 2011). The subgenus *Culex* includes most of the species of public health importance with around 16 of the 198 species recognized as vectors of pathogens to humans, with seven of the species belonging to the *Culex pipiens* complex: *Culex pipiens, Culex quinquefasciatus, Culex australicus, Culex globocoxitus, Culex molestus*, *Culex pallens* and *Culex torrentium* being the most relevant for the burden transmission of these diseases (Knight 1978, Becker et al. 2010, Harbach 2012). Although the term *pipiens* complex will apply here as well as is used in most of the recent publications, recent works have also suggested the use of the term *Culex pipiens* assemblage to avoid taxonomic connotation as the degree of relationship among these species is not yet clearly defined (Harbach 2012).

Notwithstanding their global distribution, the species within the *Culex pipiens* complex have different preferential habitats across tropical, temperate and sub-temperate regions (Figure 1.1) (Hesson et al. 2011, Russell 2012). Indeed, the geographic distribution and ecological features of these species impacts on their primary medical importance. Among species of the *Culex pipiens* complex, differences in aspects of behavior, such as distinct feeding preference (ornithophily / mammophily); and ability to complete a gonotrophic cycle without blood feeding (anautogenous / autogenous), are reflected in the vectorial capacity of each species (Vinogradova 2000, Fonseca et al. 2004, Gomes et al. 2009, Farajollahi et al. 2011).

Additionally, the apparent high adaptability of mosquitoes of the complex to a wide variety of breeding sites such as sewerage systems, polluted and fresh water, and artificial containers, as well as the ability to feed on a variety of vertebrate blood types, enables successful adaptation to both rural and urban locations (Reusken et al. 2010, Farajollahi et al. 2011). This habitat flexibility, which allows a closer connection between *Culex* mosquitoes and human populations, makes these species a highly efficient system for bridging pathogen transmission from animal reservoirs to humans (Bowman 2014).



Taken from (Farajollahi et al. 2011)

Figure 1.1: Global distribution of *C. pipiens* complex mosquitoes. *C. p. pipiens* composition may include both forms (*pipiens* and *molestus*).

1.1.2 Epidemiology of vector-borne diseases transmitted by *Culex* mosquitoes

Although highly adapted to a wide diversity of habitat conditions, *Culex* spp. composition and their public health relevance contrasts greatly among ecozones (Hesson et al. 2011). Among the vector-borne diseases with worldwide medical relevance, *Culex* species play an important role in the transmission of arboviruses and filarial worms, mainly in tropical and sub-tropical regions (Alatoom and Payne 2009, WHO 2012a, Schmidt et al. 2013).

At present, arboviruses transmitted by *Culex* mosquitoes belong almost exclusively to two genera: *Flavivirus*; which are viruses with single, positive-strand RNA (*eg* JEV and WNV) and *Phlebovirus*, viruses with single, negative-strand RNA (*eg* RVFV) (Mukhopadhyay et al. 2005, Pepin et al. 2010, Lequime and Lambrechts 2014). Among these arboviruses, only WNV has a worldwide distribution, while SLEV is endemic in the American continent and JEV and RVF are geographically spread throughout South-east

Asia, sub-Saharan Africa and Arabia (Kramer et al. 2008, Weissenböck et al. 2010, Ikegami and Makino 2011). The majority of WNV infections in the Western hemisphere have been reported in the United States. Since the virus was first detected in New York City in 1999, more than 37,000 cases have been reported, with around 7,000 classified as neuroinvasive disease, 9,000 as West Nile fever like (WNF) and 770 deaths (Hayes et al. 2005, Kilpatrick et al. 2006, Roehrig 2013).

Furthermore, *C. quinquefasciatus* is responsible for most of the urban transmission burden of lymphatic filariasis (LF), recognized as the second largest cause of disability globally and with a heavy social and economic impact in endemic regions (Chandy et al. 2011, van den Berg et al. 2013). LF, caused by three species of filarial worms *Brugia malayi*, *Brugia timori* and *Wuchereria bancrofti*, is a parasitic infection broadly spread throughout 83 endemic countries of the tropical and sub-tropical regions, resulting in one fifth of the world`s population being at risk of infection and more than 100 million people infected in Asia, Africa and the Americas (Ottesen 2006, Ichimori et al. 2014). The socio-economic and public health impact of LF in endemic regions results from a wide range of clinical symptoms including chyluria, tropical pulmonary eosinophilia and adenopathy (Huppatz et al. 2009).The large disability rate of LF as reported in Africa and Southeast Asia, ranging from 2.3 to 3.5 million DALYs (disability-adjusted life-years), reflects mostly the long term disability related to lymphoedema, elephantiasis and hydrocele, which is caused by the blocking of the lymphatic system due to deposition of adult worms, especially in the lower limbs (Chandy et al. 2011, van den Berg et al. 2013).

1.1.3 Public health intervention toward diseases transmitted by *Culex* mosquitoes

Due to the socio-economic and human health impact of vector-borne diseases transmitted by *Culex* species, development of control interventions is imperative in order to block transmission and prevent outbreaks in endemic regions. Despite the similar patterns of transmission of the arboviruses and parasite-caused diseases through bites of infected *Culex* species, distinct epidemiological control strategies have been applied for different diseases and geographic locations (Spinsanti et al. 2003, Batallán et al. 2015).

Public health risk management for arboviral diseases such as JEV and WNV have typically applied vaccination as the main strategy to prevent outbreaks in endemic regions (Schmidt et al. 2013). For these arboviruses, vaccines are available to immunize either the animal or human host, but are not suitable for vaccination of both. For example, there are four vaccines licensed for WNV; however, they are exclusively used for the epizootic host (Kramer et al. 2008). Likewise, the pillar for JEV vaccination campaigns is human immunization using one of two vaccines available (Erlanger et al. 2009), while vaccination of animal hosts (e.g. pigs) is an alternative strategy of control; although two drawbacks for swine vaccinations exist; 1) requirement of annual vaccination and 2) low effectiveness of attenuated vaccines for young animals (Wada 1988). Despite the availability of specific vaccines to prevent outbreaks of arboviruses transmitted by *Culex* spp, vector control intervention is still an imperative approach to mitigate burden transmission of arboviruses in endemic regions. Control programs against *Culex* mosquito populations include a wide range of approaches based on different methods (e.g biolarvicides, insect-growth regulators and insecticides) and strategies (e.g. indoor

residual spraying (IRS), long lasting insecticide-treated bednets (LLITNs and aerial fog) (Keiser et al. 2005, Takken and Knols 2009).

Successful campaigns of *Culex* spp control for preventing and reducing arboviruses outbreak have been broadly reported across endemic regions. For example, vector control through aerial and ground use of Ultra-Low Volume (ULV) applications with Naled (1,2-dibromo-2,2-dichloroethyl dimethyl phosphate) in Louisiana, US resulted in up to 10-fold reduction of *C. quinquefasciatus* population density over the intervention term, together with a dramatic drop in the number of WNV human cases (Palmisano et al. 2005). Also in the USA, during the 2005 WNV epidemic in Sacramento County, California, aerial spraying with pyrethrins had a strong impact on the control of a WNV epidemic, which resulted in a decrease of both vector population density (*C. pipiens* and *C. tarsalis*) and WNV infection rates by 50% between sprayed and unsprayed areas within the county (Macedo et al. 2010). Furthermore, in the same epidemic outbreak in Sacramento in 2005, a different intervention using sprays of pyrethrins combined with piperonyl butoxide (PBO), an insecticide synergist, was effective at reducing both mosquito populations and the number of human WNV positive infections (Carney et al. 2008).

Moreover, although vaccination campaigns for human and swine populations have almost eliminated the incidence of JEV in countries such as Japan, Korea and Taiwan (van den Hurk et al. 2009), vector control interventions have also played an important role to minimize the JEV transmission cycle, especially in endemic rural settlements, where immunization campaigns is a challenge to reducing human vaccination rate (Hills and Phillips 2009). Additionally, in Asian rural endemic regions, mosquito control interventions have also been a key element to prevent and reduce the density of *Culex* mosquitoes populations across regions with large rice fields; distinct control approaches

using biological larvicides and invertebrate predators to reduce vector-population density by limiting breeding sites, including changes in agricultural practices, such as wet and dry irrigation (AWDI), have reduced JEV transmission (Keiser et al. 2005, Kumari et al. 2014).

In contrast to the vaccination approach used to control the arboviruses transmitted by *Culex* mosquitoes due to the absence of vaccines for preventing filarial worm infections, anti-filarial chemotherapy was chosen as the primary intervention to reduce infective microfilaria prevalence in endemic regions (WHO 2005). This strategy is a worldwide programme recommended by the World Health Organisation (WHO), and applied through the Global Program to Eliminate Lymphatic Filariasis (GPELF) launched in 2000 (WHO 2012a, 2013b). The mainstay of the GPELF is annual mass drug administration (MDA) in endemic regions using three anti-filarial drugs: albendazole, diethylcarbamazine (DEC) and ivermectin, which kill the microfilaria in infected patients' blood and interrupt the transmission cycle (Bhullar and Maikere 2010).

Nevertheless, despite the initial recommendation of MDA as the primary intervention to eliminate LF, vector control interventions have been recognised as an important complementary strategy to achieve the target of LF elimination by 2020, with vector control measures providing a valuable contribution in both MDA and post-MDA intervention phases (Bockarie et al. 2009). Vector control interventions have played an important role in LF eradication especially in Central Africa, where due to co-infection with LF and Loa Loa, MDA could not be applied, as patients treated with Ivermectin may develop severe adverse reactions including neurologic decline and encephalopathy (Babu et al. 2006, Addiss and Global Alliance Eliminate 2010). Furthermore, combinations of MDA and vector control have also been recommended for regions with a high prevalence

of microfilaria, where use of both interventions can reduce the number of MDA rounds and the time required to achieve the microfilaria eradication threshold (Kyelem et al. 2008, Rivero et al. 2010).

1.2. Insecticide-based tools applied for vector control

Insecticide-based approaches targeting both larval and adult stages are the most widely applied vector control interventions (WHO 2012b). Despite the utility of insecticides for minimizing the burden of vector borne diseases, to date, a restricted number of insecticides belonging to four classes: pyrethroids, organochlorines, organophosphates and carbamates, have been approved for public health programmes (Malaria Vectors 2012). Furthermore, there are also limitations to their application due to their toxicity towards either humans or the environment. For example, while all four classes of insecticide are approved for indoor residual spraying (IRS), solely pyrethroids are recommended by WHO for use on bed nets (WHO 2003). By contrast, due to the toxic effect of pyrethroid insecticides on aquatic species, such as fish, their use is restricted for vector control targeting larval stages (Sanchez-Bayo 2012).

Consequently, for control interventions targeting different parts of the mosquito life cycle, specific insecticides and delivery methods are recommended by WHO to ensure both security and maximum efficacy of control actions. At present, 12 adulticide formulations; DDT, malathion, dichlorvos (DDVP), temephos, fenitrothion, bendiocarb, propoxur, cypermethrin, alpha-cypermethrin, deltamethrin, lambda-cyhalothrin and permethrin are authorised for use in either indoor or outdoor sprays or for both (Becker et al. 2010, WHO 2011). For instance, exclusively deltamethrin and cyphenothrin are

approved for both indoor and outdoor sprays, while the others have application restrictions for indoor (e.g. permethrin) or outdoor use (e.g. lambda-cyhalothrin and malathion) (Matthews 2008). Additionally, adult control interventions especially in Africa where *Culex* is co-endemic with *Anopheles* species, have also applied long-lasting insecticidal nets (LLINs), which have either deltamethrin, alpha-cypermethrin or permethrin incorporated into polyethylene or coated on to polyester during manufacturing (Bellini et al. 2014).

As an outbreak prevention strategy, mosquito larval control using synthetic insecticides relies exclusively on four organophosphate insecticides; chlorpyrifos**,** fenthion**,** pirimiphos-methyl and temephos, with the latter two recommended for application in natural and artificial breeding sites, whereas the former two are to be used only in open water bodies (Becker et al. 2010). Alternatively to syntactic insecticides, bacterial larvicides such as *Bacillus thuringiensis israelensis* (*Bti*) and *B. sphaericus* have also been applied to mosquitoes larvae control, with significant reduction of larvae density already reported in *Culex,* and *Anopheles mosquito* and *A. aegypti* after bioassays (Cyrino Zequi et al. 2014, Dylo et al. 2014, Soares-da-Silva et al. 2015). Nevertheless, a recent study also show that ingestion of *Bti* in sugar meals by adults of the *A. gambiae* complex have no effect in the survivorship rate (Terbot et al. 2015), which indicated a stage specific activity.

1.3. Molecular basis of insecticide resistance in mosquitoes

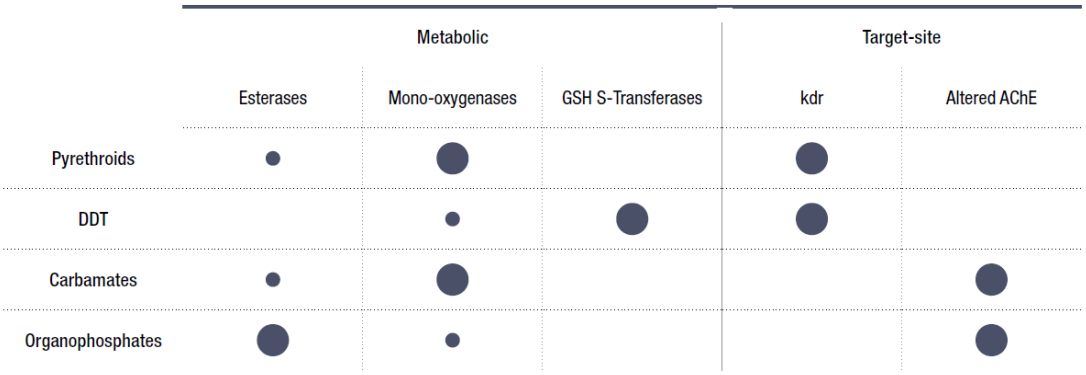
The historic and continuing use of insecticide in agricultural practices and for public health has driven the evolution of resistance, with resistance to at least one insecticide having now been reported in around 600 arthropod species (Mota-Sanchez et

al. 2002, Whalon et al. 2008, Sparks 2013). Therefore, due to the limited number of insecticides and the timeframe of around 12 years needed to develop new insecticides, characterization and understanding of the mechanisms involved in the resistance are crucial to preserve and increase the life span of existing compounds (Sparks 2013).

Insecticides belonging to all four classes recommended for vector control target the insect's peripheral and central nervous system, leading to continuous nerve excitation, paralysis and death (Coats 2012). For instance, pyrethroid insecticides induce neuronal over-excitation by delaying the inactivation of voltage-gated sodium channels (Soderlund 2012). Similarly, neonicotinoid insecticides also induce neuronal over-excitation; however, by contrast they target nicotinic acetylcholine receptors (Matsuda et al. 2009). Although structurally and biochemically distinct, these insecticides share similar modes of action. For example, organophosphates and carbamates act by inhibition of acetylcholinesterase (Ace), preventing breakdown of the neurotransmitter acetylcholine, resulting in neuromuscular overstimulation. On the other hand, both pyrethroids and organochlorines act on the sodium channel located in the neuronal membrane, keeping the channel open and thus, causing loss of nervous impulse (the knockdown phenotype) or can result in an over-stimulation causing repetitive neuron firing, with both phenotypes leading to paralysis of the animal (Ranson et al. 2011, Sanchez-Bayo 2012).

So far, most of the resistant-phenotypes reported in resistant mosquitoes rely on four main types of mechanisms of resistance; target-site insensitivity, metabolic, behavioural and cuticular, with their presence alone or in combination within individuals or populations conferring resistance to all insecticides applied in vector control interventions (Marcombe et al. 2013). Additionally, due to similar modes of action between insecticides from different classes**,** a single mechanism can also confer cross-

resistance to one or more classes of compound, although distinct mechanisms may impact differently in the resistance to insecticides from different classes (Figure 1.2) (Nauen 2007). Due to the scope of this work further details for the mechanisms of resistance mediated by target-site and detoxification enzymes are provided herein, while additional information toward behavioral and cuticular resistance are available elsewhere (Liu et al. 2006, Wood et al. 2010, Chareonviriyaphap et al. 2013).



Taken from (Malaria Vectors 2012)

Figure 1.2: Impact of molecular mechanisms in insecticide cross-resistance. GSH, glutathione; AchE, acetylcholinesterase; circle size reflects relative impact of mechanism of resistance.

1.3.1 Target-site mutations in mosquito's resistant phenotypes

The mechanism of resistance by target insensitivity relies on structural changes in the insecticide target site, which blocks insecticides from binding to its target. Most structural changes result from replacement of single amino acids (Yang and Liu 2013). At present, the insecticides available are designed to bind one out of three targets; the synaptic acetylcholinesterase *Ace-1* gene, the gamma-aminobutyric acid (GABA) receptor gene and the voltage-dependent sodium channel encoded by the sodium channel gene (Casida and Durkin 2013, Yang and Liu 2013).

The voltage-gated sodium channel (*Vgsc*) is the primary target for both pyrethroids and DDT. Insecticide target-site mutations in the *Vgsc,* are referred to as knock-down resistance *(kdr*), due to reduction of the knock-down effect (insect paralysis after short time contact with insecticide) (Ffrench-Constant et al. 2004). The *Vgsc* gene is composed of four domains (I-IV), with each containing six transmembrane helices (S1-S6). Mutations associated with resistant phenotypes are mostly found in domain II and helices S4 to S6, where changes in five different residues; Met918 in the IIS4-IIS5 linker, Leu925, Thr929 and Leu932 in IIS5 and Leu1014 in IIS6 have previously been associated with resistance to class I and II pyrethroids as well as cross-resistance to DDT (O'Reilly et al. 2006). Although all these mutations are linked to insecticide binding sites, occurrence of resistant phenotypes can result from either single mutations or in combination. Resistant mosquitoes encompassing the combination of any of these variants - M918T L925I, T929I, T929V with L1014F - are termed super-*kdr* mutations, which result in a higher level of resistance (Williamson et al. 1996, Ranson et al. 2000, Bass et al. 2004).

Among the *kdr* mutations, a substitution of leucine to phenylalanine [L to F] at the position 1014 in the 6th segment of domain II (IIS6) is the most common *kdr* mutation associated with resistance to pyrethroids and DDT in many insect species, as well as vector species (Soderlund 2008, Rinkevich et al. 2012, Liu 2015). Nevertheless, across vector species other mutations have also been linked to pyrethroid resistance such as the N1575Y in *A. gambiae*, I1011M/V, V1016G/I and F1269C in *A. aegypti*, F1534C in *A. albopictus* and L1014F/S in *Culex pipiens* (Jones et al. 2012a, Zhao et al. 2014a, Kushwah et al. 2015, Li et al. 2015).

Target-site mutations in the acetylcholinsterase gene is a common resistance mechanism to organophosphate and carbamate insecticides (Bourguet et al. 2013). These

insecticides are irreversibly competitive inhibitors of acetylcholinesterase, which is responsible for hydrolyzing the neurotransmitter acetylcholine at the postsynaptic membrane, terminating nerve impulses (Cousin et al. 2005, Alout et al. 2007a). Among arthropod genomes the number of *Ace* genes differs between species. In *Drosophila* only a single copy has been identified (called *Ace*-2) whereas most insects possess two copies known as *Ace*-1 and *Ace*-2. Although both copies of this gene are functional encoding two Ace (Ace-1 and Ace-2), only one of them is involved in resistance, with the Ace-1 proposed as the major catalytic enzyme based on higher expression and the association of insecticide resistance point mutations in this gene with the resistant phenotype, while the function of *Ace-2* is unknown (Ren et al. 2002, Mori et al. 2007).

In mosquitoes, evolution of insecticide resistance due to insensitive Ace-1 has been globally reported in diverse species such as *C. pipiens*, *C. vishnui*, *C. tritaeniorhynchus*, *C. quinquefasciatus, A. gambiae* and *A. albimanus, A. nigerimus*, *A. atroparvus* and *A. sacharovi* (Bourguet et al. 2013, Weetman and Donnelly 2015, Weetman et al. 2015). In these species, insensitive Ace-1 results from non-synonymous mutations in residues close to the organophosphate and carbamate binding site. So far, only three single point mutations have been described in resistant phenotypes; G119S, F290V and F331W (numbered according to *Torpedo californica* AChE nomenclature) (Alout and Weill 2008). While two amino acid substations F290V and F331W have been exclusively described in *C. pipiens* and *C. tritaeniorhynchus*, respectively, the G119S variant has been selected in several species including *C. pipiens*, *C. vishnui*, *A. gambiae* and *A. albimanus* (Weill et al. 2003). Ace-1 protein insensitivity due to the G119S variation, results from changes in protein structure, since the glycine is located in the

oxyanion hole of the enzyme, at the base of the active site gorge reducing the access of the insecticide to the catalytic triad (Alout et al. 2007a).

Despite these point mutations in the *Ace-1* gene, these mutations are important to mosquitos` survival under insecticide selection. Many studies have shown that such resistance-associated mutations can result in deleterious effects and fitness disadvantages due to reduction of the Ace-1 catalytic activity efficiency (Kwon et al. 2012, Lee et al. 2015). Among the *Ace-1* point mutations, the highest fitness costs have been associated with the resistant allele 119S, which accounts for a reduction between 60 and 70% of the Ace-1 enzymatic activity (Bourguet et al. 1997, Djogbenou et al. 2008). Additionally, other point mutations in the *Ace-1* gene such as G228S and F439W in *Tetranychus urticae* has also been linked to a reduction of Ace-1 catalytic efficiency (Kwon et al. 2012), while the fitness cost associated with other mutations like F290V described in *C. pipens* is not elucidated (Alout et al. 2009).

The impact of *Ace-1* insecticide resistant alleles in *Culex* mosquitoes fitness costs has been described in many instances such as; survival cost of resistant mosquitoes (Gazave et al. 2001), increase in development time and changing in morphology e.g. shorter wing length (Bourguet et al. 2004) as well as life-history trails including: larval mortality, adult female size and mating disadvantage compared to susceptible genotypes (Berticat et al. 2002, Duron et al. 2006). To compensate the deleterious effects in the reduction of Ace-1 catalytic activity driven point mutation, duplication of the *Ace-1* gene have been proposed as a compensatory adaptation mechanism by restoring the normal level of the Ace-1 activity (Alout et al. 2009, Lee et al. 2015). Indeed, *Ace-1* gene duplication has been reported in diverse arthropod species such as *Tetranychus evansi*, *Tetranychus urticae*, *Plutella xylostella*, *Aphis gossypii* (Kwon et al. 2010, Carvalho et al.

2012, Shang et al. 2014, Sonoda et al. 2014) as well as vector species as *A. gambiae* and *Culex* mosquitoes (Bourguet et al. 1996a, Djogbenou et al. 2008).

In mosquitoes of the *Culex pipiens* complex, many studies have indicated that enhancing of *Ace-1* copy number results from several independent duplications encompassing both resistant and susceptible copies of the *Ace-1* gene creating a 'permanent heterozygote' (Labbe et al. 2007a, Alout et al. 2011, Osta et al. 2012b). These not exhibit deleterious pleiotropic effects since the presence of the susceptible *Ace-1* allele in the duplicated genotype compensate the effects of the resistant-associated mutation (Labbe et al. 2007b). Consequently, the duplicated *Ace-1* genotypes have more advantages compared to non-duplicated resistant genotypes when there are changes from insecticide selection to a free insecticide environment, as resistant mosquitoes harboring the duplicated *Ace-1* susceptible copy compensate the cost associated with the resistant copy (Bourguet et al. 1996a, Labbe et al. 2007a, Labbe et al. 2007b).

In addition to the proposed compensatory adaptation mechanism of the *Ace-1* duplication, enhanced copy number of the *Ace-1* gene have also been associated with the level of insecticide resistance. For example, quantitative PCR analysis in the two-spotted spider mite, *T. urticae* revealed the presence of *Ace-1* gene copy number and level of mRNA expression ranging from 2 to 6, resulting in different levels of monocrotophos-resistant phenotype (Kwon et al. 2010). On the same way, in *Aphis gossypii* the *Ace-2* copy number and transcription level was also significantly associated with organophosphate-resistance (Shang et al. 2014). Hence, the association of *Ace-1* gene copy number and insecticide resistance need to be investigated not only as a compensatory mechanism of the fitness cost associated with resistant alleles but also as a likely alternative mechanism driving evolution of resistance.

Insecticide resistance to cyclodiene, phenylpyrazoles, pyrethroids and ivermectins have also been linked to single point mutations in the (GABA) receptor. This neurotransmitter receptor encoded by the *Rdl* (resistance to dieldrin) gene is formed by five subunits around a central transmitter-gated ion channel (Remnant et al. 2014). The resistant phenotype is associated with a single non-synonymous change from an alanine residue to a serine, or rarely to a glycine at position 302 within the second transmembrane region of the *Rdl* subunit (Ffrench-Constant et al. 2004, Ffrench-Constant 2007).

Insensitivity to insecticides due to the alanine to serine or alanine to glycine substitution in the GABA receptor results from two aspects; reducing insecticide binding and destabilization of the insecticide's preferred (desensitized) receptor conformation (Zhang et al. 1994). In mosquitoes, resistance to dieldrin has been mostly reported in *Anopheles* spp. and *Ae. aegypti* (Du et al. 2005, Wondji et al. 2011, Casida and Durkin 2013).

1.3.2 Molecular mechanisms of metabolic resistance

Metabolic resistant-phenotypes in insects are mostly mediated by three major metabolic detoxification gene families: cytochrome P450s (P450s), esterases, and glutathione S-transferases (GSTs) (Hemingway and Ranson 2000, Li et al. 2007).

1.3.2.1 Cytochrome P450s (P450s)

Among the three major detoxification gene families, cytochrome P450 is the largest gene family, with a substantial number of genes already described in the three most studied vector species; with 105 identified in *A. gambiae,* 160 in *A. aegypti* and 196 in *C.*

quinquefasciatus. In *C. quinquefasciatus* the number of predicted P450 genes is more than twice and three times compared to the number of carboxyl/cholinesterases and GSTs respectively (Komagata et al. 2010, Yan et al. 2012).

Cytochrome P450-dependent monooxygenases are hydrophobic, heme-containing enzymes, with many of them specialized for metabolism of endogenous substrates including steroids, hormones and lipids, although these enzymes also play an important role in metabolism and detoxification of xenobiotic compounds such as environmental toxins and insecticides (David et al. 2013). In insects, the large diversity of P450 genes are distributed within four clans; CYP2, CYP3, CYP4 and the mitochondrial CYP clan (Feyereisen 2012). Most of the P450 genes identified with high expression levels in insecticide resistant phenotypes belong to the *Cyp*6 family, which have been suggested to present characteristics of environmental response such as tissue, or temporal specific expression (Hemingway et al. 2004, Ingham et al. 2014). Indeed, P450 genes have been associated with resistance to all classes of insecticides, which reflects their genetic diversity, broad substrate specificity and catalytic spectrum (Feyereisen 2005). However, it is important to bear in mind that overproduction of enzymes like P450 is not always correlated with insecticide resistance as a unique regulatory transcriptional factor and can be regulated by several genes (Schuler and Berenbaum 2013).

In mosquitoes, a large number of P450 candidate genes from distinct Cyp families have been identified in pyrethroid resistant mosquitoes from *Culex*, *Anopheles* and *Aedes* as broadly reviewed by many authors (Ranson et al. 2002, Schuler and Berenbaum 2013). Although many P450 candidate genes have already been identified, only a limited number of genes have their metabolizing capability validated by heterologous expression, as described for CYP6P3 (Li et al. 2007, Djouaka et al. 2008, Müller et al. 2008), CYP6M2

(Mitchell et al. 2012), CYP6Z1 (David et al. 2005) and CYP6P9 (Riveron et al. 2013) from *Anopheles*, CYP9J32 (Bingham et al. 2011), CYP9J24 and CYP9J28 (Strode et al. 2008) from *Aedes* and CYP9M10 (Liu et al. 2011a, Wilding et al. 2012) and CYP4H34 from *Culex* (Hardstone et al. 2010, Komagata et al. 2010).

1.3.2.2 Glutathione S-transferases (GSTs)

GSTs are another gene family commonly associated with detoxification and cellular antioxidant defense, in which elevated levels of activity have been found to be associated with organophosphate, organochlorine, and pyrethroid insecticides (see for review (Li et al. 2007)). These enzymes act by three primary functions; conjugation of reduced glutathione (GSH), dehydrochlorination, or sequestration of natural and synthetic exogenous xenobiotics; the two former mechanisms having been mostly associated with insecticide resistance (Syvanen et al. 1996, Ranson et al. 2001, Vontas et al. 2001). GSTs are classified in two groups based on their cellular location; microsomal or cytosolic, with distinct GSTs divided within six classes; sigma, zeta, theta, omega, delta and epsilon. The last two are the largest classes and found exclusively in insects (Enayati et al. 2005, Li et al. 2007).

Due to broad substrate specificities of individual enzymes, elevated GST expression has been associated with resistance to insecticides from all the main classes of insecticides, e.g. organophosphate resistance in *Musca domestica* and *Plutella xylostella* and DDT resistance in *Drosophila melanogaster* (Ku et al. 1994, Syvanen et al. 1994, Tang and Tu 1994). In mosquitoes, DDT and pyrethroid resistance mediated by GSTs have already been reported in distinct vector species such as *Aedes* and *Culex* (Grant et al.

1991, Penilla et al. 2006), as well as in a vast number of *Anopheles* species including: *A. albimanus, A. annularis, A. culicifacies, A. gambiae, A. stephensi* and *A. arabiensis* (Gunasekaran et al. 2011, Mitchell et al. 2014, Sanil et al. 2014, Wilding et al. 2014). For instance, in *A. aegypti* DDT-resistant strains from three geographic locations (New Orleans, Thailand and Mexico), three Epsilon GSTs; *GSTe2*, *GSTe5* and *GSTe7* were detected with over-expression in resistant mosquitoes, while the independent partial silencing of the last two Epsilons also increased the susceptibility to pyrethroids in these strains (Lumjuan et al. 2011).

Similarly, in DDT-resistant *A. albopictus* mosquitoes from Florida and New Jersey, USA a higher over expression of GSTs was also reported as the mechanism driving the resistant in both larval and adult stages (Marcombe et al. 2014). In *A. gambiae*, *in silico* analysis of 24 GSTs, shows that the GST Delta D6 has the highest interaction affinity with DDT (Aravindan et al. 2014). Nevertheless, transcriptomic microarray-based studies have also identified high levels of expression of *AgGSTE2* and *GSTS1-2* in *A. gambiae* strains resistant to pyrethroids and permethrin (David et al. 2005), while *GSTS1-1*, *GSTS1-2* and microsomal GSTs (*GSTMIC2*) were detected with high expression level in *A. stephensi* pyrethroid-resistant strain (Vontas et al. 2007).

In *Culex* mosquitoes, genomic sequences analyses indicated a total of 40 putative GSTs, with 35 corresponding to cytosolic and 5 microsomal (Reddy et al. 2011, Reddy et al. 2012). As also reported in other insects, GSTs-resistant phenotypes are mostly driven by GSTs gene amplification and over expression (for a review see (Li et al. 2007). Higher expression of GSTs has already been associated with *Culex* mosquito resistance to DDT and pyrethroids insecticides. For example, in *C. quinquefasciatus* a theta GST was identified up-regulated by 19-fold in parathion resistant mosquitoes (Wang et al. 2015)

whereas GST over-expression was also observed in pyrethroid-resistant strains from Mobile and Huntsville, Alabama, USA (Xu et al. 2005). Furthermore, over expression of CpGSTD1 (a DDT metaboziler) was also linked to *C. pipiens* Marin DDT-resistant phenotype (Samra et al. 2012).

1.3.2.3 Esterases

Metabolic resistance through elevated esterase expression is also an important mechanism associated with resistance to organophosphates, carbamates and pyrethroids (for a review see (Hotelier et al. 2010)). As described for the other two detoxification gene families, esterases are also ubiquitous enzymes involved in the metabolism of exogenous and endogenous compounds, playing a critical function in insect development and behaviour such as reproduction and hydrolysis of pheromones and many semiochemicals (Claudianos et al. 2006). Esterases belong to the carboxylesterase multigene family, which based on sequence similarity and substrate specificity is sub-divided into eight subfamilies: α-esterases, β-esterases, juvenile hormone esterases, gliotactins, acetylcholinesterases, neurotactins, neuroligins, and glutactins, with α-esterases, β-esterases, acetylcholinesterases and juvenile hormones involved in most of the catalytic activities (Ranson et al. 2002, Yu et al. 2009). Insecticide resistance mediated by esterases is typically associated with insecticides containing ester compounds such as pyrethroids, carbamates and organophosphates making them good substrates for the esterase hydrolysis mechanism, which is capable of hydrolysing ester bonds to generate an acid and an alcohol as metabolites (Montella et al. 2012). Resistance mediated by enhancing of esterase activities have already been reported in *A. gambiae* permethrin resistant

phenotypes as well as organophosphate resistance in *A. aegypti* and *Culex* mosquitoes (Mourya et al. 1993, Vulule et al. 1999, Paton et al. 2000).

1.3.2.4 Mechanisms underlying insecticide metabolic resistance

Despite the small number of gene families thus far implicated in metabolic resistance, a vast number of molecular mechanisms trigger changes in detoxification enzyme function, expression or both (Li et al. 2007). Resistance mediated by detoxification enzymes are mostly associated with up regulation, allelic variant or gene amplification, although some of these mechanisms are not observed in all three families of detoxification genes (Bass and Field 2011). As described for target-site mutations, changes in amino acid residues within detoxification enzymes also drive insecticide resistance; however, by increasing metabolic capacity due to an increase of affinity to insecticide. Most metabolic resistance mediated through coding sequence mutations has been reported in esterase, GTSs and P450s genes (Li et al. 2007). For example, in *Drosophila*, divergence of protein sequence among copies of the *Cyp6g1* gene resulted in resistance to multiple classes of insecticide, with variants of this enzyme conferring resistance to either DDT or nitenpyram but not for both insecticides (Harrop et al. 2014). Similarly, changes in coding sequences in carboxylesterase genes resulted in an increase of organophosphate hydrolysis in many species including *Chrysomyia putoria*, *M. domestica*, *L. cuprina, Anisopteromalus, calandrae, Plodia interpunctella* and *Culex tarsalis* (Whyard et al. 1995, Campbell et al. 1998, Claudianos et al. 1999).

Nevertheless, insecticide detoxification mediated by transcriptional up-regulation is apparently a more common cause of resistance across insect species (Bass and Field

2011). Among the mechanisms linked to higher constitutive production of transcripts, most detoxification gene up-regulation is mediated by changes in *cis* and/or *trans* gene regulatory elements through mutation or insertion/deletion in promoter regions or in regulatory loci (Komagata et al. 2010, Liu 2015). Increase of detoxification enzymes mediated by trans-acting regulatory elements was associated with overexpression of GST-2 allele in *A. Aegypti* (Ortelli et al. 2003), while in *A. gambiae* the presence of *Nrf2*/*Keap* 1 and *AhR*/*ARNT* as putative transcription factor binding sites (TFBS) within the *CYP6M2* promoter is potentially associated with its up-regulation (Mohammed et al. 2014).

Metabolic resistance driven by over-expression of detoxification genes through gene amplification has also been a common mechanism associated with GSTs and esterase genes. Association of esterase gene amplification with insecticide resistance was reported in *Myzus persicae*, where resistance to organophosphates, carbamates and pyrethroid insecticides resulted from amplification of the *E4* and *FE4* genes (Field and Devonshire 1998). Similarly, in mosquitoes of the *Culex* complex (*C. quinquefasciatus* and *C. pipiens*) independent co-amplification of two esterases, *est*α2^1 and *est*$\beta 2^1$, as well as estα3 and est*β2,* also trigger organophosphate resistance (DeSilva et al. 1997). Additionally, GST gene amplification contributed to organophosphate resistant phenotypes in *M. domestica* mediated by duplication of GSTd3 and GSTd4 (Wang et al. 1991), whereas amplification of GSTd1 in *Nilaparvata lugens* conferred resistance to pyrethroids (Vontas et al. 2001).

1.4. Evolution of insecticide resistance in *Culex* mosquitoes and impact on vector control

In mosquitoes of the *Culex pipiens* complex, most reports of resistance are to pyrethroid insecticides, which might reflect the extensive use of this insecticide both for

crop-pest control and public health interventions (Van den Berg et al. 2012). Although there are heterogeneous levels of resistance among populations, resistance to diverse pyrethroid compounds including permethrin, deltamethrin, β-cypermethrin, Es-bioallethrin, λ-cyhalothrin, etofenprox, bifenthrin, β-cyfluthrin, phenothrin and resmethrin have already been reported in *Culex* populations across the Western and Eastern Hemisphere (For a review see (Scott et al. 2015).

So far, most identified pyrethroid resistant phenotypes are associated with two main mechanisms; target-site mutation in the *Vgsc* and detoxification mediated by P450s. Among the target-site mutations already associated with the *Culex* pyrethroid resistant phenotype: L1014S, L1014C, V1016G and L1014F, only the last is widely distributed across the American, African and Asian continents (Scott et al. 2015). Despite this wide geographic distribution, previous studies suggest that the L1014F has arisen at least twice as two mutant alleles - either an A-to-T or an A-to-C substitution at the same codon position resulting in the 1014F resistant allele - with the A to C variant initially identified in Sri Lanka and having only recently been detected at low frequency on the African continent on Pemba island, Zanzibar (Martinez-Torres et al. 1999, Wondji et al. 2008, Jones et al. 2012b).

To date, the other three mutations associated with pyrethroid resistance are restricted to a few *Culex* species as well as being locally geographically distributed e.g. L1014S was identified in *C. pallens* from Japan and *C. pallens* and *C. pipiens* from China, L1014C has been detected in *C. pipens molestus* from China and V1016G identified in *C. quinquefasciatus* from Saudi Arabia (Komagata et al. 2008, Chen et al. 2010, Wang et al. 2012, Liu et al. 2013). However, additional mutations like V1016G are typically linked to the L1014F mutation, as described in *C. pipiens molestus* from China, which

make it difficult to pinpoint the contribution of each mutation to the resistant genotype (Wang et al. 2012). Additionally, distinct mutations can also have different impact in resistance. For instance, in *C. pipiens pallens* from China, the L1014F mutation was reported to be associated with deltamethrin resistance, but no association was detected for the alternative mutation L1014S (Chen et al. 2010).

In addition to target-site mutations in the *Vgsc* gene, many studies have reported that resistance to pyrethroids in *Culex* is mediated by P450- dependent oxidases (Yang and Liu 2013, 2014). Indeed, a vast list of P450 genes have been identified in permethrin resistant *C. quinquefasciatus* mosquitoes, with most of them classified in the families 4, 6 and 9 (CYP4H34, CYP4C52v1, CYP4D42v2, CYP4C38, CYP4H40, CYP6Z10, *CYP6F1* ,CYP6AA7, CYP6AA7, CYP6Z15, CYP9M10, CYP9J40, CYP9J34, CYP9M10, CYP9J45, CYP9M10, CYP9J33, CYP9J43, CYP9J34, CYP9J45, CYP9J39) or other P450 gene family (CYP325K3v1, (CYP) CPIJ000926, CYP325G4, CYP306A1, CYPPAL1) (Kasai et al. 2000, Komagata et al. 2010, Gong et al. 2013).

However, so far only CYP9M10 has been associated with pyrethroid resistance in *C. quinquefasciatus* strains and natural populations from distinct geographic background including Saudi Arabia, Asia, North America and Sub-Saharan Africa (Liu et al. 2004, Wilding et al. 2012, Itokawa et al. 2013). By contrast, no difference of expression of CYP4H34 was observed in larvae and adults of two permethrin selected strains (ISOP450 and ISOJPAL), while in the same colonies permethrin resistance was associated with CYP9M10, which was over expressed 1800-fold in ISOP450 and 870-fold in ISOJPAL compared to a susceptible strain (Hardstone et al. 2010).

Although CYP9M10 permethrin-resistant phenotypes have been demonstrated in various populations, many studies have indicated the distinct molecular mechanism

underlying its over-transcription among resistant phenotypes. Two main mechanisms have already been linked to over-expression of CYP9M10. One involving a genomic tandem duplication including the CYP9M10 and other genes was reported in *C. quinquefasciatus* strains from Asia and Africa (Itokawa et al. 2010, Itokawa et al. 2013). The second mechanism involves two independent *cis*-acting mutations resulting from insertion of a transposable element upstream of the putative transcriptional start site of CYP9M10, which modulates the high level of gene expression (Itokawa et al. 2010, Wilding et al. 2012).

Before replacement of organophosphates with pyrethroid insecticides due to an increase in insecticide resistance, many organophosphate compounds including dichlorvos, malathion, diazinion, chlorpyriphos were the most widely applied insecticides for *Culex* control on the African, European, American and Asian continents (Chandre et al. 1997, Raymond et al. 2001, Jones et al. 2012b, Osta et al. 2012b). Evolution of organophosphate resistance in *Culex* mosquitoes, has been described in other vector species driven mostly by two main mechanisms involving target-site mutation and detoxification enzymes mediated by over-expression of esterase or both mechanisms (Toma et al. 2011, Karunamoorthi and Sabesan 2013, Liu 2015).

Organophosphate resistance in *C. pipiens* from France is one of the most well documented examples of evolution of insecticide resistance in *Culex* mosquitoes after four decades of control intervention (Labbe et al. 2007b). In this study, the authors clearly demonstrated that in these populations the evolution of resistance involved a dynamic process of mosquito's adaptation to organophosphate selection pressure, which included an increase in frequency of the *Ace*-1 resistant allele (119S), but also successive gene duplication events including resistant and susceptible alleles, while the duplication of the

susceptible allele was associated with reducing of the deleterious effect of the *Ace*-1 resistant allele.

The target-site mutation G119S in the *Ace*-1 gene, which is associated with reduced susceptibility to organophosphates and carbamates, has been the most widely detected in the resistant phenotype (Weill et al. 2004, Casida and Durkin 2013, Zhao et al. 2014a). Although the G119S mutation has been more frequent and broadly detected, additional non-synonymous mutations in the *Ace*-1 gene such as V185M, G247S, A328S, A391T, and T682A have also been detected in *C. quinquefasciatus* from China but not demonstrated to be involved with resistance to dichlorvos, while two of them G247S and A328S were associated with resistance to the carbamate insecticide propoxur (Zhao et al. 2014a). Two other mutations in the *Ace*-1 gene have also been identified; F290V in *C. pipiens* from Cyprus and F331W *C. tritaeniorhynchus* (according to Torpedo californica AChE nomenclature), which show insensitivity to insecticide using *in vitro* experiments (Alout et al. 2007b, Alout et al. 2007a, Alout and Weill 2008).

Despite the fitness benefit of the *Ace*-1 target-site mutations for populations under insecticide selection, in an insecticide free environment many studies have shown that resistant target-site alleles have strong deleterious effects (Berticat et al. 2002, Berticat et al. 2008). Interestingly, many studies have reported that over the course of selection and evolution of the 119S resistant allele in the *Ace*-1 gene, duplication of the *Ace*-1 gene acts like an alternative compensatory mechanism to normalise the level of the wild type enzyme avoiding the deleterious effects of the G119S mutation (Lenormand et al. 1998, Labbe et al. 2007a, Alout et al. 2009, Alout et al. 2011, Labbé et al. 2014). Organophosphate resistance phenotypes associated with *Ace*-1 gene duplication have been broadly reported in *Culex* populations, with many studies showing that the duplications

resulting from independent events within and between *Culex* species and geographic localities, as identified in *C. pipiens* from Southern France and Lebanon as well as *C. quinquefasciatus* from Martinique, Caribbean and Philippines (Bourguet et al. 1996a, Bourguet et al. 1996b, Osta et al. 2012a).

Another important mechanism associated with resistance to organophosphate insecticide involves the esterase gene duplication, which results in higher esterase expression and consequently increases its capability to bind or metabolize the insecticide (Raymond et al. 1998). Although there are many esterase allozymes, six from the esterase B locus (B1, B2, B4, B5, B6 and B7) and four (A1, A2, A4 and A5) at the esterase A locus are the alleles frequently associated with resistance, while co-amplification of two esterase genes (est$\alpha 2^1$ and est$\beta 2^1$) is the most common resistant genotype in *C. quinquefasciatus* mosquitoes (Guillemaud et al. 1996, Liu et al. 2011b, Gordon and Ottea 2012, Johnson and Fonseca 2015). Particularly, the est2 alleles associated with organophosphate resistance have the broadest distribution, already detected in resistant *Culex* populations across Europe, Asia, Africa, America and Oceania (Labbé et al. 2005). Despite most esterase-resistant phenotypes resulting from gene amplification of two or more linked esterase genes, resistance associated with single amplification or due to change in gene expression regulatory elements have also been described (for a review see (Raymond et al. 1998)).

More recently, carbamate insecticides became an alternative to the threat of the high level of pyrethroid and organophosphate resistance detected in vector mosquitoes. Indeed, although most reports of susceptibility to carbamates are from *Anopheles* especies (Yewhalaw et al. 2011, Kigozi et al. 2012, Mulamba et al. 2014), a few studies have also indicated higher susceptibility to bendiocarb than to other insecticides in *C.*

quinquefasciatus from Africa as reported in populations from Ghana and Tanzania (Jones et al. 2012b, Kudom et al. 2015). Nevertheless, since both target-site mutations and metabolic resistance (e.g. esterases and P450s) associated with organophosphate resistance also confer cross-resistance to carbamates (Li et al. 2007, Casida and Durkin 2013, Pocquet et al. 2013), it is important to bear in mind that these mechanisms of resistance might already have been present in low frequency in that population, which can jeopardise future control interventions with carbamate insecticides.

In Uganda, inferences of the impact of vector control programmes in the evolution of insecticide resistance targeted chiefly at *Anopheles* species; *A. gambiae* and *A. funestus* due to the primary importance of insecticide-based methods to control these mosquitoes population and consequently to mitigate the high prevalence of malaria throughout the country (Kigozi et al. 2012, Yeka et al. 2012).

Despite the non-primary medical role of *C. quinquefasciatus* in vector-borne diseases transmission in Uganda, the present research was conducted in this country as non-official control interventions toward *C. quinquefasciatus* mosquitoes have been applied, although insecticide-based methods are applied nationwide for the control of other vector species as well as in agricultural settlements (Kigozi et al. 2012). Hence, studying *C. quinquefasciatus* populations from this region provide a suitable fieldsite to investigate the impact of insecticide selection pressure in the evolution of insecticide resistance in non-target insect populations. Additionally, the accessibility to the infrastructure of the Ugandan *Anopheles* insecticide resistance management programme (e.g. insectary as well as the possibility of synchronise the *Culex* to the *Anopheles* collection) also facilitates the development of the present study in the selected region.

1. 5. Aims and study design

The primary objective of this work was to develop and apply DNA-based markers as well as transcriptomic profiling to enhance the understanding of the evolutionary genetics of insecticide resistance in *C. quinquefasciatus* mosquitoes. Firstly, to investigate the likely impact of insecticidal selection pressure in the mosquito population structure and genetic diversity, neutral and insecticide-associated genomic markers were applied to genotype *C. quinquefasciatus* population from Uganda collected between July and August 2012. Secondly, to infer if Ugandan resistant-phenotypes are involved in the mechanism of metabolic resistance, gene expression profiles of bendiocarb resistant *C. quinquefasciatus* were used to identify candidate genes associated with metabolic resistance. For this, the following studies were conducted:

Chapter II. Application of a bioinformatics approach to design 29 new microsatellite markers for genetic diversity, and population genetic analysis of *C. quinquefasciatus*. The characterized markers were used to develop five six-plex reactions, which were used to experimentally validate samples from two Ugandan field populations (Jjnja and Kamapala).

Chapter III. *C. quinquefasciatu*s mosquitoes collected across a transect from Eastern to South Western Uganda were utilized to infer the likely patterns of the evolution of insecticide resistance among populations through combinations of putatively selected loci (the target-site mutations 1014L in the *Vgsc* gene and 119S in the *Ace*-1 gene) and 29 neutral microsatellite markers.

Chapter IV. Development and validation of a new *Vgs*c-CNV PCR-based assay to evaluate the copy number of the *Vgsc* gene in natural populations of *Culex* mosquitoes. The *Vgsc*-CNV assay was designed to perform on three distinct genotyping platforms: standard real-time PCR (qPCR), pyrosequencing and

droplet digital PCR (ddPCR). Additionally, diagnostic sensitivity and accuracy of the newly designed *Vgsc*-CNV assay was compared across the three platforms by genotyping mosquitoes collected in Uganda.

Chapter V. A novel 8 x 60K whole-transcriptomic microarray was designed and applied to identify candidate genes associated with bendiocarb insecticide resistance in mosquitoes collected and phenotyped in Tororo, Uganda.

Chapter II

Development of Microsatellite Multiplex Panels for Population Genetic Analysis of the Lymphatic Filariasis Vector *Culex quinquefasciatus*

2.1Abstract

The lymphatic filariasis vector *Culex quinquefasciatus* has previously been subject to studies to characterize genetic diversity and structure and also the genetic basis of differences in behavior and vector competence. Although a number of microsatellite markers are available for *Culex* species, these have not been optimized for multiplexed reactions, which would reduce the time and cost of large-scale screening. We identified 29 novel microsatellites from *C. quinquefasciatus* whole genome sequence data and developed a panel of five multiplex reactions. Reproducibility and polymorphism of the markers in multiplex reactions were investigated in mosquitoes collected from two field populations from Uganda. All loci were polymorphic with 2-12 alleles per locus with the mean polymorphic information content (PIC) and expected heterozygosity (H_E) of 0.533 and 0.585, respectively. These panels of marker multiplexes will enhance efficiency of population and quantitative genetic studies of *C. quinquefasciatus*.

2.2 Introduction

Species within the *Culex pipiens* complex occur widely throughout tropical and temperate environments worldwide and are vectors of several arboviruses and pathogens such as West Nile virus (WNV) and St. Louis virus (Hamer et al. 2008, Cornel et al. 2012, Molaei et al. 2012). One member of the complex, *C. quinquefasciatus*, is the primary vector of lymphatic filariasis (LF) in India, Brazil, Asia, Africa and the Western Pacific (Bockarie et al. 2009, Behura et al. 2011, Kumar et al. 2011). Recently, the frequency of LF has increased in urban

and semi-urban areas of the developing world due to the ability of *C. quinquefasciatus* to thrive in dense urban environments (Simonsen and Mwakitalu 2013).

To date few microsatellite markers have been described for *C. quinquefasciatus* (Fonseca et al. 1998, Smith et al. 2005, Edillo et al. 2009, Hickner et al. 2010), limiting the scope of medically-important quantitative and population genetic studies of ecology, vector competence and insecticide resistance. Vector competence varies markedly, with *C. quinquefasciatus* populations in West Africa almost fully refractory to infection (Zielke and Kuhlow 1977), and evidence for vector-parasite co-adaptation in other parts of the range; yet the role of genetic factors underpinning this variation remains to be determined (Kothera et al. 2012). Moreover, population genetic analyses provide an opportunity to investigate recent invasions (Fonseca and Bahnck 2006, Bataille et al. 2009), hybridization events (Fonseca et al. 2004, Gomes et al. 2009) and to evaluate the efficiency of vector control strategies in endemic areas (Cartaxo et al. 2011).

Here, we apply a bioinformatics approach to identify 29 new microsatellite loci for population genetic analysis of *C. quinquefasciatus*. In addition, we develop *in silico* five six-plex reactions, and validate these experimentally in samples from two Ugandan field populations.

2.3 Materials and methods

2.3.1 Samples and species identification

Characterization of all putative microsatellite markers was carried out by genotyping *C. quinquefasciatus* samples from two laboratory colonies (CqSF from Recife Brazil (Wilding et

al. 2012) and ISOP450 (Hardstone et al. 2007) maintained at the Liverpool School of Tropical Medicine (LSTM) and field mosquitoes from two regions of Uganda (Jinja; 00° 25' N, 33°12' E and Kampala; 00°20' N, 32°30' E) collected in July 2012. Genomic DNA of individual mosquitoes was isolated using a DNeasy kit (Qiagen). The field samples were confirmed as *C. quinquefasciatus* through a diagnostic PCR assay prior to microsatellite genotyping (Smith and Fonseca 2004).

2.3.2 Microsatellite isolation and primer design

A total of 180 randomly selected *C. quinquefasciatus* genome supercontigs were downloaded from VectorBase (www.vectorbase.org) and analyzed using SciRoKo 3.4 (Kofler et al. 2007) to identify perfect di- or tri- nucleotide motifs with the number of repeats ranging from 5 to 20. For each candidate microsatellite, approximately 200 bp of pre- and post-motif flanking sequence was isolated and target loci with flanking sequences containing microsatellites sequences were excluded. To reduce the likelihood of physical linkage, only one microsatellite per supercontig was chosen for primer design using primer 3.0 (Rozen and Skaletsky 2000), targeting primer GC content between 50% and 70% and melting temperature between 60 and 65 °C. All primers were checked for unique binding using BLAST against the *C. quinquefasciatus* genome in VectorBase. Only primer pairs from which at least one of the primers was unique in the whole genome were selected for PCR. Furthermore, the primer sequences were BLASTed against the GenBank nucleotide database to ensure that they had not been described previously.

2.3.3 Microsatellite reactions

Initially, thirty-six primer sets were designed and tested individually using eight samples from the two laboratory colonies CqSF (n = 4) and ISOP450 (n = 4). PCR reactions were performed in 25 µl volumes containing 1X buffer, 2mM $MgCl_2$, 0.2mM of each dNTP, 1.25 units of *Taq* polymerase (Fermentas), 0.5 µM of each primer and 2 µl of genomic DNA. The PCR conditions consisted of denaturation for 3 min at 95°C, followed by 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, and a final extension at 72 °C for 10 min. PCR products were separated on 2% agarose gel in 1X TAE buffer, stained with ethidium bromide and visualized using UV light. PCR reactions that showed non-specific fragments at the initial conditions were optimized by iteratively increasing the annealing temperature in steps of 2 °C up to a maximum of 65 °C.

2.3.4 Mutiplex development and amplification

Following initial PCR optimization, 30 microsatellite primer pairs that consistently amplified single fragments of expected fragment size (within the resolution of agarose gel) in all samples of both laboratory colonies were tested for compatibility in PCR multiplex reactions using Multiplex Manager 1.0 (Holleley and Geerts 2009). Multiplex panels were selected to have 6 loci per reaction, three fluorescent dyes, at least 30 bp between loci labeled with the same dye and a complementary threshold of 15 (maximum number of AT or CG matches between two primers) to prevent potential hairpins, or dimers between any primers within each reaction.

Forward primers of each set were labeled with Beckman-Coulter D2, D3 or D4 fluorescent dyes; however, D3 and D4 were preferentially selected due to their higher signal intensity.

Initially, the primer sets for each multiplex reaction were divided in two pairs of primers labeled with different dyes and then used to amplify 2 pools containing equivalent amounts of eight DNAs from each laboratory and field sample to characterize the allelic range of each locus to ensure that multiplexed loci labeled with the same marker had non-overlapping allelic ranges.

Additionally, four samples from each population and laboratory colony were used to amplify the complete multiplex panel to detect the possible presence of false peaks arising from dye spectral overlap and stutter peaks. Finally, five multiplex panels were used to genotype 35 individuals from each field sample to characterize polymorphism and screen for the presence of possible null alleles.

Multiplexes were amplified in 25 µl reaction volumes that included 1X Type-it multiplex PCR Master Mix (Qiagen), 0.2 µM of each primer and 2 µl of genomic DNA. Amplifications were carried out under the following conditions: initial heat activation for 5 min at 95 °C followed by 24 cycles of 30 s at 95°C, 90 s at 60°C, 30 s at 72°C and a final extension step of 30 min at 60°C. For fragment analysis, 2 µl of PCR multiplex reaction were added to a mix of 37.5 µl of Sample Loading Solution and 0.5 µl of 400-bp size Standard (Beckman-Coulter) followed by a denaturation step of 5 min 95°C before fragment analysis on Beckman-Coulter CEQ8000. Genotypes were scored by allelic size using Beckman-Coulter CEQ 2000 DNA analysis system software and manually verified.

2.3.5 Data analysis

Observed and expected heterozygosities were calculated using *GenAlEx* (Peakall and Smouse 2012). Linkage disequilibrium among marker pairs and deviation from Hardy-Weinberg equilibrium for each locus were assessed using the exact tests in GENEPOP (Rousset

2008). Polymorphism information content (PIC) was calculated using CERVUS v3.0.3 (Kalinowski et al. 2007). The influence of stuttering and large allele dropout on genotypes was analyzed using Micro-Checker (Van Oosterhout et al. 2004).

2.4 Results and discussion

Scanning of 180 *C. quinquefasciatus* supercontigs identified 214 putative microsatellite sequences with di- and tri- nucleotides repeats. Thirty six perfect di- or tri- nucleotide, single-locus repeats were selected for primer design and characterization. Initial screening of the designed primers by fragment analysis on agarose gel electrophoresis showed 30 of the primer pairs with consistent amplification, while the remaining six primer sets were discarded due to either no amplification (MCQ 30, MCQ 40 and MCQ 43) or amplification of non-target regions (MCQ 7, MCQ 17 and MCQ 44) under different reaction and amplification conditions. BLAST analysis against the GenBank nucleotide database of all markers revealed that one locus (MCQ 16) despite differing in primer sequences matched a microsatellite region previously described (GenBank Accession number DQ388495.1).

Thirty loci were selected for multiplexing in five six-plex reactions (Table 2.1), which all amplified under the same PCR conditions. All multiplex panels were free from spectral overlap, allele overlap, and excess peaks (Figure 2.1). For three of the loci (MCQ 4, MCQ 5 and MCQ 21), Micro-Checker suggested mis-scoring of stutter peaks in samples from Jinja. However, close examination of the electropherograms did not support this suggestion.

Table 2.1: Characteristics of polymorphic microsatellites from *Culex quinquefasciatus* collected in two field populations from Uganda.

Multiplex	Locus	Primer sequences (5`-3`)	Dye	Repeat motif	size range (bp)	No of alleles	PIC	H_E	Population		GenBank accession no.
									Jinja	Kampala	
									F_{IS}	F_{IS}	
Reaction 1	MCQ24	F: CTTCTAGGGTTGGCGTTGAG R: TCGTGATCTGCATCGGTAAT	[D4]	$(AC)_8$	127-139	6	0.673	0.717	0.108 ^{NS}	-0.069	GF112168
	MCQ16	F: ACGCCGTCAGAAACACATTA R: GAGCTGTGTGACAAGCATGG	[D4]	$(AC)_{13}$	197-209	7	0.658	0.688	0.094	0.127	GF112169
	MCQ19	F: GTCCCAGGATCGTAGCTCAG R: CGTTCCCCTAGAGTTGGTTG	[D4]	$(GT)_8$	250-261	6	0.464	0.492	-0.052	0.119 ^{NS}	GF112166
	MCQ45	F: GCGCTAGGGACCATACACAA R: TGGTTTCTAACTGGGGCATC	[D3]	$(AG)_{14}$	100-115	6	0.351	0.374	-0.062 ^{NS}	0.056	GF112160
	MCQ3	F: GGGCCAGAGGAAAGTGAGAT R: CCCGAAACCTTAAGCACATC	[D3]	$(GT)_8$	206-214	6	0.383	0.412	0.240 ^{NS}	0.334 ^{NS}	GF112143
	MCQ25	F: CTAGAGGAAAAGCGGTGCAT R: CTGGCTGTCCACACATCAAT	[D3]	$(GT)_{10}$	254-266	5	0.478	0.554	0.386 ^{NS}	0.272	GF112150
Reaction 2											
	MCQ28	F: TTTGGCAAATAGCCTTCTGG R: GGTGTCGATGTGAGGGGTTA	[D4]	$(TG)_8$	116-126	4	0.604	0.659	0.658*	0.488*	GF112170
	MCQ39	F: TCTTTAGCAGCGCCTGTATG R: ACAACACGTAACCTCGCTGA	[D4]	$(GT)_9$	186-200	5	0.467	0.502	0.090	-0.067	GF112157
	MCQ10	F: AGCGAGTGCGACGAATAAAG R: CAGCGTGGAAGACAAGTTCA	[D4]	$(CGA)_8$	306-320	5	0.583	0.636	0.398*	0.221	GF112145
	MCQ2	F: TGATTGAGTCATGGTGCAGAG R: ACCCTTAAGCCTTCCGTGTA	[D3]	$(AG)_{20}$	135-155	2	0.340	0.434	0.060	0.328 ^{NS}	GF112142
	MCQ4	F: TTCATCTCTATCCGGTTGTGG R: CTAGCCTGGCAAGAACCAAC	[D3]	$(TG)_8$	258-262	3	0.580	0.654	0.083	0.020	GF112162
	MCQ29	F: CCCTTGTGGGAAGTAGTTGG R:TGACACTTCCTCGAGACACG	[D2]	$(AC)_8$	119-127	4	0.602	0.662	-0.127	0.158	GF112171
Reaction 3											
	MCQ1	F: TACCGAGAGGTTTGCAGGAC R: GCGTACCAGGATCGTCTCAT	[D4]	$(AG)_{12}$	133-145	4	0.445	0.521	0.504*	0.415 ^{NS}	GF112161
	MCQ22	F: TCAAATCTGGGTCACAATGC R: TGACAACTTCCCCAGAGAGG	[D4]	$(GT)_{17}$	179-215	12	0.612	0.666	0.359 ^{NS}	0.060	GF112148

	MCQ37	F: AATCCAAACGACGCAAGAAC R: GGCGCTAGAAGTAGCCTTCA	[D4]	(AC)8	245-251	4	0.378	0.420	0.102	-0.097	GF112156
	MCQ36	F: GGATGGACTCCACGAAATGT R: GGGTTTCATGGTGTCTACGG	[D3]	$(CA)_8$	148-152	3	0.339	0.406	-0.087	-0.019	GF112155
	MCQ23	F: TCTTTAGCTTGCAGGGCCTA R: TTCTGACAGCTGCACTCACC	[D3]	$(CT)_8$	244-251	4	0.474	0.539	0.598*	0.367*	GF112149
	MCQ11	F: CATGAGCACGTGTCTTCTCC R: CATCATAATCGGCGCCTAAC	[D2]	$(TG)_8$	251-265	5	0.462	0.558	-0.022	-0.017	GF112146
Reaction 4											
	MCQ8	F: CCCCAACATTAAACCCTCTTT R: TTTCTGTACACCTCGCACCA	[D4]	$(CA)_{13}$	213-221	4	0.618	0.683	0.221	0.066	GF112163
	MCQ9	F: TTAGCGGAGCAGCTGTGTAG R: GTGCCTCAAGAGTCCATCGT	[D4]	$(GA)_{10}$	271-281	8	0.659	0.706	0.058	0.190 NS	GF112164
	MCQ31	F: AATGAAGGAACCTCGCGTAA R: ATTTAGATGCGACCGCAGAA	[D4]	$(TG)_8$	158-170	5	0.675	0.714	-0.107	-0.015	GF112152
	MCQ42	F: AAGGGTCACAACCCACTTCA R: TGGTGGGGACACATGTTAAA	[D3]	$(CT)_{13}$	122-136	8	0.501	0.528	-0.036	-0.053 NS	GF112159
	MCQ13	F: ACAGAGCTGCCTTTTGCAGT R: CCAGCTGCCAATTTCATTCT	[D3]	$(AC)_9$	248-256	5	0.552	0.622	0.175	0.123	GF112165
	MCQ41	F: GAAGGCACTTCCTGTCTCGT R:TGGTCGTAACAATCCCCTT	[D3]	$(AG)_8$	194-202	5	0.600	0.666	-0.068	0.024	GF112158
Reaction 5											
	MCQ33	F: ATCCGCTCGACAAATAATGG R: ACATGGAACGACGTCGAAAT	[D4]	$(CA)_9$	110-114	3	0.337	0.369	0.795*	0.465*	GF112153
	MCQ20	F: TGTATGATGCTGTGCGATGA R: CAAACCTTGCCAAAGAGTGC	[D4]	$(AG)_9$	198-202	3	0.539	0.611	0.129	0.355 NS	GF112147
	MCQ21	F: CAGCTCGGCAATAGAAAACC R: TCTGTCTCTGTCTGCCTTGC	[D4]	$(CT)_{10}$	241-271	8	0.589	0.617	0.414*	0.208	GF112167
	MCQ26	F: ACCTGTCACTCGAGCCATTC R: GTGCGACATCCGATACTGAA	[D4]	$(CT)_9$	145-167	9	0.826	0.844	-0.033	0.103	GF112151
	MCQ34	F: CAGTGGGGAAAGAAACCAGA R: TTAAGCCAATCTGCGTTGTG	[D3]	$(CA)_9$	172-190	7	0.581	0.621	0.409*	0.271*	GF112154
	MCQ5	F: GGAAATCCATTGGACAGGAA R: ACATCGTCGGAGGAACAGAG	[D2]	$(AC)_9$	198-206	5	0.630	0.679	0.230 NS	0.370*	GF112144

Polymorphic Information content (PIC), expected heterozygosity (H_E) and Weir and Cockerham's (1984) inbreeding coefficient (FIS). NS: not significant; *Significant departure from Hardy-Weinberg after Bonferroni correction (adjusted significance [5%] threshold = 0.001666) of the probability values using Fisher`s method.

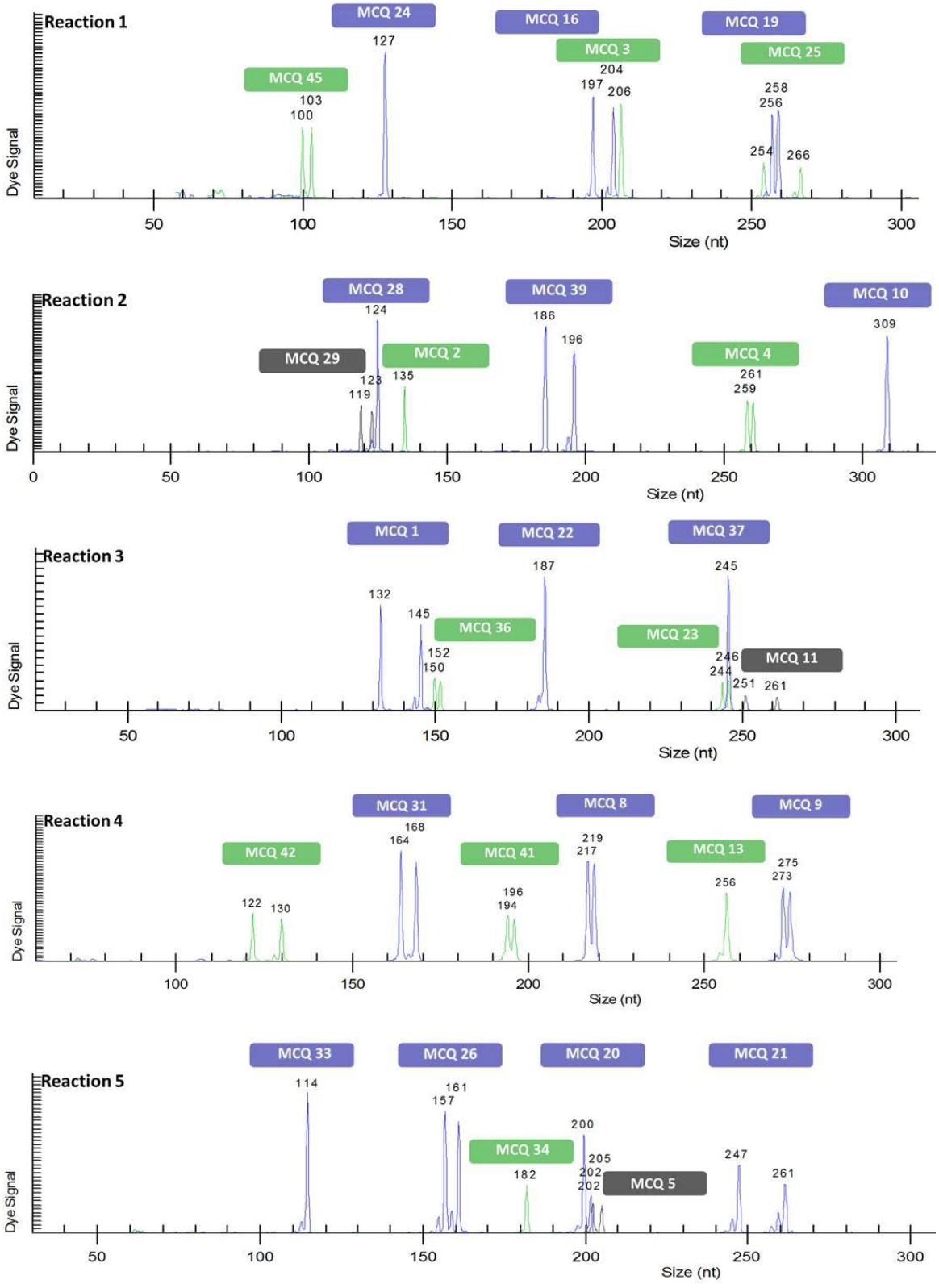


Figure 2.1: Electropherograms of the five multiplex panels depicting amplification profile and loci size distribution. Horizontal axis shows the size in base pairs (bp). Numbers above the peaks are the allele size.

Polymorphism and reproducibility of the microsatellite markers was surveyed in 70 *C. quinquefasciatus* individuals collected in Uganda. Amplification success was 100% for 21 of the 30 markers, while for the remaining nine loci amplification was between 77.14% and 98.6%. The lowest success rate (77.14%) was observed in multiplex 5 primarily as a result of amplification failure of loci MCQ33 and MCQ34 (Figure 2.2).

All 30 loci were polymorphic (0.95 criterion), with 2-12 alleles observed per locus (average 5.367 SD 0.388), while the observed and expected mean heterozygosities were 0.484 (SD 0.029) and 0.585 (SD 0.022) respectively. Four out of 5 panels exhibited the same proportion of alleles (mean = 5.74), while the mean of expected heterozygosity in reaction 2, reaction 4 and reaction 5 was higher than 0.6 (Figure 2.2). Furthermore, polymorphic information content (PIC) also showed a high level of genetic variability ranging from 0.337 (MCQ 33) to 0.826 (MCQ 26).

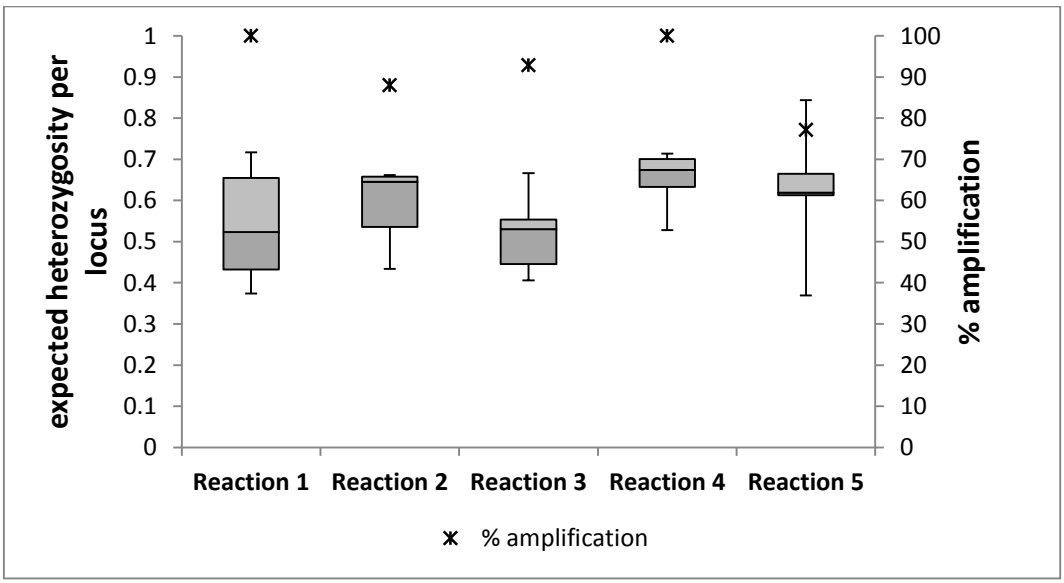


Figure 2.2: Proportion of amplification and expected heterozygosity in each six-plex panel. % amplification: proportion of samples per reaction that amplified for all loci. Box plot includes expected heterozygosity from the 25th to the 75th percentile; the horizontal line within the box represents the median value. Lines outside the box represent the lowest and highest value.

Samples from Jinja and Kampala exhibited seven and five significant deviations from Hardy-Weinberg equilibrium (HWE) respectively after Bonferroni correction (Table 1), with four markers (MCQ 28, MCQ 23, MCQ 33 and MCQ 34) showing significant HWE deviation in both populations. For these loci the HWE deviation was associated with positive F_{IS} values (between 0.271 and 0.795) reflecting heterozygote deficiency, probably due to the presence of null alleles. Owing to very large diversity in mosquitoes and insects, it is common for some loci to exhibit null alleles. Results from the linkage disequilibrium test (Fisher`s exact test) detected significant linkage disequilibrium between only loci MCQ 26 and MCQ 31 (Jinja) and MCQ 26 and MCQ 41 (Kampala), suggesting broad-scale independence of markers.

In this study we describe 29 new microsatellite markers, which have been combined into five multiplex reactions, thereby significantly enhancing the genotyping efficiency for genetic diversity studies of *C. quinquefasciatus*. Though *in silico* methods for isolation of microsatellite loci have significantly increased the number of markers developed in different species (Lovin et al. 2009, Abreu et al. 2012, Dobes and Scheffknecht 2012), the high frequency of primer site and flanking region mutations still impedes the development of markers which amplify consistently in wild populations (Carlsson 2008, Hickner et al. 2010). It remains to be investigated as to whether these markers will be useful in other members of the complex as large variation in flanking regions between other species within the *C. pipiens* has been described (Smith et al. 2005).

The development of these six-plex suites is especially important since most of the previously published microsatellite markers applied for *C. quinquefasciatus* genotyping have been amplified in simplex reactions (single locus) and analyzed by pooling PCR products from several loci (Fonseca et al. 1998, Huang et al. 2008), which is a limitation for high throughput screening. Previous studies (Masi et al. 2003, Porta et al. 2006, Eusemann

et al. 2009) have shown that the optimization of parameters such as optimal annealing temperature and primer concentration are critical but time-consuming steps for multiplex development. However, here we demonstrated that by combining *in silico* characterization of primer compatibility and a commercial multiplex amplification kit the optimization process proved straightforward, without apparently compromising amplification quality or specificity. Although these multiplex have been designed for a specific dye system, it can be readily converted to other systems using the design software described.

Chapter III

Local selection in the presence of high levels of gene flow: Evidence of heterogeneous insecticide selection pressure across Ugandan *Culex quinquefasciatus* populations

3.1 Abstract

Despite the role of insecticide based-approaches to control *Culex quinquefasciatus* populations, few studies so far have been conducted to understand the impact of insecticide selection pressure in the process of local evolution of resistance in natural populations. Herein, *C. quinquefasciatus* mosquitoes collected in Uganda, where no direct vector control interventions for this species have been conducted recently, was used as a model to determine if is possible to detect heterogeneities in selection pressure driven by the use of insecticides targeting other insect species with medical or agricultural interest. Genetic diversity and structure of the local populations were assessed through microsatellite markers, and the impact of insecticide pressure from distinct classes was investigated by applying two parallel genotyping assays to type the target-site mutations;*Vgsc*-1014L and *Ace1*-119S. Among the populations, no significant differences in genetic diversity indices were observed by the microsatellite markers with H_E ranging from 0.597 to 0.612, with the highest and lowest allele frequency detected in samples from Kanungu (4.96) and Kampala (5.42), respectively. The microsatellite data also indicated low genetic differentiation among populations (F_{ST} = 0.019, P = 0.001), although a pattern of genetic structure was detected, which grouped the four populations within three clusters. In contrast to microsatellite markers, the genotyping of insecticide-resistance markers indicated a heterogeneous distribution of resistance alleles between Uganda Central to Eastern and Southwest populations. In populations from the central region, a frequency of 62% and 32% for the *Kdr* and *Ace-1* resistant allele were observed, respectively. Conversely, in the populations from Eastern and Southwest a higher frequency of *Kdr* alleles (100% and 97%) was detected, while the frequency of *Ace-1* resistant alleles corresponded to 12% on both regions. Taken together, the pattern of microsatellite genetic diversity and insecticide selected markers suggest that despite the absence of official vector control against Ugandan *C. quinquefasciatus*, populations are under heterogeneous selection pressure imposed by insecticides from distinct classes with variation in level of exposure.

3.2 Introduction

Culex quinquefasciatus, also known as the southern house mosquito, is widely distributed throughout many regions of Africa, the Americas, Asia and Australia (Farajollahi et al. 2011). *C. quinquefasciatus* is a primary vector of arboviruses such as West Nile virus (WNV) and St. Louis encephalitis virus (SLEV) in temperate regions (Diaz-Badillo et al. 2011, Molaei et al. 2012) and in many tropical/sub-tropical regions it is implicated in the transmission of lymphatic filariasis (LF) (Bockarie et al. 2009, Simonsen and Mwakitalu 2013). On the African continent, LF is predominantly transmitted by *Anopheles* mosquitoes in rural areas, while in urban and coastal areas of East Africa, *Culex* species are the main vector (Bockarie et al. 2009).

Since LF is the second largest cause of long term disability among the Neglected Tropical Diseases (NTDs), efforts to eliminate LF in endemic regions by 2020 have been intensified through the Global Program to Eliminate LF (GPELF) (Katiyar and Singh 2011, Rebollo and Bockarie 2013). Although Mass Drug Administration (MDA) has been the primary intervention of the GPELF to block LF transmission, vector control measures have also been advocated for endemic areas where MDA faces challenges such as adverse reactions due to co-infection with LF and Loa-loa. In regions with high LF prevalence, vector control is expected to reduce the number of MDA rounds required to achieve the elimination threshold (Sunish et al. 2007, Bockarie et al. 2009, Kelly-Hope et al. 2013).

Vector control relies chiefly upon the use of insecticides, either through insecticide treated nets or indoor residual spraying of insecticides onto the interior surfaces of dwellings. Development of resistance to these insecticides has been the major impediment to effective control. In contrast to other vector species, few studies have addressed mechanisms and distribution of resistance in *C. quinquefasciatus* (Jones et al. 2012b, Scott et al. 2015), likely

due to the reliance upon MDA, rather than vector control, as the primary anti-LF intervention (Kolaczinski et al. 2007). In Uganda, a country with high rates of malaria transmission and with LF prevalence concentrated in the North and West ranging from 0.4-30.7%, *Anopheles* is incriminated as the primary vector (Onapa et al. 2001, Kolaczinski et al. 2007) and vector control is targeted chiefly at *Anopheles*. Whilst there is no official vector control programme targeting *Culex* populations, insecticide resistance may be indirectly selected through the use of insecticides for the control of vector species such as *Anopheles* or agricultural pests or directly through control of nuisance biters, principally in urban areas (Norris and Norris 2011, Nkya et al. 2013).

Indeed, due to the high prevalence of malaria across the country, two vector control interventions have been used (Kigozi et al. 2012, Yeka et al. 2012); distribution of long-lasting pyrethroid insecticide treated nets (ITNs) and focal indoor residual spraying (IRS) in regions of high malaria prevalence especially in the Mid-North region (Yeka et al. 2012, Uganda Bureau of Statistics 2015) but also in the extreme South-West (Bukirwa et al. 2009). Targeting *Anopheles* mosquitoes, during malaria control interventions is also likely to impose selection pressure on sympatric *Culex* mosquitoes as demonstrated by other studies (N'Guessan et al. 2009, Norris and Norris 2011).

In addition, heterogeneity in selection pressure and geographical spatial structure could result in a heterogeneous pattern of insecticide resistance at both a micro- and macro-geographic scale (Lenormand 2002, Barbosa and Hastings 2012). Consequently, distinguishing the effect of local selection from population genetic background is not easily achieved (Kawecki and Ebert 2004). For example, frequencies of adaptive selected markers for insecticide resistance, such as *kdr* or *Ace-1* alleles, if approximately without deleterious fitness effects, may occur at similar frequencies in insecticide-treated and non-treated areas through extensive gene flow as well as

through direct selection pressure (Endersby et al. 2008). Conversely differential resistance allele frequencies could result from differences in either/or selection pressures or genetic drift. Population genetic inferences combining neutral and adaptive loci might allow us to infer differential selective pressure across an area. Since allele frequencies at neutral loci are influenced mostly by effective population size and migration, these markers have become a powerful tool for detecting the influence of demographic and colonization effects on population diversity and structure (Selkoe and Toonen 2006, Hoffmann and Willi 2008). By contrast, changes in the frequency of insecticide resistant alleles such as 1014L and 119S have been studied extensively and are associated with insecticide selection pressure in vector populations worldwide (Weill et al. 2004, Donnelly et al. 2009, Tantely et al. 2010, Mawejje et al. 2013). Studying both resistance marker frequencies and neutral markers from the same samples promises to yield important insights about heterogeneities in selection pressure.

Hence, since *C. quinquefasciatus* has not been linked to LF transmission in Uganda and MDA is applied as the main strategy to mitigate the burden of LF transmission, no direct vector control intervention for *C. quinquefasciatus* has been conducted recently throughout the country (although malaria control interventions may indirectly target *Culex*). In this study, we utilized Ugandan *C. quinquefasciatus* collections as a model to determine through combinations of putatively selected and neutral markers, if it is possible infer heterogeneities in insecticide selection. Population genetic diversity and structure were assessed using microsatellite markers, whilst the impact of insecticide pressure from distinct classes in the evolution of resistance was investigated by applying two parallel genotyping assays to type the target-site mutations 1014L in the VGSC gene and 119S in the *Ace*-1 gene.

3.3 Materials and methods

3.3.1 Area of study and mosquito collection

C. quinquefasciatus were collected from four districts of Uganda (Figure 3.1) with similar levels of insecticide-treated bed nets (ITNs) coverage (Uganda Bureau of Statistics 2015). Three of these districts are the main study sites of the Ugandan ICEMR (International Centers of Excellence for Malaria Research) (ICEMR 2015). A total of 162 resting adult mosquitoes of both sex were collected by aspiration from inside houses between July and August 2012. Collection points were located in Tororo and Kanungu districts with populations of 526 and 252 thousand people, respectively and more than 80% of the population dwelling in rural places. In Jinja and Kampala, with populations of 468 thousand and 1.5 million people, respectively samples were obtained from peri-urban places in Jinja (63% of rural houses) and in urban centres from Kampala (Uganda Bureau of Statistics 2014).

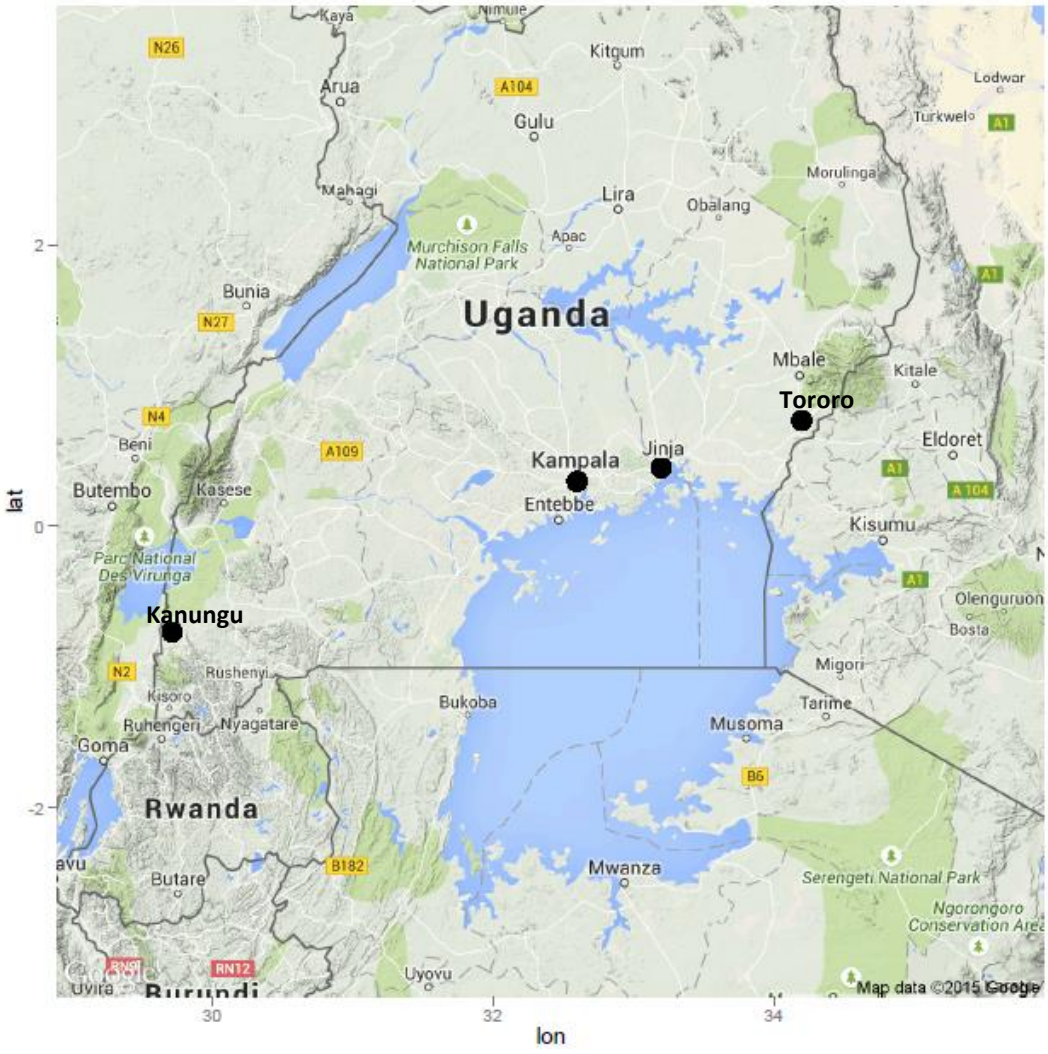


Figure 3.1: Geographic distribution of field-collected Ugandan *C. quinquefasciatus* mosquitoes.

3.3.2 DNA isolation and Species identification

Genomic DNA from individual mosquitoes was isolated using a DNeasy kit (Qiagen) following the manufacturer's recommendations and then re-suspended in 200 µl of distilled water. All samples were confirmed as *C. quinquefasciatus* by a diagnostic PCR assay (Smith and Fonseca 2004).

3.3.3 Allelic discrimination assays of insecticide target-site mutations

For the initial design of target-site mutation genotyping assays, partial genomic fragments spanning the location of the *G119S* mutation in the *Ace-1* gene and the *L1014F* mutation (*kdr*) in the *Vgsc* gene were amplified from genomic DNA isolated from mosquitoes collected from four different regions (Figure 3.1). PCR reactions were performed in a final volume of 20 µl including 1µl of genomic DNA, 1X Phusion HF buffer, 200 µM of each dNTP, 0.02 U/µl of Phusion Hot start II DNA polymerase and 0.4 µM of each specific primer for the *Ace-1* and *Vgsc* gene (Table 3.1). Amplification was performed with cycling conditions of 98 ºC for 30 sec, followed by 30 cycles of 98 ºC for 10 sec, 56 ºC for 15 sec and 72 ºC for 15 sec with a final extension of 72 ºC for 10 min.

PCR products were purified using the GeneJET PCR purification kit (Thermo Scientific) and then cloned into the pJet 1.2 vector using the CloneJet PCR cloning kit (Thermo Scientific). Clones were screened using PCR and DNA extracted for sequencing from positive colonies using the GeneJET Plasmid Miniprep kit. After sequencing, sequences were aligned and manually edited in CodonCode Aligner (CodonCode Corporation). Sequence regions conserved across haplotypes were used to design custom primers and TaqMan probes using the Custom TaqMan® Assay Design Tool (Life Technologies, UK) and pyrosequencing assay using the PyroMark assay design software 2.0 (Qiagen).

The *Ace-1* TaqMan assay was designed to genotype the SNP (GGC/AGC) in the first base of the codon at the position 119 in the *Ace-1* gene. TaqMan allelic discrimination reactions were performed in a final volume of 10 µl using 1 µl of genomic DNA, 1X SensiMix II probe (Bioline), 900 mM of each primer and 200 nM of probes (Table 3.1). A Stratagene MX3005P,

with cycling parameters of 95 °C for 10 min and 40 cycles of 92 °C for 15 sec and 60 °C for 1 min was used.

The *Kdr*-pyrosequencing assay to type the L1014F mutation in the exon 20 of the *Vgsc* gene was developed to detect two synonymous resistant alleles (TTC and TTT) and the wild-type allele (TTA). PCR reactions to amplify a 105 bp fragment was performed in a total of 25 µl containing 10ng of gDNA, 200 µM of each dNTP, 1X of the 10X PCR buffer, 2.0 mM of $MgCl_2$, 0.6 units of HotStarTaq DNA polymerase (Qiagen) and 0.4 µM of each specific primer (Table 2). After initial denaturation at 95 °C for 15 min, PCR amplification was performed for 40 cycles of 94 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 30 sec, followed by a final extension step at 72 °C for10 min. For genotyping by pyrosequencing, single-stranded PCR products were obtained using the PyroMark Q24 Vacuum Prep Workstation and then used in pyrosequencing reactions performed using the PyroMark Gold Q96 reagent kit (Qiagen). Sequencing primer and dispensation order are described in Table 3.1.

Table 3.1: Primers and probes for screening the *Ace-1* and *Kdr* mutations in the *Ace*-1 and *Vgsc* genes, respectively in *Culex quinquefasciatus* mosquitoes.

Locus	PCR primers	Target-site SNP assay
		TaqMan allelic discrimination
***Ace*-1**	Cx_*Ace*-1-F: 5`- CGACTCGGACCCACTCGT - 3`	Primer F: 5` - TCCAGCGTGGCAGTCC - 3`
	Cx_*Ace*-1-R: 5` - CCTACCTCAGTGCCAGGTTC - 3`	Primer R: 5` -GCCGTCATGCTGTGGATCTT - 3`
		Wild-type/probe: 5` - VIC-AGTAGAAGCCACCCCC - 3`
		SNP/probe: 5` - FAM-AGTAGAAGCTACCCCC - 3`
Kdr		**Pyrosequencing**
	Cx-*Kdr*-F: 5` - CCTCCCGGACAAGGACCTG - 3`	Primer F: 5` - CTTGGCCACCGTAGTGATAGG - 3`
	Cx-*Kdr*-R: 5` - GGACGCAATCTGGCTTGTTA -3`	Primer R: 5`Biotin - GCTGTTGGCGATGTTTTGACA - 3`
		Seq. primer: 5` - CCGTAGTGATAGGAAATTT - 3`
		Dispensation: 5`HGTCGTGAGT ATTCCAGCGT GAAGTC - 3`

3.3.4 Detection of *Ace-1* gene duplication by haplotype diversity

The absence of *Ace-1* homozygous resistant genotypes observed from the TaqMan results indicated a likely gene duplication of *Ace-1* in all four populations studied (Djogbénou et al. 2009, Alout et al. 2011). To investigate the possible presence of multiple gene copies, a partial fragment of *Ace-1* was PCR amplified from 12 individuals from Kampala. After cloning, between six and eight colonies from each individual were sequenced in order to detect the presence of >2 alleles, which is indicative of gene duplication (Labbe et al. 2007a).

Sequences from each individual were aligned in CodonCode Aligner software version 4.2.2 with ClustalW (Larkin et al. 2007) and visualized using Jalview (Waterhouse et al. 2009). MEGA 5.1 (Tamura et al. 2011) was used to analyse haplotype variability by calculating the number of polymorphic sites and nucleotide diversity (π nucleotide diversity index). Frequency and relationships between haplotype were visualised by a minimum spanning network tree generated using the program PopArt available at **http://popart.otago.ac.nz**.

3.3.5 Microsatellite genotyping

Microsatellite genotyping was conducted using a novel panel of 30 microsatellite loci developed for this study (see Chapter II). Microsatellite loci were isolated by scanning 180 *C. quinquefasciatus* supercontigs downloaded from VectorBase (Megy et al. 2012) with SciRoKo (Kofler et al. 2007) to identify perfect di- or tri- nucleotide motifs with repeat number ranging from 5 to 20. Genotyping was performed using five six-plex assays with PCR reactions conducted in 25 µl volumes containing: 1X Type-it multiplex PCR Master Mix (Qiagen), 0.2 µM of each primer and 2 µl of genomic DNA. PCR cycling conditions followed the QIAGEN Type-it Microsatellite procedure and amplified fragments were genotyped using a Beckman-Coulter CEQ8000 capillary electrophoresis system with a 400 size standard kit. Genotypes were

sized using the Beckman-Coulter CEQ 2000 DNA analysis system software and manually verified.

The microsatellite genotype data was analyzed with the program Micro-Checker (Van Oosterhout et al. 2004) to detect possible scoring errors (stutter peaks and allele drop-out) and null alleles. Then, allelic frequencies, observed (H_O) and expected (H_E) heterozygosities were calculated using the GenAIEX 6.5 (Peakall and Smouse 2012) . Allele richness, adjusted to the smallest sample size, was estimated in FSTAT (Goudet 2001), and Polymorphic Information Content (PIC) calculated using Cervus 3.0 (Kalinowski et al. 2010). Genotypic frequencies were tested for deviation from Hardy-Weinberg equilibrium (HWE) for each locus by the exact probability test available in GENEPOP 4.3 (Rousset 2008), followed by a sequential Bonferroni correction. Detection of loci under selection were conducted using coalescent simulations for each loci comparing levels of genetic differentiation (F_{ST}) and genetic diversity (heterozygosities) within and between population as described by Excoffier et al. (2009) implemented in Arlequin 3.5.1 (Excoffier et al. 2005).

Genetic differentiation among populations was estimated by pairwise F_{ST} using Arlequin 3.5.1 (Excoffier et al. 2005). In addition, an AMOVA analysis was also carried out in Arlequin to estimate the level of differentiation among populations from different clusters based on F_{ST} values. The pattern of migration under an isolation-by-distance model was tested with a Mantel's test using Rousset's genetic distance ($F_{ST}/(1-F_{ST})$) and geographic distance in the isolation-by-distance program (Jensen et al. 2005). A Bayesian analysis to infer the population structure without prior information of the geographic distribution of samples was carried out using STRUCTURE 2.3 (Pritchard et al. 2000). To identify the optimal number of clusters (K) in these populations, twenty independent runs were conducted for each K value (ranging from $K = 1$ to $K = 7$) with 10,000 interactions and 100,000 replications. The most likely K value was

calculated for each run by the log likelihood (LnP(D)) method and results compiled using CLUMPP (Jakobsson and Rosenberg 2007). Population structure was also evaluated by Discriminant Analysis of Principal Components (DAPC) using the adegenet R package (Jombart 2008). To identify optimal number of cluster for the DAPC clustering, k-means values were sequentially tested and then compared using Bayesian Information Criterion (BIC), with the lowest value of BIC used as likely number of population clusters.

3.4 Results

3.4.1 Frequency of target-site resistant alleles

Genotyping of two target-site mutations G119S and L1014F in the *Ace-1* and *Vgsc* genes respectively was conducted utilizing TaqMan allelic discrimination and pyrosequencing assays, respectively. The *Ace-1* resistant allele (AGC) was observed in all populations with a frequency ranging from 12-33% (Figure 3.2A). Despite the high frequency of the 119S allele we observed complete absence of homozygous resistant mosquitoes. The frequency of heterozygotes was almost three times higher in Kampala and Jinja compared to the other two populations (Table 3.2, Figure 3.2B). Among the four populations, a moderate *H*e (0.323 $\pm$ 0.065) was observed with a marked excess of heterozygotes (F_{is} = -0.362), whilst within populations, genotype frequencies were not significantly different from Hardy-Weinberg expectation for both the Kanungu and Tororo populations (Table 3.2). Additionally, no significant difference in allele frequencies was detected between Jinja and Kampala (P = 1.0) and Kanungu and Tororo (P = 1.0).

For the *kdr* mutations (L1014F) we detected a high frequency of resistant alleles ranging from 62 - 100% (Figure 3.2A). The *kdr* mutation in *C. quinquefasciatus* has two alternative non-

synonymous substitutions (TTT and TTC) at the third position of the codon. The pyrosequencing genotyping identified 11 individuals harbouring all three *kdr* alleles instead of the two expected from diploid organisms. With the exception of Kanungu, mosquitoes with tri-allelic genotypes were observed in all populations with a frequency of 4.0 - 10.8% (Figure 3.3), which was not used for the calculation of resistant allele frequency. Excluding individuals with a tri-allelic pattern to allow the use of standard methods to infer departures from Hardy-Weinberg, our analysis detected significant deviation only in mosquitoes from Tororo with $F_{IS} < 0$, indicating an excess of heterozygotes (Table 3.2).

Nevertheless, between populations significant differences in allele's frequencies was detected with the exception of Jinja and Kampala ($P = 0.63$). The TTC allele was observed at a high frequency (41-69%) in all populations with the exception of Kanungu, where a higher frequency of the TTT resistant allele (55%) was detected, coupled with an absence of the wild-type allele (Figure 3.2A). In all populations, we observed a low frequency of susceptible homozygotes (ranging from 0 to 14%), while the majority of resistant alleles were observed in heterozygotes (Figure 3.2B).

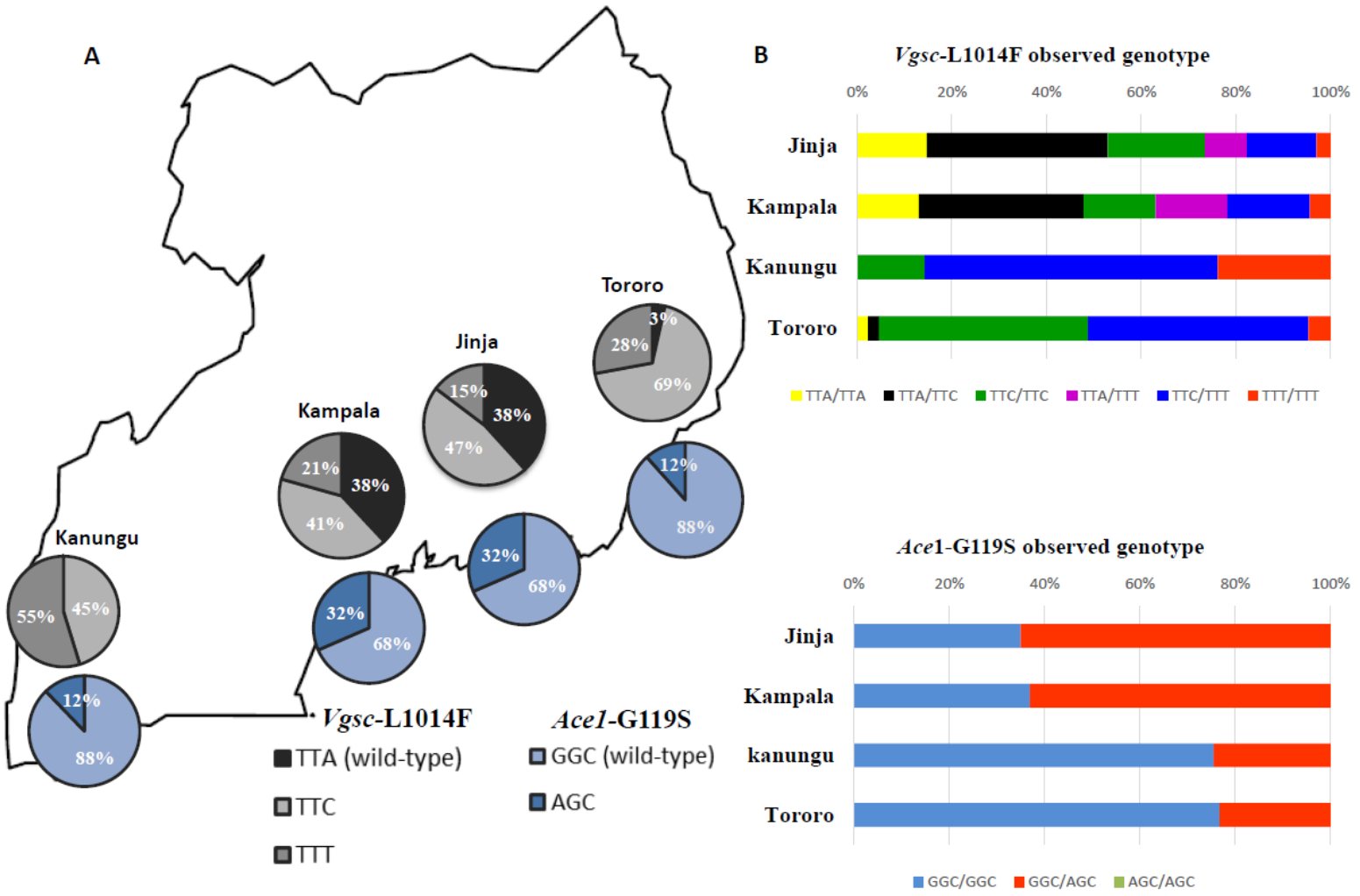
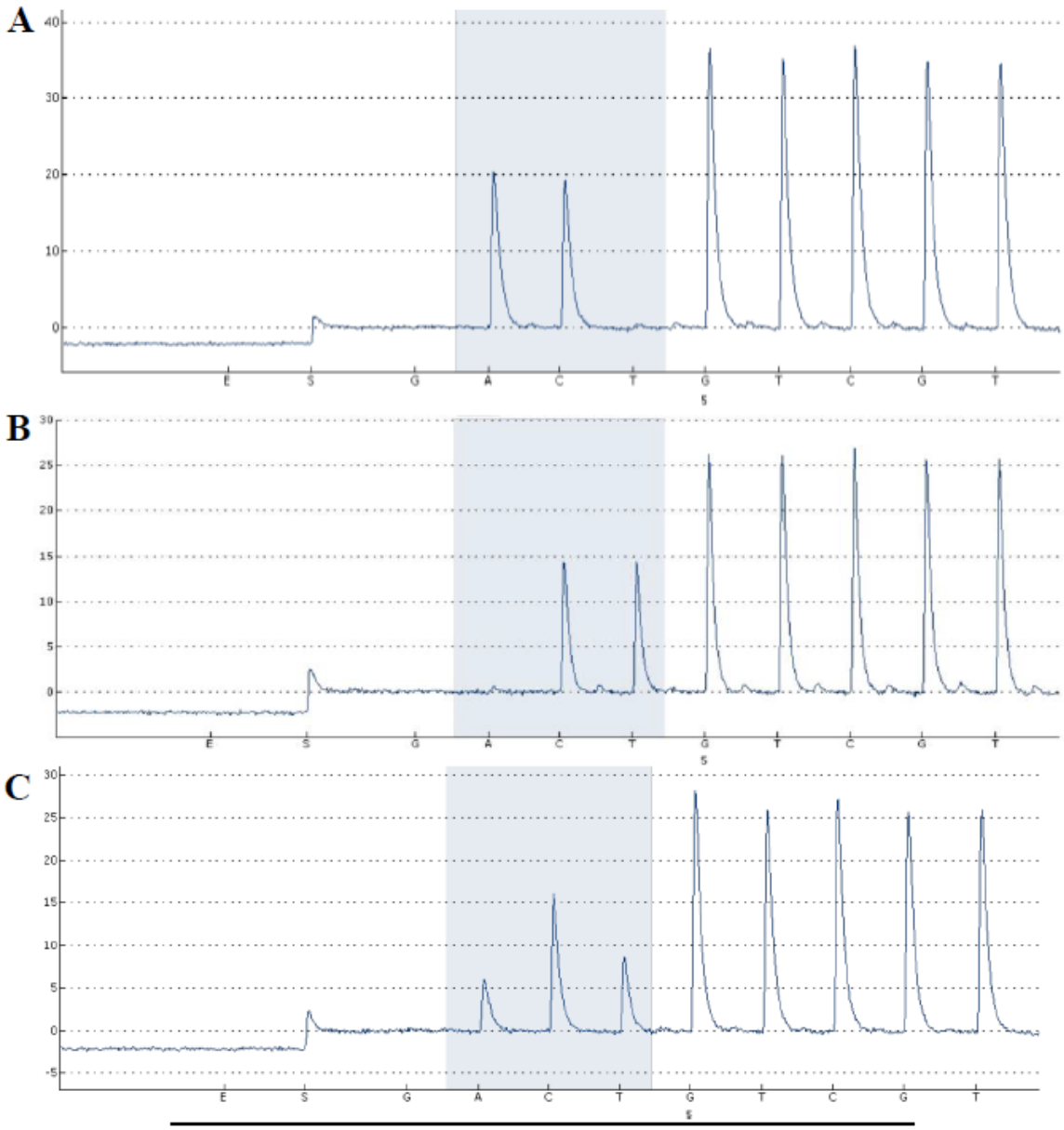


Figure 3.2: Genetic variability for the L1014F and G119S target-site mutations in *C. quinquefasciatus* mosquitoes from Uganda. A) Geographic distribution of target-site mutations. Pie charts depict the relative frequencies of L1014F and G119S mutation in the *Vgsc* and *Ace-1* gene, respectively. B) Target-site mutations genotypic frequency.

Table 3.2: Ugandan *Culex quinquefasciatus* populations and genotypes of the target-site mutations G119S and L1014F

	***ace*1-G119S locus**							***Vgsc*-L1014Flocus**									
	Genotype							**Genotype**									
Population	N	GG	GS	SS	H_E	F	HW[a]	N	LL	F^C L	F^C F^C	F^TL	$F^C F^T$	F^T F^T	H_E	F	HW[a]
Jinja	40	14	26	0	0.439	-0.481	0.002**	34	5	13	7	3	5	1	0.611	-0.011	0.945^{NS}
Kampala	46	17	29	0	0.432	-0.460	0.002**	46	6	16	7	7	8	2	0.649	-0.050	0.954^{NS}
Kanungu	45	34	11	0	0.215	-0.139	0.350^{NS}	42	0	0	6	0	26	10	0.495	-0.249	0.106^{NS}
Tororo	43	33	10	0	0.206	-0.132	0.388^{NS}	43	1	1	19	0	20	2	0.450	-0.085	0.000***

N is the number of mosquitoes analysed; H_E is the expected heterozygosity and F is the fixation index. HW[a], *P*-value of χ^2 tests for Hardy-Weinberg equilibrium.
GG, GS and SS correspond to homozygous and heterozygous genotypes for *ace1*-G119S locus.
F^C; codon TTC, F^T; codon TTT, L; codon TTA are distinct codons at the *Vgsc*-L1014 locus.
NS, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$



D

Population	N	>2 alleles genotypes
Jinja	38	4(10%)
Kampala	46	5 (10.8%)
Kanungu	47	0
Tororo	45	2(4%)

Figure 3.3: Genotyping by pyrosequencing of the *Vgsc*-L1014F mutation in *C. quinquefasciatus.* A and B) Allelic *Vgsc*-L1014F pyrogram showing two heterozygous genotypes: wild-type/resistant and resistant/resistant C) *Vgsc*-L1014F allelic genotyping with a pattern of tri-allelic pyrogram indicating likely gene duplication due to simultaneous presence of A, C and T alleles, instead of two alleles expected for a diploid genome. D) Frequency of tri-allelic genotype across Ugandan population.

3.4.2 *Ace-1* haplotype diversity and identification of allelic copy variation

A 535 bp fragment of the *Ace-1* gene was sequenced from 12 individuals (*N* = 57 sequences; GenBank accession numbers: KT591708-KT591765) with nine haplotypes detected displaying a total of ten polymorphic sites (Figure 3.4A) with frequencies ranging from 0.017-0.397, with the resistant allele detected in four haplotypes: A, E, H and I accounting for 40% of total haplotypes identified. The minimum spanning network tree (Figure 3.4B) shows that three resistance bearing haplotypes (A, H, I) differ from each other by a single mutational step with Hap A the most common. The remaining 119S (E) is three mutational steps away and is likely to have an independent origin. For 119G wildtype haplotypes there was no predominant haplotype. In three out of 12 individuals studied, we identified mosquitoes with three to five haplotypes belonging to different cluster from the neighbour-joining dendrogram (Figure 3.5), which indicates the presence of copy number variation for the *Ace-1* gene in the populations studied.

A

Position	12	33	69	75	87	114	189	277	**355**	462	Total of Variation	Haplotype freq
Consensus	A	Y	R	G	A	T	G	G	G	T		
Haplotype												
A		A	C						A		3	0.397
B		G	T				A				3	0.190
C		A	C								2	0.121
D		G	T	A		C					4	0.0172
E		G	T	A					A		4	0.0172
F		G	T	A	C	C					5	0.0517
G		G	T			C				G	4	0.172
H	G	A	C						A		4	0.0172
I		A	C					T	A		4	0.0172

B

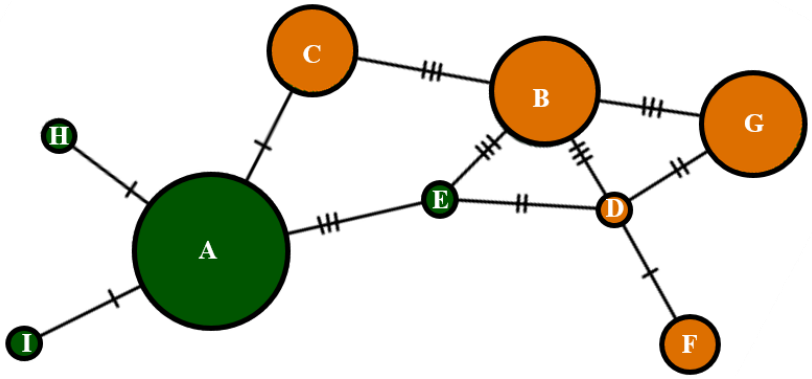


Figure 3.4: *Ace-1* gene haplotype diversity based on sequences obtained from *C. quinquefasciatus* mosquitoes sampled in Uganda. A) polymorphic sites identified in a 535 bp partial fragment (intron 2 and exon 3) of *Ace-1*. Bold number indicates the position of the G119S mutation in exon 3. B) Minimum spanning Network built using sequences of a partial fragment of the *Ace-1* gene. Each haplotype is represented by a circle, whose size is proportional to the number of individuals showing that haplotype. Haplotypes are coloured to differentiate haplotypes containing the susceptible allele (Orange) and resistant allele (green). Hatch marks represent mutational steps separating observed haplotypes.

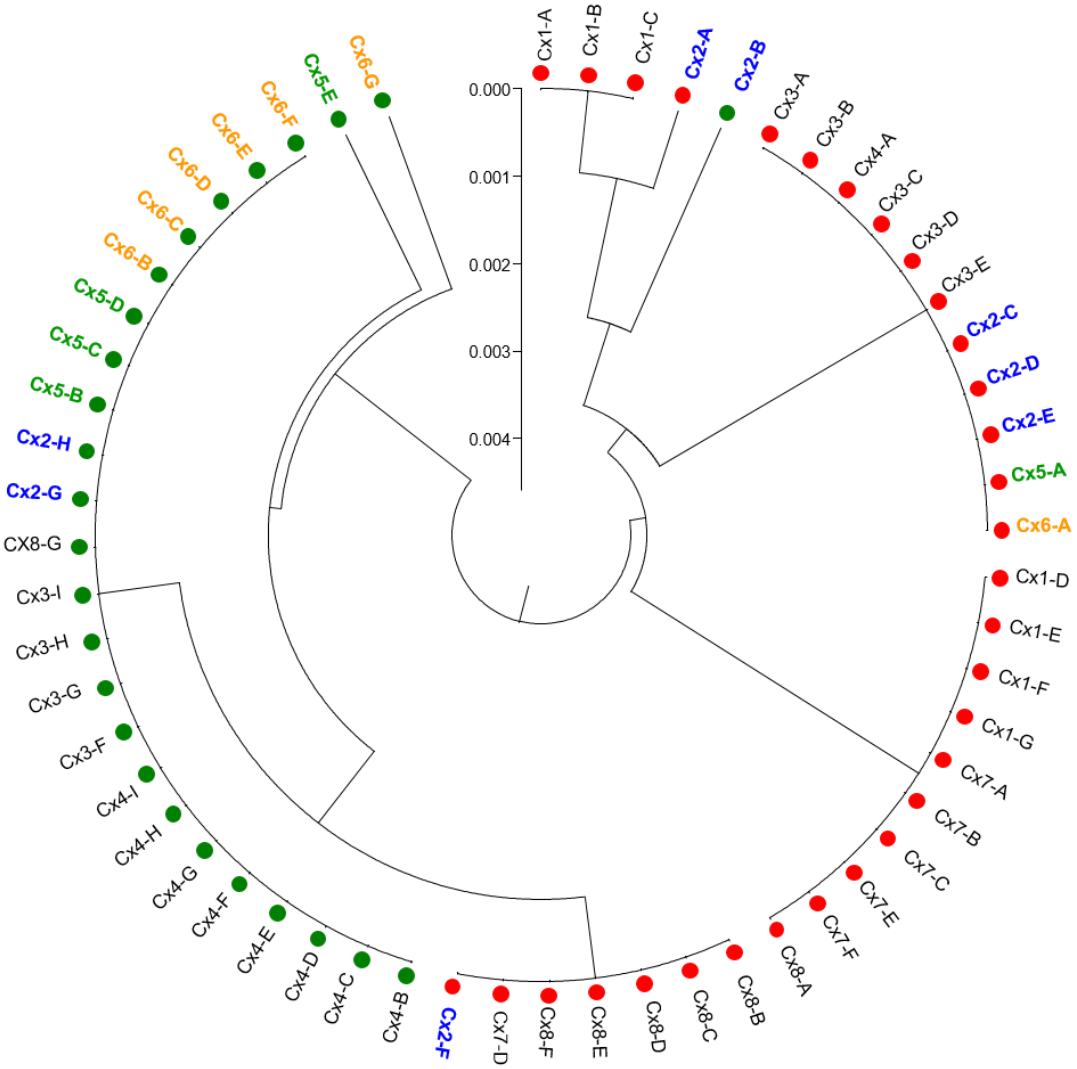


Figure 3.5: Dendrogram constructed using partial sequence of the *Ace*-1 gene from *C. quinquefasciatus* individuals with a likely duplicated haplotype. Red and green dots correspond to susceptible and resistant G119S haplotypes, respectively. Haplotypes are labelled Cx followed by a number corresponding to an individual identifier and a letter from A to I to identify an individual sequenced colony from that individual. Haplotype labels highlighted in blue, green or orange are individuals with more than two haplotypes.

3.4.3 Microsatellite genotyping

3.4.3.1 Genetic diversity

A total of 186 alleles were detected across the 30 microsatellite loci analysed (Appendix3-A) with 19 private alleles detected in 17 of the 30 loci. The number of unique alleles in the samples from Kampala was twice that detected in any other population (Figure 3.6A); however, the presence of these eight private alleles are unlikely to significantly influence the genetic structure analysis since only two alleles have a frequency higher than 1%. The allelic richness and Polymorphic Information Content (PIC) across markers varied from 2.88 (MCQ 2) to 11.14 (MCQ 21) and from 0.332 (MCQ 2) to 0.825 (MCQ 26), respectively (Appendix3-A).

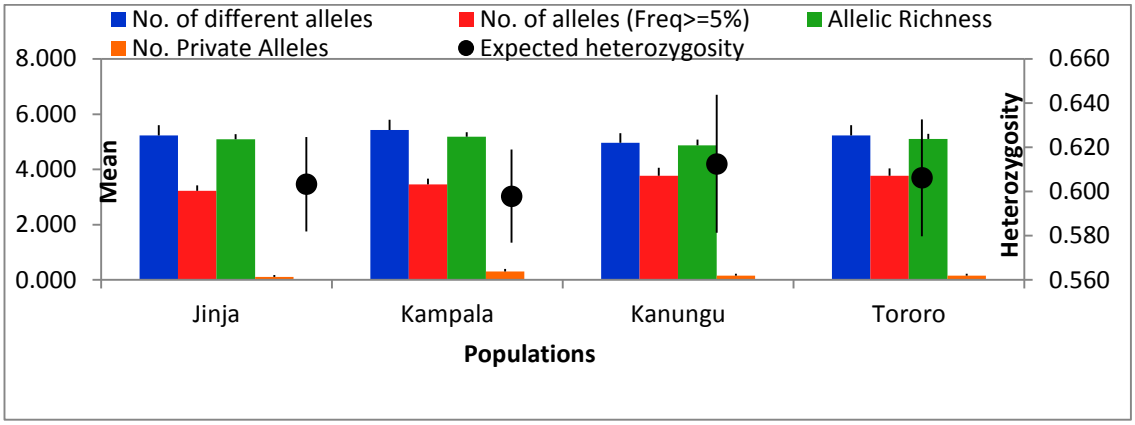
The Hardy-Weinberg exact test performed in individual locus/population indicated that 26 out of 120 instances did not conform to HW equilibrium after multiple corrections. However, only two loci (MCQ 28 and MCQ 33) showed departure from HW equilibrium across all populations (Appendix3-B). The number of loci that were not in HW equilibrium in each population ranged from five to eight (Table 3.3). For all loci with departure from HW equilibrium, significant deviation was associated with a positive inbreeding coefficient (F_{IS}), revealing deviation in the direction of heterozygote deficiency.

Analyses performed with Micro-Checker indicated a high frequency of null alleles at loci MCQ 28 (0.12) and MCQ 33 (0.22), which could likely be responsible for the heterozygote deficiency at these loci. Although the MCQ 1 and MCQ 34 loci were within HW expectation in at least one population, these loci failed to amplify in more than 10% of the samples also suggesting the presence of null alleles and them not used on the genetic diversity analysis.

Allelic diversity, expected heterozygosity (H_E) and allelic richness within populations were calculated using those 26 loci conforming to HW equilibrium and exhibiting an absence or low frequency of possible null alleles. Levels of genetic diversity were similar across populations (Figure 3.6A, Appendix 3B). The mean number of alleles across all loci varied from 4.96 in samples from Kanungu to 5.42 in samples from Kampala, whilst the highest allelic richness (Rs = 5.17) was detected in Kampala; nevertheless, no significant difference in index of genetic diversity was observed among the populations (Figure 3.6A). Average H_E across all samples ranged from 0.597 in Kampala to 0.612 in Kanungu with a median of 0.604 (Appendix 3B). From the 26 microsatellite loci tested for deviation from a neutral-equilibrium model based on the F_{ST} outlier approach, only the marker MCQ24 showed signs of possible divergent selection (Figure 3.6B).

The analysis of the genomic region (supercont 3.264) containing this microsatellite locus with sign of non-neutrality shows that it is close to 16 genes, most of which are described as hypothetical proteins of unknown function; however, one gene (CPIJ010175) belongs to the P450 family (CYP9J48) suggesting that the departure from neutrality of MCQ24 might reflect genetic hitchhiking from insecticide selection nearby the marker genomic region. Although further investigation is needed to confirm the possible link of *Cyp9j48* with insecticide resistance, the identification of this gene is especially important as in *A. aegypti*, few candidate genes from the family *Cyp9* have been associated with permethrin and deltamethrin insecticide resistance, with two genes *Cyp9J24* and *Cyp9J28* have their permethrin metabolizer confirmed by heterologous expression (Strode et al. 2008).

A



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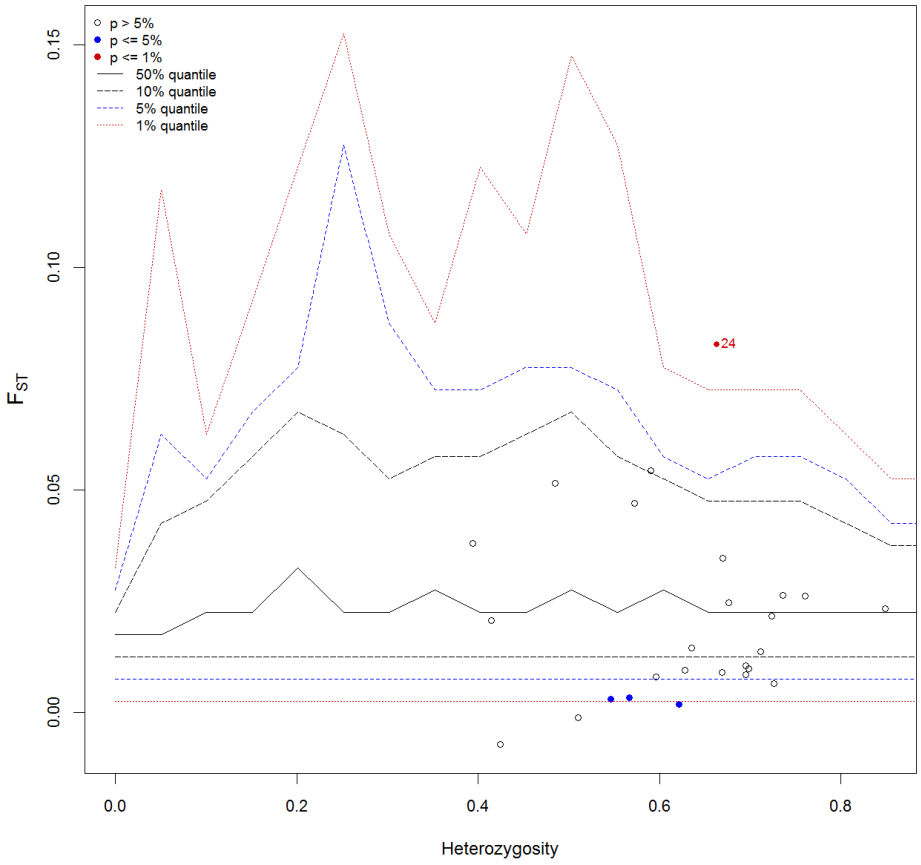


Figure 3.6: Characterization of microsatellite markers. A) Genetic diversity estimates across Ugandan *C. quinquefasciatus* populations determined from data of 26 microsatellite markers. Allelic richness was calculated based on sample size of N=33. B) Test of microsatellite markers deviation from neutral-equilibrium model using F_{ST} outlier approach. Each dot represents a microsatellite locus and red dots identify candidate loci under positive selection.

Table 3.3: Sample sizes and indices of genetic diversity at 26 microsatellite loci for four Ugandan *C. quinquefasciatus* populations.

Location	N	H_E	H_O	F_{IS}	HW
Jinja	41	0.603 (0.108)	0.541 (0.162)	0.116 *	8[b]
Kampala	44	0.597 (0.107)	0.531 (0.128)	0.123 *	5[b]
Kanungu	39	0.612 (0.159)	0.549 (0.199)	0.115 *	8[b]
Tororo	38	0.606 (0.134)	0.511 (0.172)	0.170 *	5[b]

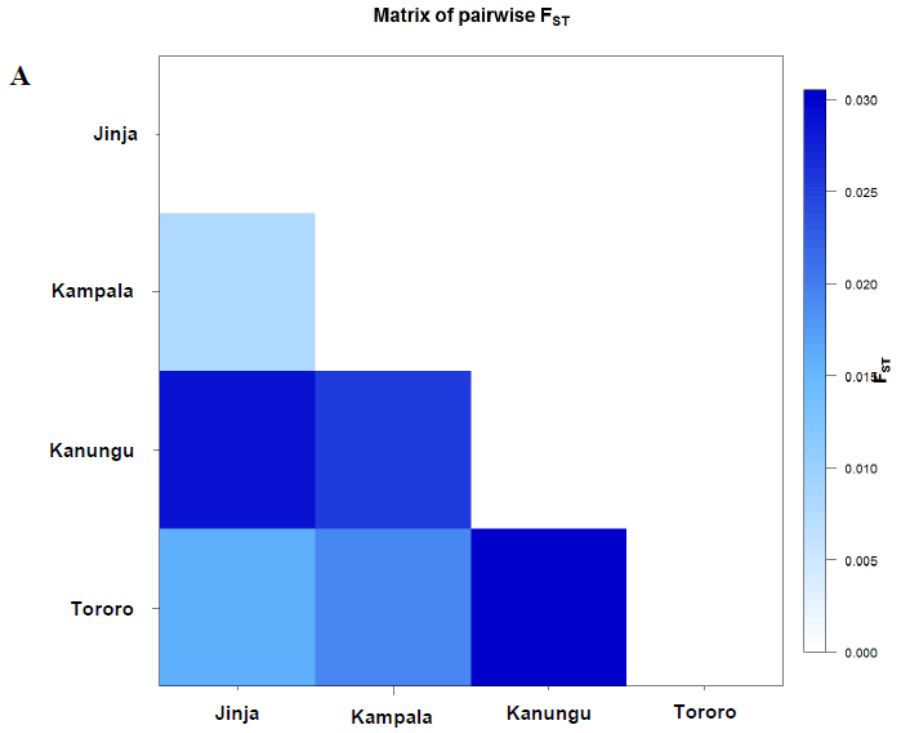
N, Sample size; Ne, Estimate of effective population size; H_E, mean expected heterozygosity; H_O, mean observed heterozygosity; F_{IS}, inbreeding coefficient; HW, Hardy-Weinberg equilibrium.
[a] Linkage disequilibrium method
[b]Number of loci showing departure from Hardy-Weinberg equilibrium after multiple test correction
*$P > 0.05$

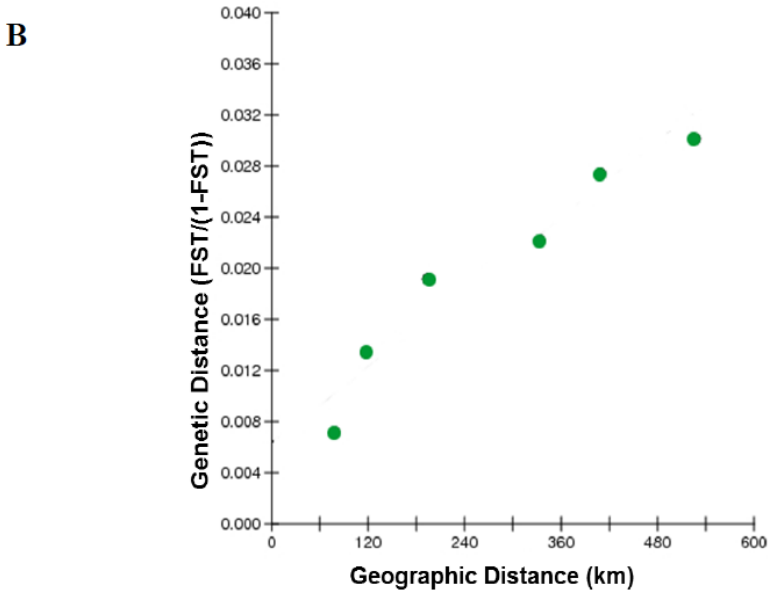
3.4.3.2 Population structure

Pairwise F_{ST} estimates were used to measure the genetic differentiation among the four Ugandan populations. The overall F_{ST} value was relatively low but statistically significant (F_{ST} = 0.019, P = 0.001), while the pair wise F_{ST} values between populations ranged from 0.007 to 0.029 (Figure 3.7A), with all figures significant at $P < 0.05$. Additionally, a pattern of migration positively correlated with the geographic distance between populations was detected (R^2 = 0.963, P = 0.047, Figure 3.7B).

The DAPC and Structure analysis (Appendix 3C) also indicated population structure, which grouped the four populations within three clusters, with individuals from Jinja and Kampala belonging to the same cluster, which was distinct from the other two samples (Figure 3.7C-D). AMOVA analysis carried out using the three geographic clusters indicated by the Bayesian and DAPC results showed that the majority of variation exists within individuals of (97.60%), while the variation among

geographic clusters and populations of the same cluster are 1.62% and 0.78% respectively.





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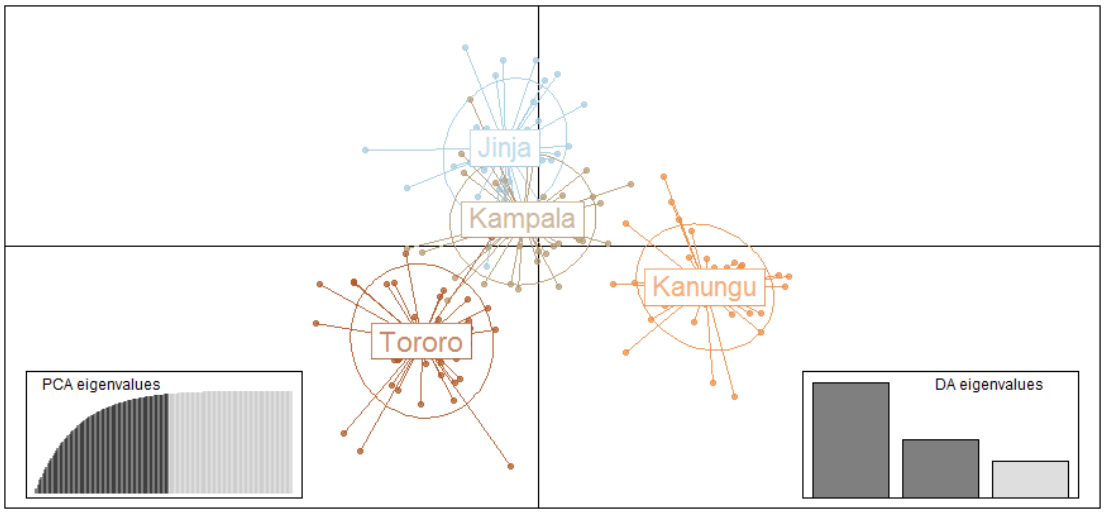
C



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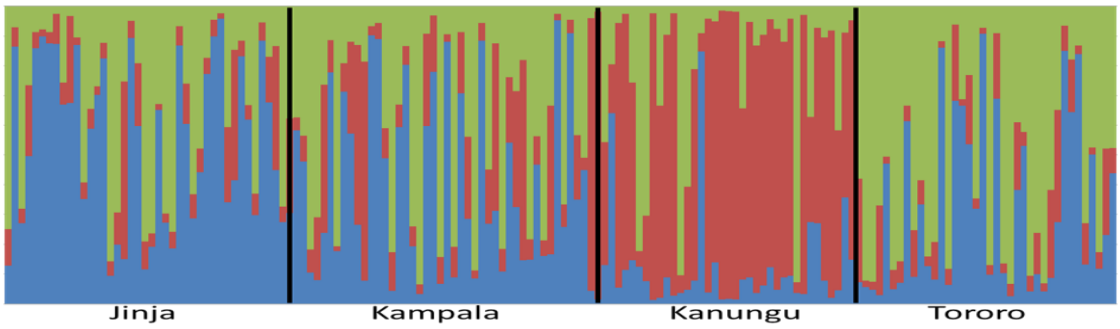


Figure 3.7: Genetic differentiation estimates among the four *C. quinquefasciatus* populations based on allele frequencies of 26 microsatellite markers. A) Pair wise *F*st matrix among the four populations B) Regression of population pairwise linearized *F*st values with geographic distances. C) First and second PCs of the DAPC. Inferred populations clusters are indicated by ellipses, which model 95% of the corresponding variability. D) Bayesian cluster analysis. Diagrammatic representation of population clusters for the most likely *K* (*K* = 3), where each vertical bar represents an individual and each colour represents the probability of belonging to one of the three clusters.

3.5 Discussion

3.5.1 Pattern of insecticide target-site mutations

Based on the assessment of the two target-site mutations, L1014F (*kdr*) and G119S (*Ace-1*), our data provides evidence of intense insecticide selection pressure on

C. quinquefasciatus populations across Uganda's Eastern-Southwest regions. Genotyping results indicated a very high frequency of both *kdr* and *Ace-1* resistant alleles (Figure 3.2), with the frequency of *kdr* significantly higher than *Ace-1* mutation in all populations. A higher frequency of *kdr* compared to the *Ace-1* resistance mutation has been also reported in *Culex* and other vector mosquitoes in the African continent (Corbel et al. 2007, Yewhalaw et al. 2011, Namountougou et al. 2012), which might reflect the historical application of DDT and pyrethroid insecticides for both vector and pest insect control or the deleterious effects of the 119S resistant allele in the absence of insecticidal pressure (Djogbénou et al. 2009, Sparks 2013, Hemingway 2014).

In Uganda, vector control interventions with pyrethroids, DDT and carbamate insecticides have been regularly applied through the Uganda National Malaria Control Program (UNMCP), which includes national scale distribution of ITNs and IRS in regions with a high malaria prevalence (Yeka et al. 2012). Additionally, these insecticides have been also applied for the tsetse and tick control to reduce sleeping sickness and tick-borne diseases transmission (Bardosh et al. 2013). Remarkably, a higher frequency of pyrethroid/DDT resistant markers (*kdr*) in our study has also been described for *Anopheles* populations from the same geographical regions (Ramphul et al. 2009, Verhaeghen et al. 2010).

Nevertheless, although previous studies have indicated a possible influence of control intervention (ITN and IRS) targeted at *Anopheles* on insecticide resistance in *C. quinquefasciatus* (Norris and Norris 2011, Jones et al. 2012b), with our study design it is not possible to directly associate the UNMCP intervention with the evolution of resistance in *Culex* populations. Furthermore, since insecticide is applied to control other NTDs as well as in agriculture practises and domestic use (Kotlyar 2010,

Wielgosz et al. 2014) in Uganda, pinpointing the likely insecticide exposure source and the impact on the evolution of resistance in the studied populations is difficult.

Interestingly, the *kdr* genotyping also indicated a high frequency of both variants (TTT and TTC) of the 1014F mutation in all populations, contrasting with previous reports on the geographic distribution of 1014F in *Culex* mosquitoes worldwide, which exclusively detect the TTT allele (Martinez-Torres et al. 1999, Sarkar et al. 2009, Ponce et al. 2015). To date, the TTC variant has been detected at high frequency only in *C. quinquefasciatus* from Sri Lanka, which was previously argued as the most likely geographic origin (Wondji et al. 2008), while only recently having been detected in African *C. quinquefasciatus* from Zanzibar yet at very low frequency (Jones et al. 2012b). The present data also show a significant difference in frequency of the *kdr* resistant alleles within populations, with the TTC variant being predominant in all populations with the exception of Kanungu. I do not have a conclusive explanation for the difference in frequency of TTT and TTC within Ugandan populations and between Uganda and other African populations.

Even so, at least two possible scenarios with relevance to resistance management and understating of evolution of resistance can be raised. First, the allelic frequency might reflect differences in allelic effect. Although in *C. quinquefasciatus* both 1014F alleles are synonymous, differences in frequency due to higher adaptability of one haplotype over the others cannot be discounted as previous work in *Musca domestica* shows difference in fitness advantage in distinct haplotypes bearing the same *kdr* mutations (Rinkevich et al. 2013b).

Another explanation involves a colonization process with genetic drift. Since the TTT allele has been detected worldwide (see reference (Martinez-Torres et al. 1999, Sarkar et al. 2009, Ponce et al. 2015) this may indicate an ancient origin/colonisation

or dispersion, which suggests that the TTC allele was introduced in Uganda from another geographical region with a large founder effect followed by genetic drift that shifted the TTT and TTC allele frequency, although a *de novo* origin and dispersion cannot be discarded. Additionally, it is also possible that the TTC allele arose on the African continent but has not been detected in other populations due to the *Culex* species *kdr* genotyping methodologies employed in previous studies.

Interestingly, *kdr* genotyping by pyrosequencing indicated that 6.25% of the 176 individuals genotyped displayed three alleles simultaneously at position 1014 (Figure 3.3) suggesting a duplication of the *Vgsc* gene in the studied populations. In *C. quinquefasciatus Vgsc* gene duplication was first reported in mosquitoes from the USA (Xu et al. 2011), while in this study analysis (see Chapter IV) using quantitative PCR methods to screen the same individual indicated the presence of copy number variation (CNV) for the *Vgsc* gene, with copy number ranging from two to four. Recently, *Vgsc* gene duplication was also detected in *A. aegypti* from Brazil, indicating that gene duplication in target-site genes might be a common event (Martins et al. 2013). Although detection of gene duplication for genes such as *Ace-1*, esterase and the resistance to dieldrin gene, *Rdl* (Labbe et al. 2007b, Djogbénou et al. 2009, Remnant et al. 2013) have already been demonstrated to be involved in insecticide resistance, a definitive association of *Vgsc* gene duplication with insecticide resistance is currently unproven.

Genotyping of the *Ace-1* mutation indicated a high frequency of resistant alleles in all four populations, with heterozygosity in Jinja and Kampala almost twice as high compared to the other populations. In no populations were homozygous resistant genotypes detected, while significant departures from Hardy-Weinberg was detected in all populations possibly due to the absence of one of the genotypic classes (Table 3.2,

Appendix 3D). The absence of homozygous *Ace-1* resistant genotypes was not surprising as the same pattern has been previously reported in both *Culex* and *Anopheles* populations (Djogbenou et al. 2008, Edi et al. 2014, Labbe et al. 2014). In both species, this pattern has been linked to the deleterious effects of the resistant homozygous genotype due to changes in acetylcholinesterase kinetic properties and mutation fitness cost in an insecticide-free or reduced insecticide exposure environment. However, the departure from Hardy-Weinberg in our surveyed Ugandan mosquitoes might not be linked exclusively to intensity of selection pressure, but could also reflect *Ace-1* gene duplication involving the wild type allele, thus creating a permanent heterozygosis to normalise ace-1 enzyme activity level (Kwon et al. 2010, Alout et al. 2011, Weetman et al. 2015). Indeed, our haplotype analysis identified mosquitoes harbouring *Ace-1* gene duplication in three out of 12 individuals investigated, with these mosquitoes exhibiting either three or five distinct haplotypes simultaneously.

3.5.2 Population structure and heterogeneous evolution of insecticide resistance

To infer the impact of indirect insecticide selection on genetic diversity and population structure in a non-target species such as *C. quinquefasciatus* mosquitoes from Uganda, we performed a population genetic study combining neutral markers (microsatellites) and genotype data from two markers (1014F and 119S) linked to resistance to four classes of insecticide applied in public health programs (Ffrench-Constant 2007, Rivero et al. 2010). The data described here indicated a contrasting genetic pattern between the microsatellite and insecticide selected markers. For both markers linked to resistance, it was not able to identify a clear spatial geographic spread, but did detect heterogeneity in allelic frequencies when comparing Ugandan

Central to Eastern and Southwest populations. By contrast, no significant difference in genetic diversity indices was observed from neutral markers among the populations.

The asymmetric distribution of insecticide resistant alleles strongly indicates that *C. quinquefasciatus* populations across this Eastern/South-West transect of Uganda are under heterogeneous insecticide selection pressure. This evidence is supported by the homogeneity of genetic diversity observed with neutral markers across the populations, suggesting that demographic effects, genetic drift, effective population size and migration are not the main factors driving the heterogeneous distribution of resistance alleles. The homogeneous microsatellite genetic diversity observed in the studied populations contrasts with an expected reduction in genetic diversity between populations under different strength of selection pressure as reported in *C. quinquefasciatus* from Recife, Brazil after vector control intervention with *Bacillus sphaericus*, a bio-larvicide (Cartaxo et al. 2011). As indicated by these authors, discrepancies between neutral and selected markers might be linked to differences in the marker's genetic properties that modulate changes in allele frequencies, including aspects such as quick recolonization and drastic bottleneck effects, difference in intensity of local selection with frequent gene flow or low impact of insecticide treatment on effective population size (Ne) (Kawecki and Ebert 2004, Paris et al. 2010).

Markedly, our data indicate that both the Tororo and Kanungu populations from outside the Central-Eastern region (based on four administrative regions in Uganda) have experienced a higher or longer-term selection pressure with pyrethroid insecticides and/or DDT than the other populations, as suggested by the fixed or close to fixation *kdr* mutation. On the other hand, the highest frequency of the *Ace-1* mutation was observed in a geographic distribution pattern opposite to that of *kdr*. Not

surprisingly, similar processes of local adaptation reflecting a heterogeneous evolution of insecticide resistance in sympatric populations have also been observed in all the main vector species worldwide (Sunil et al. 2013, Ambrose et al. 2014).

Interestingly, most studies reporting heterogeneous patterns of insecticide resistance at a micro-geographical scale have linked the local evolution of resistance to challenges for implementation of efficient control intervention in highly fragmented habitats including overlapping of urban and rural patches or due to heterogeneous urbanization structure, which could result in a mosaic of landscapes with sharp shifts of insecticide selection pressure in local and metapopulation levels (Pocquet et al. 2013, Yadouleton et al. 2015).

The different distribution of *kdr* alleles between the districts might reflect the combination of approaches to reduce *Anopheles* populations, which include national distribution of ITN and IRS only in highly endemic and epidemic-prone regions. For example, across Uganda irregular spraying with insecticides such as lambda-cyhalothrin, DDT and alpha cypermethrin has been reported since 2006 when IRS was re-introduced for vector control (Yeka et al. 2012). Although equally distributed in urban and rural regions, differences in usage of ITN might also be linked to the pattern of *Culex kdr* distribution as indicated by the Uganda Bureau of Statistics (Kudom et al. 2015), which shows householders that own at least one ITN differ for example by 44% between the East Central and West Nile regions. Additionally, it is also important to consider that in non-urban districts, other sources of insecticide exposure due to agriculture practices, domestic usage as reported in other studies (Nalwanga and Sempebwa 2011, Uganda Bureau of Statistics 2012) or the concomitant tsetse fly vector control intervention in rural Uganda (Bardosh et al. 2013) might also play an important role in the evolution of *C. quinquefasciatus* resistance.

Surprisingly, the data from the present study also identifies a widespread distribution of the *Ace-1* G119S mutation, with a higher frequency in mosquitoes from the Uganda Central region. The high frequency of the *Ace-1* allele strongly indicates that the mosquitoes collected in our study have been exposed to organophosphates and/or carbamates insecticides, possibly for purposes other than public health as no official intervention with insecticides from these classes for vector control were conducted in the studied districts. For example, in *C. quinquefasciatus* populations from Ghana and Benin, the high frequency of *Ace*-1 was attributed to the use of these insecticides by farmers for pest control or pollutants in mosquito breeding sites (Pocquet et al. 2013, Yadouleton et al. 2015).

Alternatively, the high frequency of *Ace-1* mutation might be linked to the replacement of pyrethroids and DDT for carbamate IRS throughout Ugandan Northern regions in 2010, which reduced malaria transmission and indicated that *Anopheles* populations were susceptible to bendiocarb (Yeka et al. 2012). Although it is not possible make a direct link to the start of bendicoarb IRS action with the high frequency of *Ace*-1 mutation in *C. quinquefasciatus* from Kampala and Jinja, an increase of *Ace-1* resistant allele in these districts, driven by migration of bendiocarb-resistant mosquitoes from North to Central Uganda, is also a feasible possibility.

In spite of probable intense gene flow between populations supported by the low pairwise F_{ST} observed, our data indicated the populations are structured in three demes. Certainly, the population structure observed might reflect our study design with asymmetric geographic distance between collections points, which could result in the very strong pattern of isolation-by-distance (IBD) detected. Nevertheless, it is important to notice that factors other than geographic distance, such as environmental heterogeneity and associated selection as well as serial sequential founder effects, may

also cause similar patterns of spatial autocorrelation (Orsini et al. 2013). For example, in *Aedes rusticus*, comparisons among areas treated and not-treated with *Bacillus thuringiensis israelensis* (Bti) showed more intense IBD among treated than among non-treated sites (Nabyonga et al. 2013). These authors argue that the difference in observed IBD instead of spatial distribution could result from differences in population size after insecticide application or lower migration capability of selected mosquitoes imposed by a fitness cost of the resistance mechanism. Consequently, it is also possible that the structured distribution observed in Ugandan populations might reflect the impact of heterogeneous insecticide selection pressure and not necessarily the geographic distribution as the closest populations (Jinja and Kampala) show higher frequencies of the *Ace-1* mutation whereas the most distant (Kanungu and Tororo) have the higher frequency of *kdr* resistant alleles.

3.6 Conclusion

Taken together the genetic diversity of neutral markers and two insecticide resistance target-site markers shown in this study provide evidence that despite the absence of a direct vector control intervention targeting *C. quinquefasciatus* populations in Uganda, those populations have been submitted to an intense or long term insecticide selection evidenced by a high frequency of 1014F and 119S resistant alleles. These results are a warning of the importance of monitoring the evolution of resistance in non-target mosquito populations in areas with frequent usage of insecticide, as increase of resistance in vector species with secondary importance might also contribute to increased transmission burden of vector-borne disease and nuisance biting. In light of the unstructured distribution of insecticide resistance alleles despite the likely high gene flow and low mosquito population differentiation, the

population genetics study also suggests that *C. quinquefasciatus* populations from different Ugandan districts are under heterogeneous selection pressure imposed by insecticides from distinct classes with variation in the level of exposure creating a pattern of local adaptation. Consequently, the results shown here indicate that public health or agriculture insecticide usage could be driving the evolution of resistance in non-target species such as *C. quinquefasciatus* in Uganda, thus representing a threat to the local burden of transmission of vector-borne disease. Lastly, the results also demonstrate that combining both newly resistant marker assays and the developed microsatellite multiplex-panel is an applicable tool for detecting and monitoring the pattern of local evolution of resistance in *C. quinquefasciatus* under control interventions, which could provide new insight for developing more specific and effective control programs.

Chapter IV

Comparative Analysis of PCR-based Methods for Inference of Copy Number Polymorphism in the Voltage-gated Sodium Channel Gene of *Culex quinquefasciatus* Mosquitoes

4.1 Abstract

Although, the evolution of insecticide resistance in insects has mostly been associated with variation in the gene(s) encoding the insecticide target-site or alterations in detoxification gene expression, in mosquitoes of the *Culex pipiens* complex in many instances the genetic basis of resistant phenotypes have also been related to an increase in copy number of insecticide-resistance associated genes. Recently, in *Culex quinquefasciatus* mosquitoes it was reported that there is a variation in copy number of the para-type sodium channel genes (*Vgsc*), which is the target-site for the pyrethroids and organochlorines insecticides. For further investigation of the *Vgsc* gene copy number in *C. quinquefasciatus*, in the present study a novel *Vgsc*-copy number (CN) assay was designed and applied to infer the *Vgsc* gene CN in Ugandan field-collected mosquitoes. Additionally, the applicability of distinct genotyping platforms and efficiency of the *Vgsc*-CN assay were assessed by comparing the predicted CN of mosquitoes genotyped through three platforms and four different methods: standard quantitative PCR (qPCR) using absolute quantification (qPCR-Std) and ΔΔCt method, pyrosequencing by RQPS method and droplet digital PCR (ddPCR) through absolute quantification. Quantification of the *Vgsc* copy across all platforms and methods indicated the presence of CN variation in around 10% of the mosquitoes assayed, with variation in CN corresponding to three or four copies per diploid genome. Under the experimental conditions applied herein, the ddPCR and Real-time PCRs methods performed more precisely and yielded similar prediction of the *Vgsc* CN, while the lowest concordance among all methods was observed for the RQPS methods. Hence, due to evident variation in copy number of the *C. quinquefasciatus Vgsc* gene, the developed and validated *Vgsc*-CN assay described here will facilitate the monitoring of the *Vgsc* gene CN in wild populations as well as can provide a greater opportunity for future studies to elucidate associations between the *Vgsc* CN and level of insecticide resistance.

4.2 Introduction

The evolution of insecticide resistance involves a complex interaction of many types of genetic variation; although known resistance mechanisms in mosquitoes are associated mainly with variation in the gene(s) encoding the insecticide target-site, or alterations in detoxification gene expression (Corbel et al. 2007, Berticat et al. 2008). Nevertheless, multiple studies have now shown that gene duplication can also play a very important role in the evolution of insecticide resistance (Kwon et al. 2012, Harrop et al. 2014, Shang et al. 2014).

Evolution of insecticide resistance through changes in gene copy number in contrast to others sources of genetic variation such as single nucleotide polymorphisms (SNPs), can drastically increase gene divergence and function by modifying gene structure, gene dose and expression level of genes linked to resistance (Kondrashov et al. 2002, Zhong et al. 2013, Zhao et al. 2014c). Gene duplication or deletion are also important sources of intraspecific diversity. One such example is through copy number variation (CNV), also known as copy number polymorphism (CNP) as detected in frequency >1% at population level, which encompasses insertion/deletion of genomic regions >1Kb (Schrider and Hahn 2010, Severson and Behura 2012).

Previous studies in organisms including humans, insects and plants have revealed links between CNV and phenotypic variation; ranging from localised plant adaptation; to a wide array of human conditions and diseases such as schizophrenia and intellectual disability (Langer et al. 2014, Mikhail 2014, Mukherjee et al. 2015). In the silkworm *Bombyx mori in silico* analysis indicated that 1.4 % of the duplicated genome, included genes associated with immunity, detoxification and reproduction (Zhao et al. 2013). In mosquitoes, species of the *Culex pipiens* complex provide well-characterized examples of how CNVs can be associated with adaptations to insecticide pressure. For example, amplification of the carboxylesterase alleles A2, B2, A5 and B5 has been associated with resistance to organophosphates through

elevated expression and insecticide detoxification (Coleman and Hemingway 1997, Buss and Callaghan 2004). Additionally, duplication of the *Ace-1* locus has been linked to organophosphate and carbamate insecticide resistance in both *Culex pipiens* (Labbe et al. 2007a) and *Anopheles gambiae* (Weetman et al. 2015). The duplication brings a wild-type and resistant allele onto the same chromatid and is thought to partially compensate for the deleterious effects of resistant alleles in the absence of insecticide (Kondrashov and Kondrashov 2006, Kondrashov 2012, Long et al. 2013).

C. quinquefasciatus, a mosquito with a broad distribution in tropical and subtropical regions is the main vector of lymphatic filariasis as well as West Nile virus (WNV) and St. Louis encephalitis virus (SLEV) (Kramer et al. 2008, Ichimori et al. 2014). Recently, variation in the copy number of the para-type sodium channel genes (*Vgsc*) has been described in field-collected mosquitoes from the United States of America, using Southern blot and PCR methods (Xu et al. 2011). The results of this study indicated that the *C. quinquefasciatus* genome contains at least two copies of the sodium channel gene. Although CNV has been described for both target-site and metabolic genes and is associated with resistance to insecticides, readily applicable molecular diagnostic tests to investigate the occurrence and evolution of CNV in natural populations is lacking (Santolamazza et al. 2008).

Discovery methods for identifying CNVs such as microarrays and next-generation sequencing are laborious (Alkan et al. 2011), which limits the identification and development of diagnostic methods. Nevertheless, developing high-throughput and cost effective PCR-based approaches for large scale population genotyping will become imperative for monitoring and elucidating the role of CNV in the evolution of resistance and on vector control.

In the present study, we developed and validated a new *Vgsc*-CNV PCR-based assay to score the number of copies of the *Vgsc* gene in natural populations of *Culex* mosquitoes. Four *Vgsc*-CNV assays were designed for three distinct genotyping platforms: standard quantitative

PCR (qPCR) and pyrosequencing, both commonly applied for genotyping SNPs, as well as the newly developed droplet digital PCR (ddPCR) platform (Pinheiro et al. 2012). Additionally, the *Vgsc*-CNV assay sensitivity and accuracy was compared across the three platforms by genotyping field-collected mosquitoes from Uganda.

4.3 Material and methods

4.3.1 Sample collection and DNA isolation

Indoor resting adult *C. quinquefasciatus* mosquitoes were collected from four Ugandan towns/cities: Jinja, Kampala, Kanungu and Tororo (see Chapter III, Figure 3.1) between June and July 2012. Adults were sexed using antenna morphology with only males selected to characterize the *Vgsc* gene dose since gravidity in females can affect CN estimation. Samples were stored on silica gel prior to DNA isolation using a DNeasy kit (Qiagen) following the manufacturer's recommendations. DNA concentration from each mosquito was quantified by PicoGreen (Life Technologies) and then normalized to approximately 10 ng/µl. Before CN analysis all adult mosquitoes were confirmed as *C. quinquefasciatus* by a diagnostic PCR method (Smith and Fonseca 2004).

4.3.2 Characterization of *Vgsc* haplotype diversity

4.3.2.1 Kdr mutation (L1014F) allelic discrimination assays

Two assays to genotype the L1014F *kdr* mutations in exon 20 of the *Vgsc* gene (see below), which has been implicated in resistance to pyrethroids and organochlorine insecticides were designed and applied in parallel to detect two non-synonymous variants; one to genotype TTA/TTT alleles and the other to detect TTA/TTC variants. Primer sets and TaqMan probes were designed using the Custom TaqMan® Assay Design Tool (Life Technologies).

TaqMan allelic discrimination reactions were carried out using approximately 20 ng of gDNA, 1X SensiMix II probe (Bioline), 0.4 µM of each primer (*Kdr*-F: 5`-CTTGGCCACCGTAGTGATAGG-3`and *Kdr*-R: 5`-GCTGTTGGCGATGTTTTGACA-3`) and 0.1 µM of each probe (Probe-TTC-allele: 5`- FAM-CACGACGAAATTT-3` or Probe-TTT-allele: 5`-FAM-TCACGACAAAATTT-3` and Probe-TTA-allele/wildtype: 5`-VIC-ACTCACGACTAAATTT-3`), in a final volume of 10 µl. The PCR was performed on a Stratagene MX3005P with cycling parameters of 95 °C for 10 min followed by 40 cycles of 95 ºC for 10 sec and 60 ºC for 45 sec.

4.3.2.2 Haplotype diversity characterization

To investigate the haplotype diversity and the number of distinct haplotypes present in each individual, a partial fragment of approximately 676 base pairs (bp) of the *Vgsc* gene spanning intron 19 and exon 20 including the position of the *kdr* mutations (L1014F) originally described in houseflies (Williamson et al. 1996) and then other insects (O'Reilly et al. 2006) was used. Identification of CN using haplotype diversity assumed that each individual mosquito carrying >2 distinct haplotypes exhibited copy number variation, as described by Labbé (Labbe et al. 2007a). The number of distinct haplotypes per individual was characterized by cloning and sequencing eight clones of the PCR per individual.

The partial fragment of the *Vgsc* gene was amplified by PCR in a reaction volume of 25 µl including approximately 25 ng of gDNA, 1X Phusion HF buffer, 200 µM of each dNTP, 0.02 U/µl of Phusion Hot start II DNA polymerase and 0.4 µM of each specific primer *Vgsc*-F: 5`-CCTCCCGGACAAGGACCTG-3` and *Vgsc*-R: 5`-GGACGCAATCTGGCTTGTTA-3`. Amplification was performed with cycling conditions of 98 ºC for 30 sec, followed by 30 cycles of 98 ºC for 10 sec, 56 ºC for 15 sec and 72 ºC for 15 sec. with a final extension of 72 ºC for 10 min. PCR products were purified using the GeneJet PCR purification kit (Thermo Scientific)

and cloned into the pJet 1.2 vector using the CloneJet PCR cloning kit (Thermo Scientific). Individual plasmids were isolated using the GeneJet Plasmid Mini Kit and sequenced (Source Biosciences).

Sequence traces were edited in CodonCode Aligner software version 4.2.2. Multiple sequence alignments were performed with ClustalW and then visualized using Jalview software (Waterhouse et al. 2009). Haplotype diversity was visualized using a Neighbour-Joining tree build using the software MEGA 5.1 (Tamura et al. 2011) with frequency and relationships between haplotypes were visualized by a haplotype network generated using the program TCS version 1.21 (Clement et al. 2000).

4.4.3 *Vgsc* gene CN assignment by PCR-based assay

The *Vgsc*-CN PCR-based methods described here were designed to perform on three platforms using four distinct CN calculation methods as described in Figure 4.1. The CN assessment is based on a partial fragment of exon 20 of the *Vgsc* gene (CPIJ007595-RA) normalized to a fragment of exon 1 of the *cAMP-dependent protein kinase A (Pka)* gene (CPIJ018257-RA), a single copy housekeeping gene in the *Culex* genome (endogenous control).

The assays based on real-time and ddPCR platforms employed a TaqMan-CNV method, which consists of a duplex PCR reaction using a pair of unlabeled primers for each gene and a FAM-MGB probe for the *Vgsc* gene and a VIC-MGB probe for the reference gene (*Pka*). The CN quantification by pyrosequencing was conducted using the Reference Query Pyrosequencing (RQPS) method described by Liu et. al (Liu et al. 2010) with minor modifications. Briefly, the *Vgsc*-RQPS method utilizes an engineered plasmid (probe) encompassing a 100 bp fragment from both the *Vgsc* and *PKA* genes linked to any gene fragment (stuffer DNA) with no homology to the reference or query gene. On each fragment a

SNP was introduced that differs between the RQ-probe allele and the gDNA allele. gDNA of each mosquito with unknown CN was mixed with the RQ-probe and then co-amplified in a simplex PCR reaction for each *Vgsc* and *Pka* gene followed by pyrosequencing analysis.

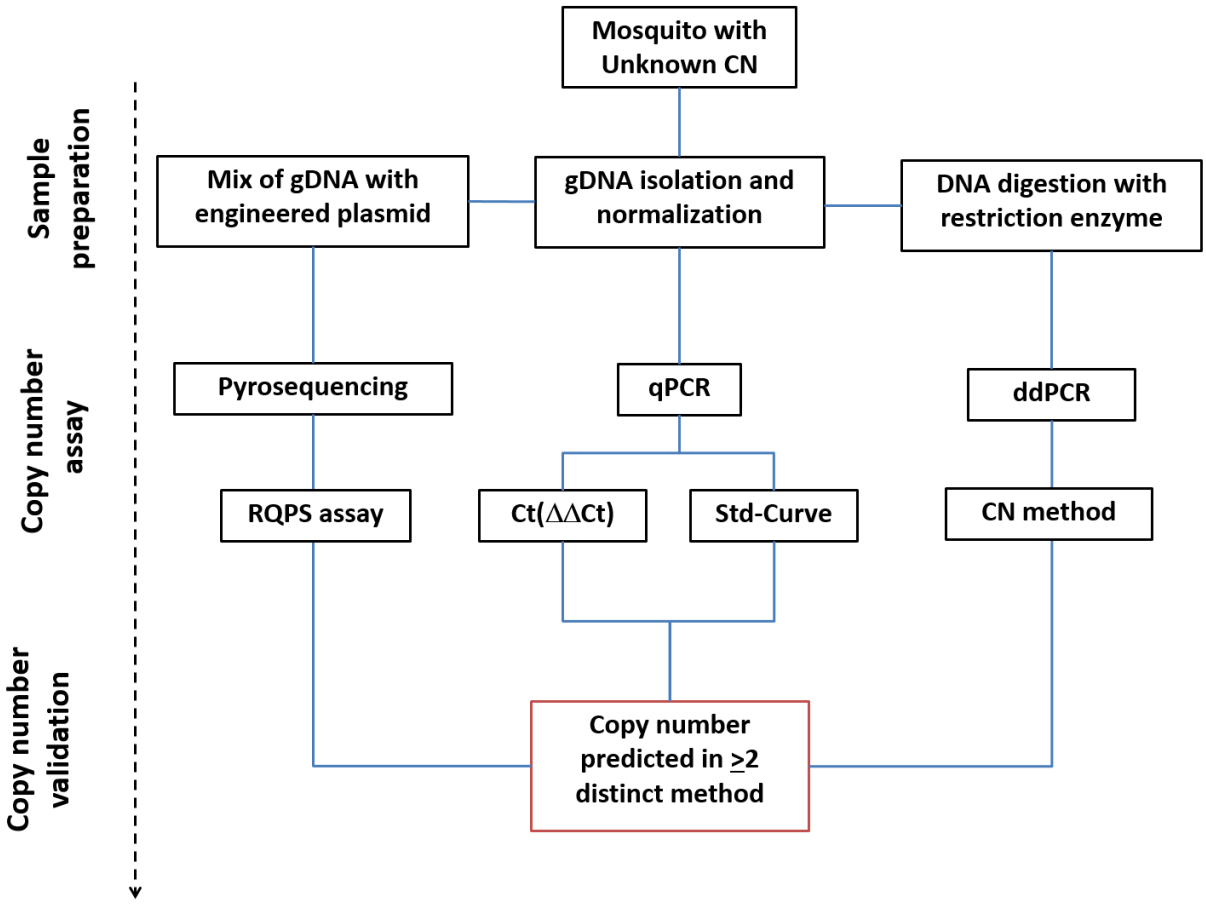


Figure 4.1: Schematic depicting the different approaches applied for genotyping and validation of the *Vgsc* gene copy number in *C. quinquefasciatus* mosquitoes. qPCR-Std-curve and ddPCR-CN method applied an absolute quantification measurement, while qPCR-ΔΔCt and pyrosequencing- RQPS use a relative CN quantification. RQPS; Reference Query pyrosequencing and ddPCR Droplet Digital PCR. Ct, intersection between an amplification curve and a threshold line.

4.4.3.1 *Vgsc* -CN primer design and validation

All primer and probe binding sites of the exon 20 of the *Vgsc* gene were selected using the sequence alignment from the haplotype diversity analysis to identify conserved regions (Figure 4.3). *Vgsc*-CN assay primers and probes used on the qPCR and ddPCR assay were

designed using the primer express version 2.0 Software (Applied Biosystems). Primers and probes for the *Vgsc* gene were: *Vgsc*/CN-F: 5`-TGCCACGGTGGAACTTCA-3`; *Vgsc*/CN-R: 5`-CACCCGGAACACGATCATG-3`; *Vgsc*/CN-Probe:5`-FAM-GACTTCATGCACTCAT-MGB-3`, while for the *PKA* reference were: *PKA*/CN-F: 5`-GACTGGTGGGCATTAGGTG TTC-3`; *PKA*/CN-R: 5`- TCAGCAAAAAAAGGTGGATATCC-3`; Probe: 5`-VIC-GTGTACGAGATGGCAGC-MGB-3`.

For the pyrosequencing assay, PCR primer sets and sequencing primers that co-amplify the genomic and RQ-probe sequences for either *Vgsc* and reference gene were also designed using the PyroMark assay design software 2.0 (Qiagen). For the *Vgsc* gene, PCR reactions were performed using the primers: *Vgsc*/Py-F: 5`-CGAATCCATGTGGGACTGC–3` and *Vgsc*/Py-R: 5`Biotin- CTATCACTACGGTGGCCAAGAAGA-3`, whereas for the *PKA* gene the primers used were: *PKA/*Py-F: 5`-GGAAACAACGCAACTTCAACA-3` and *PKA/*Py-R: 5`Biotin- TCTTCTTTAGCTTGATCCAGGAAT-3`.

The efficiency of primers and probes designed for qPCR and ddPCR were determined by using a standard curve for three replicates across five doubling dilutions from an initial concentration of approximately 20 ng/µl of gDNA. Primer specificity was tested by melt curve and electrophoresis on a 2% agarose gel. Duplex-PCR reaction conditions were experimentally determined by primer-limiting analysis to identify the optimal primer and probe concentrations that provide a constant Ct value (threshold cycle) among primer/probes titration with primer efficiency on duplex-PCR reaction not differing by more than 5%.

4.4.3.2 Copy number assignment using qPCR

Absolute and relative quantification methods were used in parallel to quantify the *Vgsc* CN. For both quantification methods qPCR reactions were performed in triplicate in a final volume of 20 µl including around 10 ng of genomic DNA, 1X TaqMan gene expression master

mix (Applied Biosystems), 0.4µM and 0.2 µM of each primer and probe as described previously. Two samples assayed earlier were used as positive controls of PCR reproducibility. Amplification was conducted using the Applied Biosystems 7500 Fast PCR-Real time systems with the following condition: 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 94 °C for 15 s and 60 °C for 1 min.

For absolute quantification, a plasmid containing the sequences spanning primer and probe binding sites for both genes used in the qPCR assay was created (see appendix 4-A). The purified plasmid concentration was measured using picogreen and then a 10-fold serial dilution ranging from 3 x 10^5 to 10^1 copies/µl of the *Vgsc-Pka* plasmid DNA was used to generate standard curves by plotting C_t values versus log copies for both *Vgsc* and *Pka* gene. Absolute copy number was calculated by determining the number of *Vgsc* and *Pka* copies per haploid genome interpolated from the standard curve for each sample and then the ratio (*Vgsc* /*Pka*) of copies/µl was multiplied by two to obtain the diploid genome CN. To increase the precision of the quantification, plates were used where the standard curve had $R^2 \geq 0.98$. The relative quantification between the *Vgsc* and *Pka* gene was assessed based on Ct values collected using a 0.2 threshold and automatic baseline. The CN analysis was carried out using the CopyCaller software v2.0 (Applied Bisystems), which applies a comparative (ΔΔCt) method.

4.4.3.3 Copy number assignment by ddPCR

For the ddPCR assay, roughly 10 ng of gDNA was digested with 0.2 units of *Alu*I (NEB) for 15 min at 25 ºC. *Alu*I was selected since its restriction sites were identified nearby the upstream and downstream position of the PCR primers for both the *Vgsc* and reference gene. Digested gDNA was assayed in a duplex ddPCR reaction in a final volume of 20 µl including: 1X ddPCR supermix and 0.4 µM of each primer and 0.2 µM of each probe described in the primer design section. The total volume of each ddPCR PCR mix was transferred to the sample

wells on the eight-channel droplet generator cartridge (Bio-Rad) while 70 µl of droplet generation oil (Bio-Rad) were loaded on each oil well channel. Lastly, 40 µl of the partitioned droplet PCR mix were transferred to a 96-well plate and then amplified to end point using a thermal cycler. The amplification condition was determined by serial dilution of the *Vgsc -Pka* plasmid DNA to identify the required input gDNA concentration while a temperature gradient ranging from 55 ºC to 65 ºC was conducted to detect assay amplitude with a well-defined separation between positive and negative droplet populations (Appendix 4-B).Thermal cycling conditions were: 95 ºC for 5 min, 95 ºC for 30 sec and 57 ºC for 1 min (40 cycles) and 98 ºC for 10 min.

After PCR amplification, the PCR product was loaded on the QX100 droplet reader (Bio-Rad), for simultaneous two-colour detection of the droplets. Data analysis of the ddPCR reads was carried out using QuantaSoft analysis software version 1.6.6 (Bio-Rad). Absolute quantification of the *Vgsc* gene CN for each sample was then calculated in reference to the *Pka* gene event number.

4.4.3.4 Copy number assignment using Pyrosequencing

Relative quantification analysis by the *Vgsc*-RQPS method required the construction of a plasmid, here denominated as RQ-probe, which contained partial sequences of the *Vgsc* and *Pka* gene with an introduced SNP for differentiating RQ-probe alleles from gDNA alleles. The RQ-probe design, cloning and purification details are described in the appendix 4-C.

For each sample tested, two mixtures of RQ-probe/gDNA were prepared using molar ratios of 1:1 and 2:1 in a final volume of 10 µl. Simplex PCR reactions for the *Vgsc* and *Pka* gene were performed in a total of 25 µl using 3 µl of each RQ- probe/gDNA molar ratio mix in parallel, 200 µM of each dNTP, 1X of the 10X PCR buffer, 2.0 mM of $MgCl_2$, 0.6 units of HotStarTaq DNA polymerase (Qiagen) and 0.4 µM of each primer described previously. After

initial denaturation at 95 °C for 15 min, PCR was performed for 40 cycles of 94 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 30 sec, followed by a final extension step at 72 °C for 10 min.

Single-stranded PCR products for analysis by pyrosequencing were obtained using the PyroMark Q24 Vacuum Prep Workstation. Pyrosequencing reactions of the *Vgsc* PCR products were performed using the sequencing primer: 5`-TGCTGGTGGGCGACG-3` and dispensation order: 5`-GTGATCTG-3`, whereas for the *Pka* PCR amplification used the sequencing primer: 5`-CCGCAGAAAGTGTAAAA-3` and the following dispensation order: 5`- TCGATCTG-3`. Pyrosequencing reactions were performed using the PyroMark Gold Q96 reagent kit (Qiagen) following the manufacturer's guidelines.

CN prediction was calculated comparing the amplification ratios of the *Pka* reference gene (RQprobe-*Pka*/ gDNA-*Pka*, alleles) and *Vgsc* (RQprobe- *Vgsc* / gDNA- *Vgsc*, alleles) by linear regression, with differences of the amplification ratios reflecting variation of gene copy number. The linear regression for the slope of the curve was multiplied by two to acquire the predicted CN in the diploid genome. Further details of the data analysis are described by Liu et al. (Liu et al. 2010).

4.4. Results

4.4.1 *Vgsc* gene haplotype diversity and genotype constitution

The potential for a gene duplication event in the *Vgsc* gene in Ugandan *C. quinquefasciatus* mosquitoes was suggested by abnormal TaqMan genotyping results for the 1014F mutation located in exon 20. Two parallel assays for detecting the 1014F mutations were designed to genotype the wild type codon (TTA) and the two alternative resistant codons, (TTT or TTC), which both result in a pyrethroid and DDT resistance associated change from Leucine to Phenylalanine (Wondji et al. 2008, van den Berg 2011).

The TaqMan applied two fluorogenic probes that produce a fluorescent signal proportional to the number of allele specific SNPs amplified in the qPCR reaction. In diploid individuals with single copy loci individuals may be assigned to one of three clusters of fluorescence: one for each homozygous genotype and the other for heterozygotes. However, in this study genotyping of approximately 190 mosquitoes showed the presence of four well separated clusters instead of the normal three. We assumed that the additional cluster represented two populations within the heterozygous cluster (Figure 4.2A). We hypothesized that the asymmetry within the putative heterozygous cluster may be due to gene duplication, which causes a shift in the fluorescence ratio between the FAM/ VIC probes since individuals with CNV can possess >2 alleles.

To further investigate if the TaqMan results were linked to variation in primer/probe sequence binding sites or due to variation in the number of alleles, we carried out a haplotype diversity analysis using 68 sequences from 12 individuals (GenBank accession numbers: KR061912-KR061979) of a partial (677bp) fragment of the *Vgsc* gene. Sequence analyses indicated the occurrence of five distinct haplotypes over all samples with frequencies ranging from 0.073 to 0.456 based on 18 segregating sites (Figure 4.2B). Haplotype C, which displays the (TTC) 1014F mutation was observed at the highest frequency (46%). The haplotype network also indicated a possible two origins for the TTT mutation due to the presence of five mutation between the two TTT resistant haplotypes (Hap A and Hap B), which surprisingly was observed in little variation in the wildtype alleles, suggesting a selective sweep in the wildtype possibly driven by the independent L-F duplication events.

Haplotype analysis indicated no variation in the binding site of the primer/probes sequence in the mosquitoes studied (Figure 4.3). Moreover, haplotype analysis indicated that the individual Cx8 possesses three distinct haplotypes; one for the resistant allele TTT and one for the TTC (Figure 4.2C), which supports the hypothesis that gene duplication can exist for

the *Vgsc* gene in Ugandan Culex mosquitoes. Additionally, this analysis also shows that there are many distinct possible genotype combinations for the five haplotypes identified, with one individuals (Cx3) having two haplotypes for the TTA susceptible allele (Figure 4.2C).

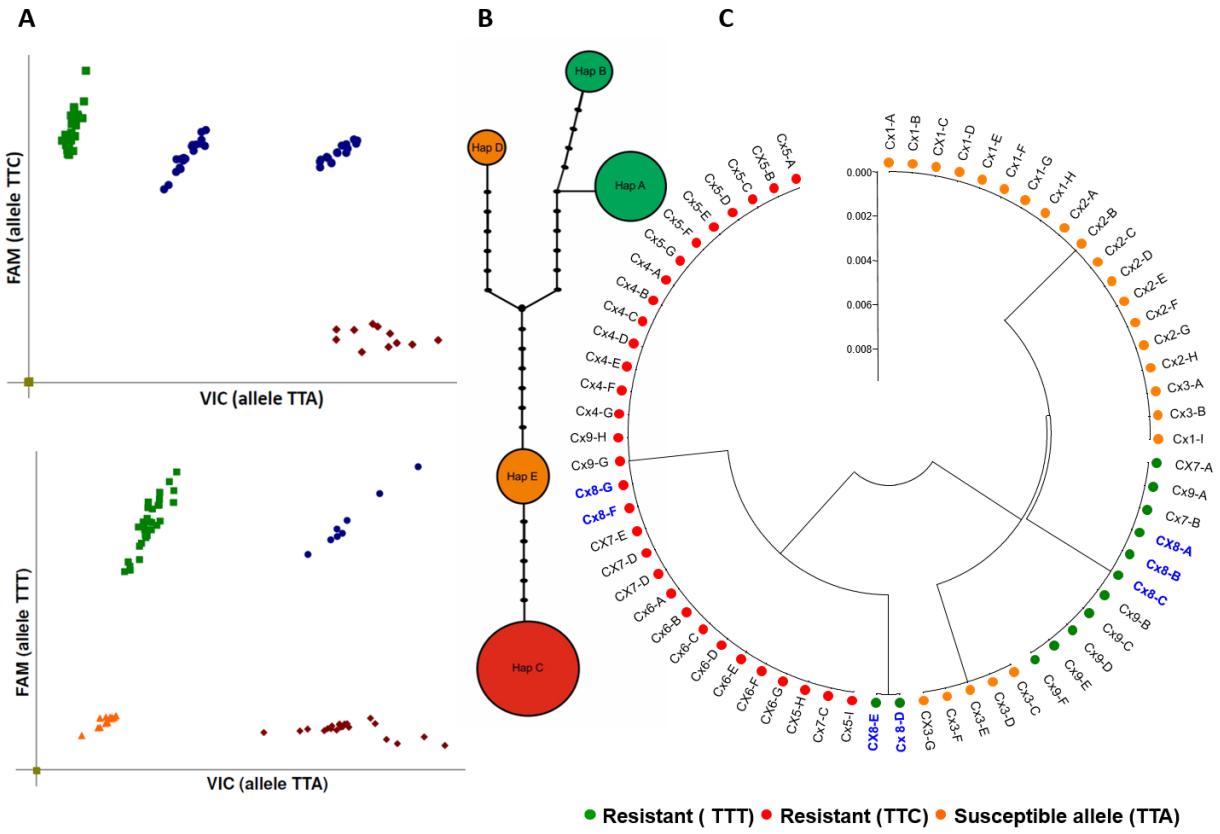
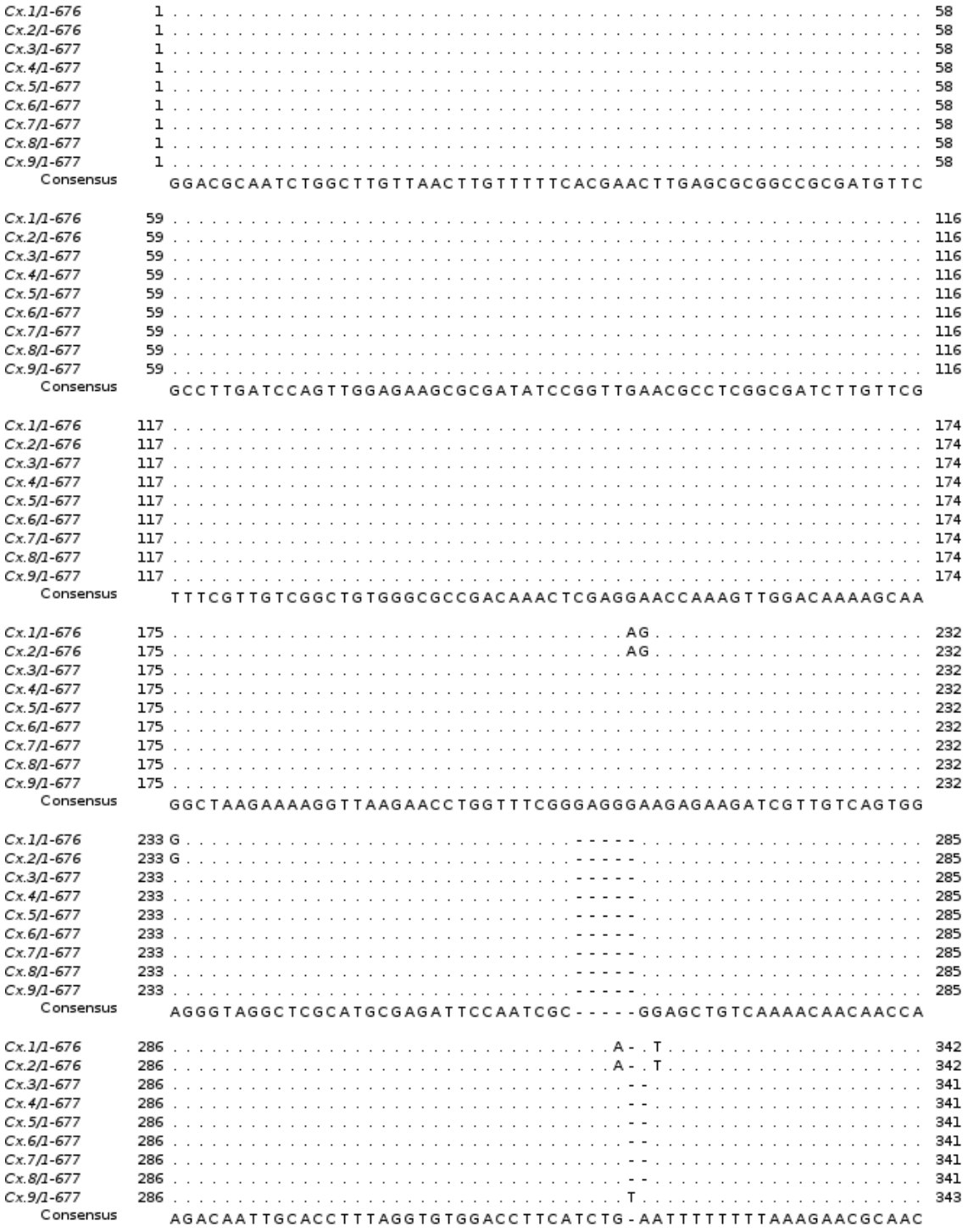


Figure 4.2: Haplotype diversity based on partial sequence of the *Vgsc* gene from Ugandan *C. quinquefasciatus* A) Scatter plot of TaqMan-based allelic discrimination of the TTA/TTC codons for 1014F mutation B) Haplotype network of the *Vgsc* gene. Circles denote the relative number of samples represented in each haplotype. The branches between black dots represent mutational steps separating observed haplotypes. C) Dendrogram of individuals with a likely duplication of the *Vgsc* gene. (Cx number) corresponds to an individual and the following letter from A to I means the number of distinct colony sequenced per sample. Individuals highlighted in blue indicate mosquito with more than two haplotypes. Green, red and orange dots correspond distinct alleles for the mutation 1014F.


Cx.1/1-676 1 58
Cx.2/1-676 1 58
Cx.3/1-677 1 58
Cx.4/1-677 1 58
Cx.5/1-677 1 58
Cx.6/1-677 1 58
Cx.7/1-677 1 58
Cx.8/1-677 1 58
Cx.9/1-677 1 58
Consensus GGACGCAATCTGGCTTGTTAACTTGTTTTTCACGAACTTGAGCGCGGCCGCGATGTTC
Cx.1/1-676 59 116
Cx.2/1-676 59 116
Cx.3/1-677 59 116
Cx.4/1-677 59 116
Cx.5/1-677 59 116
Cx.6/1-677 59 116
Cx.7/1-677 59 116
Cx.8/1-677 59 116
Cx.9/1-677 59 116
Consensus GCCTTGATCCAGTTGGAGAAGCGCGATATCCGGTTGAACGCCTCGGCGATCTTGTTCG
Cx.1/1-676 117 174
Cx.2/1-676 117 174
Cx.3/1-677 117 174
Cx.4/1-677 117 174
Cx.5/1-677 117 174
Cx.6/1-677 117 174
Cx.7/1-677 117 174
Cx.8/1-677 117 174
Cx.9/1-677 117 174
Consensus TTTCGTTGTCGGCTGTGGGCGCCGACAAACTCGAGGAACCAAAGTTGGACAAAAGCAA
Cx.1/1-676 175 AG 232
Cx.2/1-676 175 AG 232
Cx.3/1-677 175 232
Cx.4/1-677 175 232
Cx.5/1-677 175 232
Cx.6/1-677 175 232
Cx.7/1-677 175 232
Cx.8/1-677 175 232
Cx.9/1-677 175 232
Consensus GGCTAAGAAAAGGTTAAGAACCTGGTTTCGGGAGGGAAGAGAAGATCGTTGTCAGTGG
Cx.1/1-676 233 G ----- 285
Cx.2/1-676 233 G ----- 285
Cx.3/1-677 233 ----- 285
Cx.4/1-677 233 ----- 285
Cx.5/1-677 233 ----- 285
Cx.6/1-677 233 ----- 285
Cx.7/1-677 233 ----- 285
Cx.8/1-677 233 ----- 285
Cx.9/1-677 233 ----- 285
Consensus AGGGTAGGCTCGCATGCGAGATTCCAATCGC-----GGAGCTGTCAAAACAACAACCA
Cx.1/1-676 286 A-T 342
Cx.2/1-676 286 A-T 342
Cx.3/1-677 286 -- 341
Cx.4/1-677 286 -- 341
Cx.5/1-677 286 -- 341
Cx.6/1-677 286 -- 341
Cx.7/1-677 286 -- 341
Cx.8/1-677 286 -- 341
Cx.9/1-677 286 T 343
Consensus AGACAATTGCACCTTTAGGTGTGGACCTTCATCTG-AATTTTTTTTAAAGAACGCAAC

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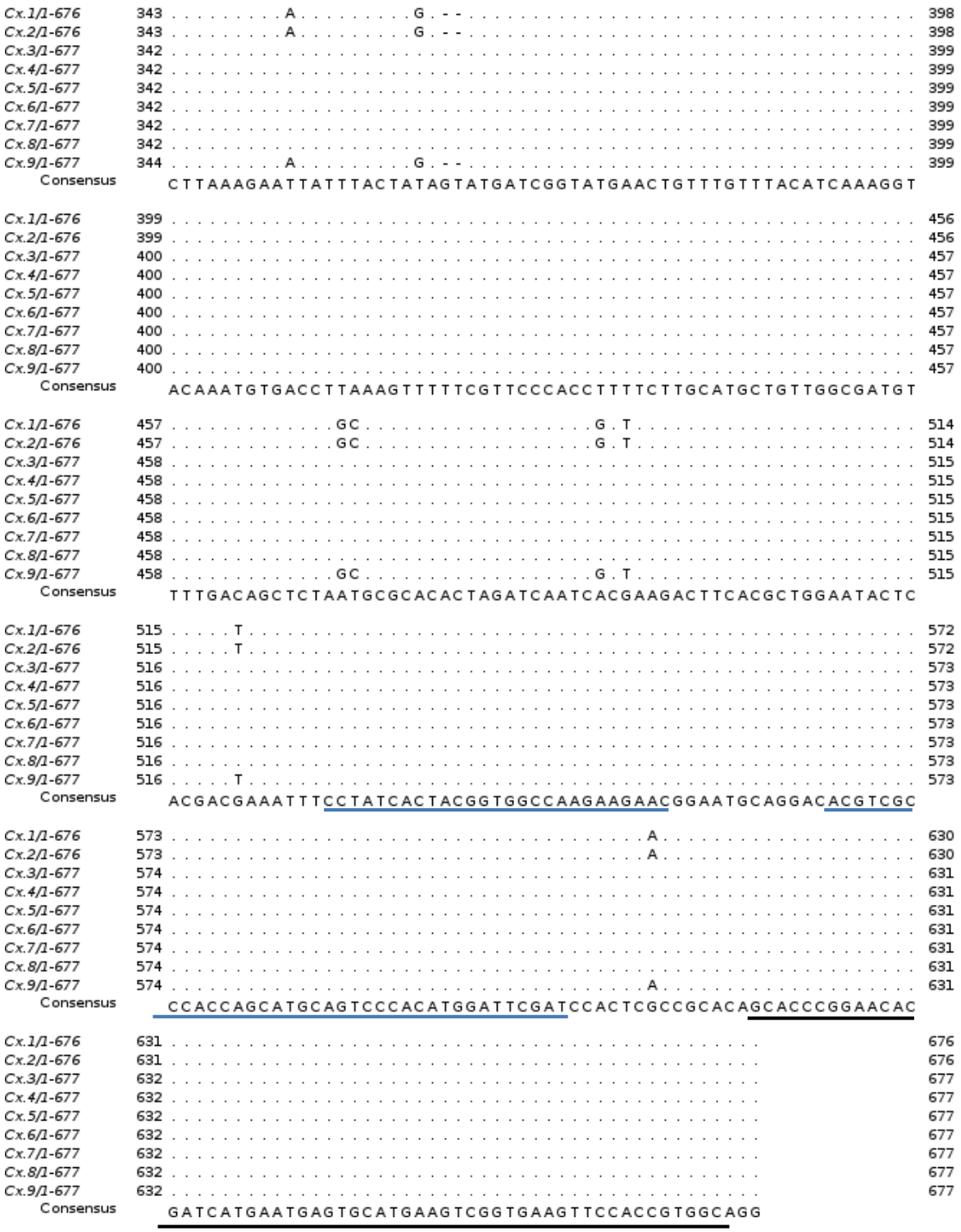


Figure 4.3: Multiple sequence alignment of the partial fragment of the *Vgsc* gene. Alignment was performed using the ClustalW multiple sequence alignment program. Underlined regions in blue and black correspond to primer and probe biding sites of the QRPS and qPCR and ddPCR assays, respectively

4.4.2 Copy number assignment of the *Vgsc* gene based on PCR-methods

4.4.2.1 Primer design

Titration experiments with varying primer concentrations found that 400 nM of primers yielded the closest Ct value comparing reactions for the *Vgsc* and *Pka* gene based on SYBR-GREEN detection. 200 nM of probe was selected as this concentration yielded early Ct values that remained constant as compared to using higher concentrations of the probe (Appendix 4-D). Primer efficiency for qPCR and digital droplet PCR (ddPCR) was evaluated using a standard curve performed on duplex PCR reactions run in triplicate for both genes. Amplification efficiency was similar for both *Vgsc* and *PKA* genes suggesting no evidence of reagent competition or primer/probe interactions, as indicated by PCR efficiencies of 98.7% and 101.9%, and correlation coefficients of approx. 0.98 (Figure 4.4A). The specificity of the primer sets was verified on both agarose gel and melt curves, which showed single bands and melting peaks. Reproducibility of the duplex *Vgsc*-CN assay was confirmed by running three experiments on consecutive days indicating strong correlation between the first run and the second and the first and third, R^2 = 0.879 and 0.980, respectively (Figure 4.4B).

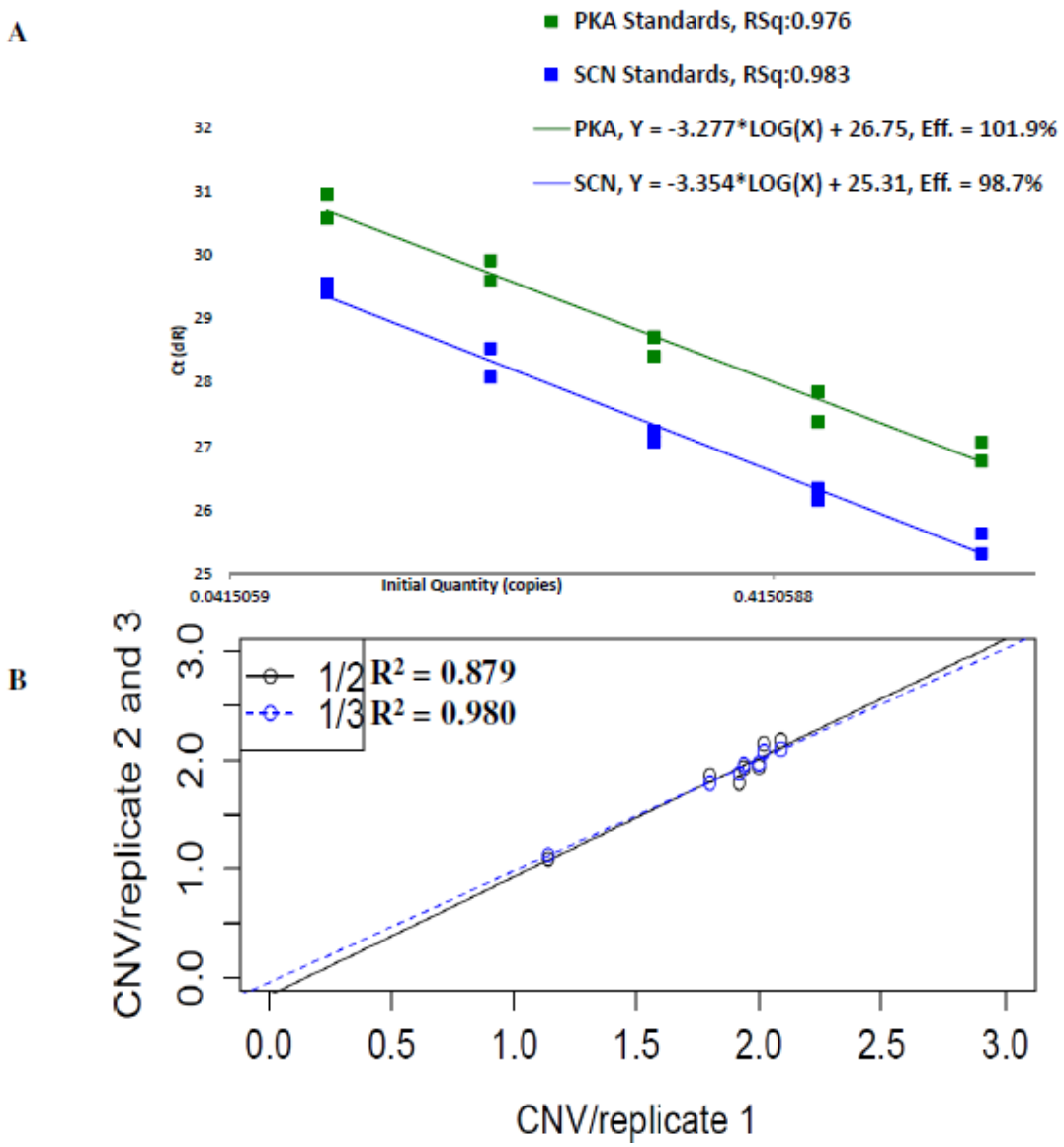


Figure 4.4: Copy number variation assay validation. A) Efficiency of primers and probes on duplex PCR reactions assessed by standard curve with 2-fold DNA dilution. B) *Vgsc*-CN assay intra-experiment reproducibility comparing triplicates of gDNA with unknown CN in three independent experiments.1/2 and 1/3 correspond to correlation between experiments 1 and 2 and 1 and 3.

4.4.2.2 qPCR

For absolute quantification analysis by the qPCR-standard method, 92 samples were compared to a relative standard curve constructed with a plasmid encompassing a single copy of the *Pka* and *Vgsc* gene. The resulting standard curve was linear in the range tested, with a PCR efficiency close to 100% differing by only 2% between both genes (Figure 4.5). The standard curve covers Ct values ranging from 24 to 34, which interpolate a genomic DNA

concentration around 10 ng/µl. The concentration of the *Vgsc* and *Pka* gene was determined from the relative standard curve in copies per microliter and was between 1115.65 and 939150.7 for the *Vgsc* gene and 31.25 and 669886.3 for the *Pka* gene. The ratio of *Vgsc* /*Pka* ranged from 0.8 to 1.70 with predicted CN between 2 and 4 (Figure 4.6A).

The predicted CN as determined with the qPCR-ΔΔCt method indicated that individuals had 2-4 copies per diploid genome (Figure 4.6A), with confidence intervals for the calculated CN higher than 0.99 in 78.9% of the individuals. The Ct values observed for the *Vgsc* and *Pka* gene ranged from 26.45 to 29.45 and 28.11 to 30.33, respectively with very little variation on the average Ct and a low standard deviation for the *Vgsc* (27.66, SD=0.26) and *Pka* gene (28.85, SD=0.23) across independent experiments.

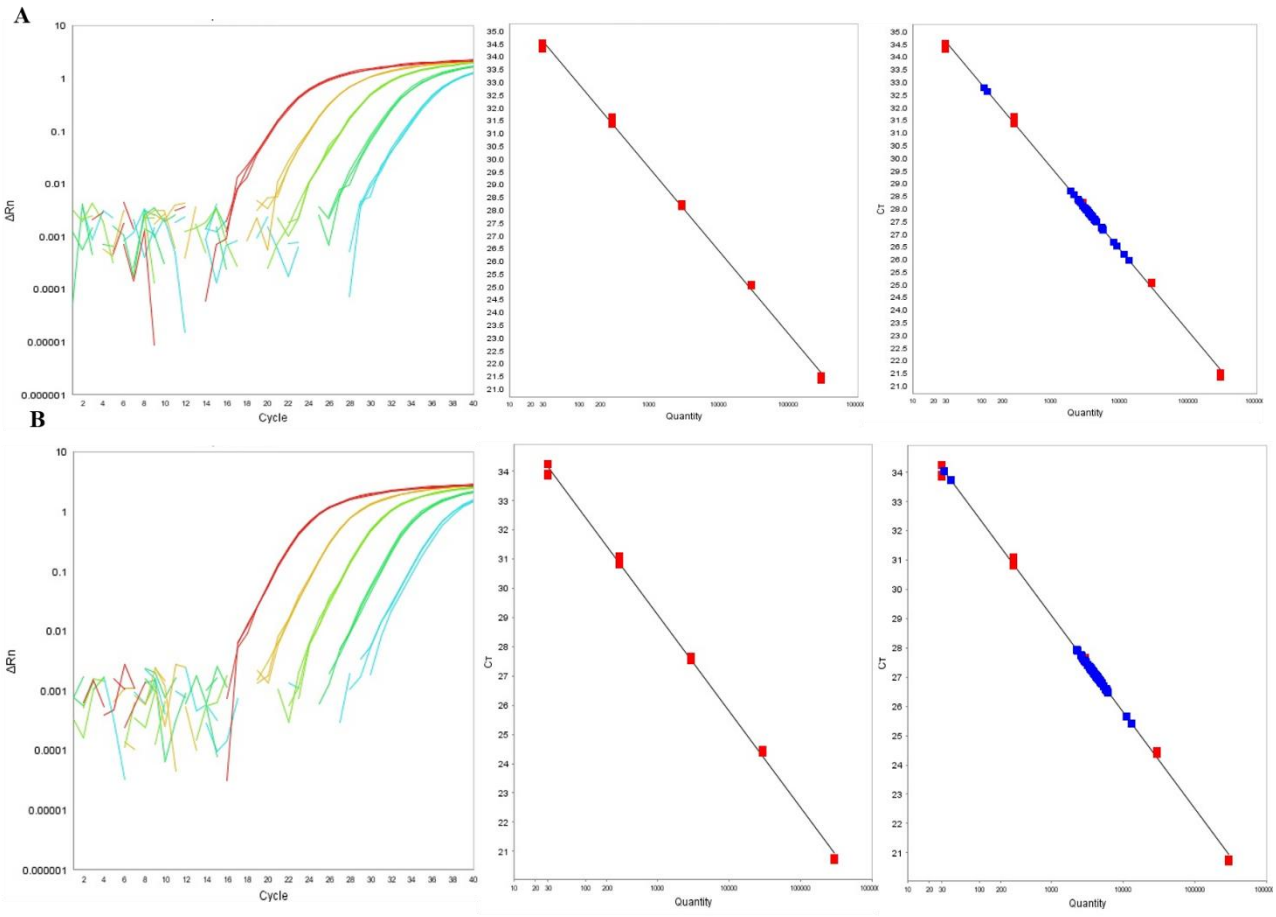


Figure 4.5: qPCR amplification of the *Vgsc-Pka* plasmid serial dilution for absolute quantification using the qPCR-Std method. A and B panels, shows the standard curve amplification for the *Vgsc* and *Pka* gene, respectively. Red square on the standard curve correspond to the five points of the serial dilution ranging from 3×10^5 to 10^1 copies/µl, whereas blue square correspond to quantification of samples with unknown copy number.

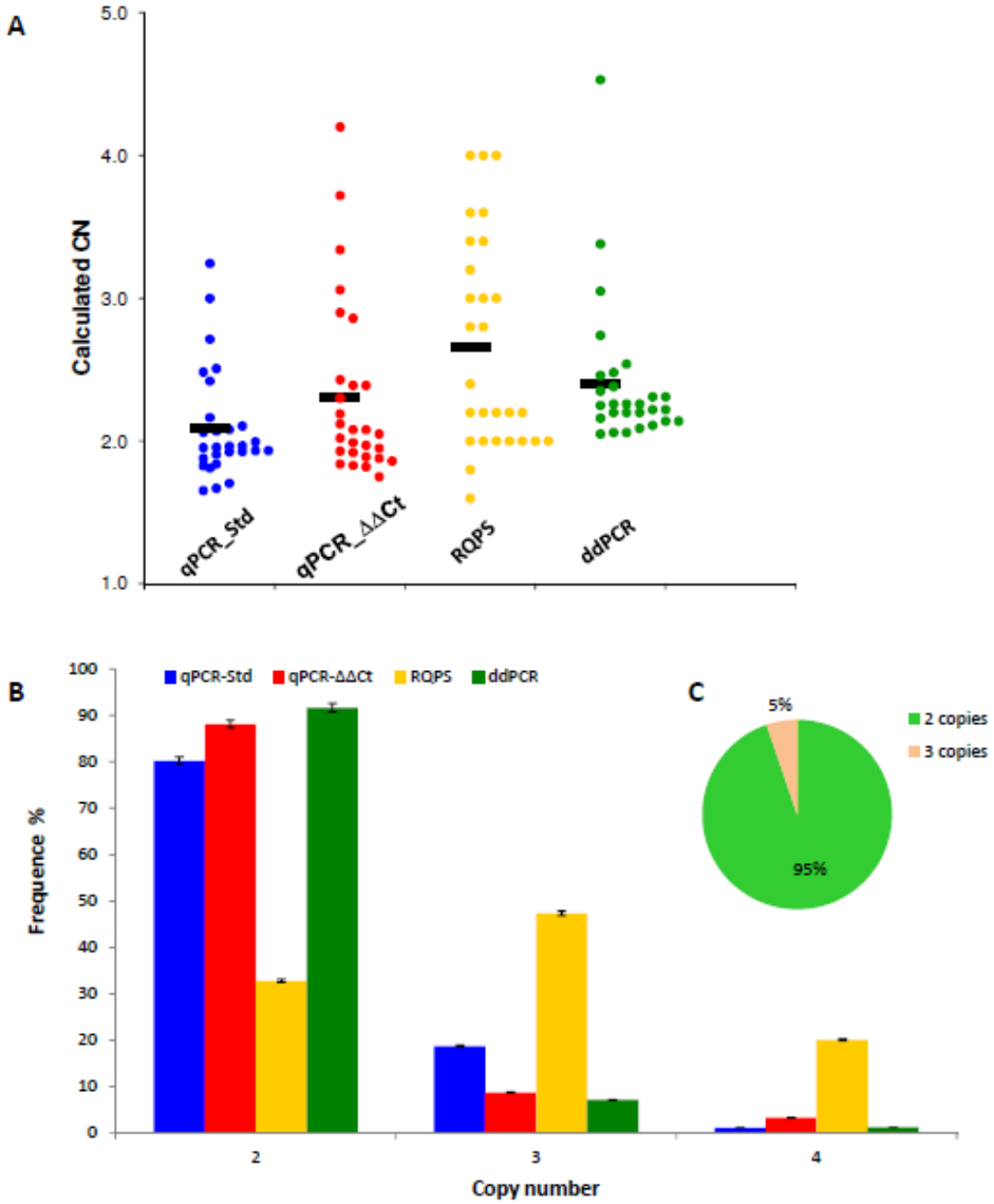


Figure 4.6: CN prediction across different methods. A) Scatterplot of predicted CN per individual and genotyping method. Each dot represents the CN predicted for each individual, predicted CN correspond to calculated number without correction to expected CN. The black lines shows the mean of predicted CN. B) Direct comparison of the predicted CN frequency across different methods applied. C) Merged CN frequency from different platforms applying the criteria of overlapping prediction of CN by $\geq$ 2 distinct methods. Error bars shows the 95% confidence intervals.

4.4.2.3 ddPCR

The genotyping of 92 individuals from four Uganda populations by ddPCR indicated the presence of CN ranging from 2-4 copies for the *Vgsc* gene per diploid genome (Figure 4.6A). The mean number of droplets analysed for the replicate reactions varied between 13,860 and 24,821 droplets with the total number of FAM/VIC positive droplets no less than 10%. Between replicates the 95% confidence interval of the calculated CN completely overlapped in most of the samples genotyped indicating strong reproducibility of the assay (Figure 4.7A). ddPCR results also indicated that the experimental conditions applied for the *Vgsc*-CN assay were efficient for complete separation of fluorescence amplitudes between positive and negative droplets as well as to identify four well distinct populations of droplets FAM+/+ , VIC +/+, FAM/VIC +/- and FAM/VIC -/- (Figure 4.7B).

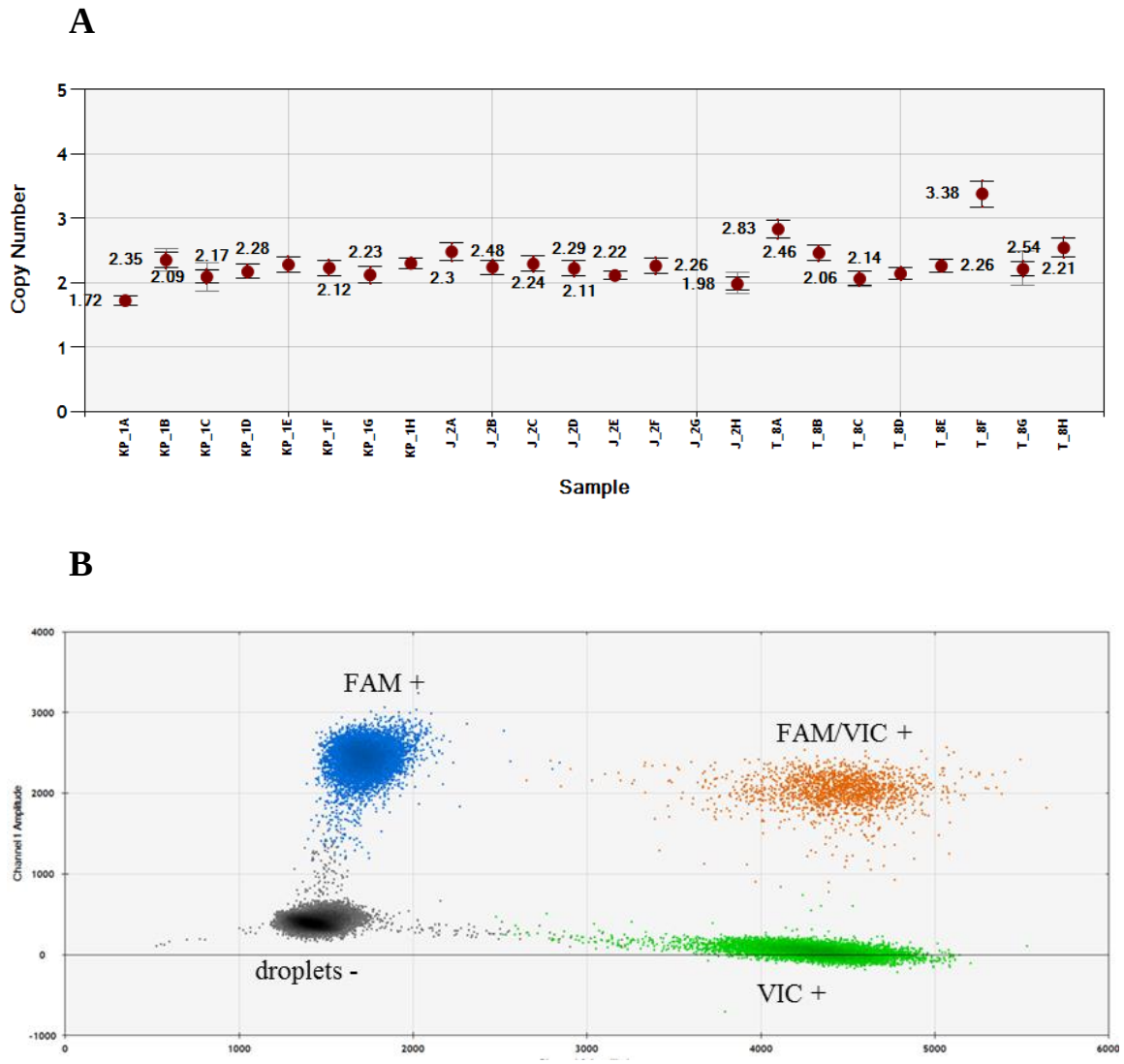


Figure 4.7: ddPCR CN prediction in Uganda *Culex quinquefasciatus* mosquitpes A) Merged of replicate calculated CN. The error bars represent Poisson 95% confidence interval. B) 2-D amplitude plot of the *Vgsc* and *Pka* showing satisfactory PCR condition to identify four well defined droplets populations.

4.4.2.4 *Vgsc*-RQPS

The assessment of the *Vgsc* CN using the *Vgsc*-RQPS method was carried out by comparing the peak ratio of the RQ-probe allele A; *Vgsc* and C; *Pka* in relation to the complementary gDNA alleles T and G using a linear regression where the intercept was set at zero. The CN of the samples was inferred by multiplying by 2 the slope of the linear regression ($y = kx$; where k is the slope) with assay quality verified using R^2 value. In total, 92 samples were genotyped with a CN (diploid) of 3 being the most frequent (in 47.3% of individuals)

whereas CNs of 2 and 4 were observed in 33% and 20% of the samples (Figure 4.6A). Application of a threshold of $R^2 \geq 0.8$ to assess the assay accuracy in the experimental conditions resulted in only 59.78% of the samples (N=55) fitting the criterion. The other samples retained very low or negative R^2 values, which indicated that the experimental conditions or assay design has low precision and reproducibility.

4.4.3 Comparison between the calculated CN across the different methods

To compare the predicted CN of the *Vgsc* gene across the four different approaches: qPCR (ΔΔCt and qPCR-Std-curve method), RQPS and ddPCR although 92 mosquitoes were genotyped for each method for assay corporation the number of samples was normalised to 55, which was the total of samples genotypes by *Vgsc*-RQPS assay that fit the accuracy criterion described previously. On the combined data set including mosquitoes from all the study regions (Jinja, Kampala, Kanungu and Tororo) the predicted CN across the four methods ranged from 2 to 4 (Figure 4.6B) with two copies being the most frequent CN observed by qPCR-ΔΔCt, qPCR-Std-curve and ddPCR (85.9%, 79.3% and 91.7%, respectively), whereas three copies was observed in higher frequency (47%) in mosquitoes genotyped using the QRPS method. At a population level, CNP was observed at a frequency of 4.7% in Kampala and 10% in Kanungu but this was not a consistent trend across the different methods. The qPCR methods and ddPCR showed higher similarity of predicted CN distribution compared to RQPS (Figure 4.6B), which in most of the cases increased the predicted CN by 1.

Since analytical variation associated with PCR based methods such as amplification efficiencies between reactions can result in confounding results, validation of predicted CN is required to avoid misidentifying CNVs. To address this issue and to increase the accuracy of the assays described herein the predicted CN was assessed in two steps. First, direct comparisons of the calculated CN between the ddPCR (assumed herein as standard due to the

high precession described elsewhere (Hindson et al. 2013) and the other methods was conducted to identify discrepancies for posterior CN validation by re-genotyping. Using this approach, deviation was highest for *Vgsc*-RQPS and lowest for qPCR-Std-curve (Figure 4.8). For the qPCR-ΔΔCt and qPCR-Std-curve method 28.3% and 6.5% of the samples respectively, were re-genotyped where most of samples had a predicted CN which increased by 1 copy indicating genotyping inaccuracy. In contrast, since there was a large variation for most of the samples assayed in the RQPS method, 50% of the samples were randomly selected to be repeated with no reduction in discrepancy compared to ddPCR prediction. Secondly, to minimize the number of false positives; predicted CN from the different methods were merged into one final CN calling for each sample. Using the criterion that the likely CN per individual must be identical for $\geq$ 2 CN methods, we identified that most individuals have single copies, while in only 5% of genotyped mosquitoes CN was detected (Figure 4.6C).

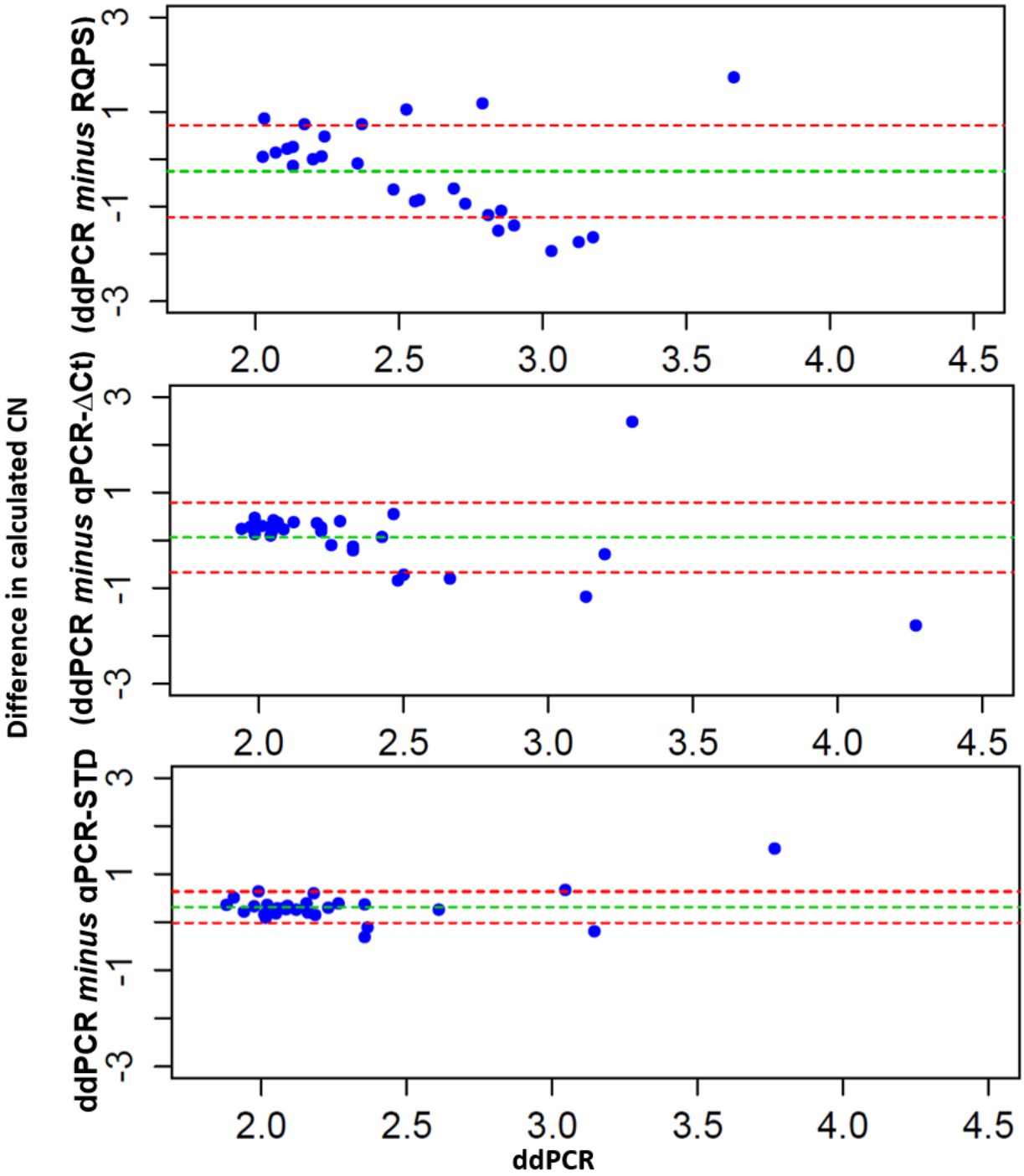


Figure 4.8: Bland-Altman plot showing the difference between the predicted CN of qPCR methods and RQPS against the ddPCR CN prediction. Each blue dot represents individuals CN difference. Dash green line correspond to mean difference, while the dash red lines shows the 95% limits of agreement.

4.5 Discussion

Mutations in the sodium channel gene (*Vgsc*), a target-site for pyrethroids and DDT insecticides, have been described as a major threat to the success of control strategies for vector-borne diseases. Various independent SNP mutations, or the combination of several mutations in the *Vgsc* gene, such as the knockdown resistance (*kdr*) or super-*Kdr* mutations, have been the most extensively described variants associated with a reduction in sensitivity of the *Vgsc* gene to insecticides (Li et al. 2012, Silva et al. 2014). The evolution of insecticide resistance to pyrethroids is especially worrying for mosquito control programs since there is a limited number of insecticides approved for public health campaigns. Moreover, pyrethroids are the only insecticide authorized for use on impregnated bed nets.

Monitoring the frequency of markers associated with the resistance can be crucial for increasing the lifespan of approved insecticides, which allows for more sustainable control programmes. In general, most of the worldwide studies of pyrethroids and DDT resistance in mosquitoes have focused on detecting the resistant allele(s) of the mutation 1014F, which is often observed at high frequency in resistant populations (Scott et al. 2015). Herein, we investigate two possible mechanisms that could be related to variation in the level of insecticide resistance associated with the *Vgsc* gene in *C. quinquefasciatus* mosquitoes, haplotype diversity and genomic variation by gene copy number.

To characterize the haplotype diversity of the *Vgsc* gene, we used the approach of sequencing multiple clones from the same individual instead of sequencing PCR products from single mosquitoes since this method allows one to infer not only the number of distinct haplotypes in the population but also an individual's haplotype diversity. Surprisingly, we identified a high difference in the frequency of haplotypes encompassing the resistant alleles; with the C haplotype being more frequent than the other resistant haplotypes (Figure 4.2B). The difference in resistant haplotype frequency was not expected since both alleles TTT and TTC

are synonymous mutations and the mosquitoes studied were collected in a region with similar selection pressure. With our data it is not possible to infer directly the reason for the increased prevalence of one resistant haplotype as compared to others. Nevertheless, these data do provide an opportunity to investigate distinct mechanisms involved in the insensitivity of the *Vgsc* gene in *C. quinquefasciatus*. For instance, in a very simple scenario the low frequency of the resistant haplotypes A and B could result from recent migration events; however, it was not possible in our analysis to confirm this hypothesis since our data lacked haplotype information from surrounding mosquito populations. Although local haplotype variation is plausible, the idea is in contrast to results of low haplotype diversity in *An. gambiae* that show a single haplotype for the 1575Y mutation on the *Vgsc* gene in a mosquito population from West and Central Africa (Jones et al. 2012a). Another hypothesis is based on the high level of haplotype diversity, since more than 10 single nucleotide polymorphisms were identified between resistant haplotypes as well as the absence of a complete separation in the resistant haplotypes (Figure 4.2C), which likely indicate an independent origin for these haplotypes or that the haplotypes occurring in low frequency correspond to additional variation of the ancestral *Kdr* resistant alleles, although an increase of diversity due to hybridisation of *C. quinquefasciatus* and *pipiens* could also been involved.

The analyses of haplotype diversity applied here also provides an opportunity to explore additional features of the *Vgsc* gene diversity at the genotype level. In our analysis, we identified two individuals out of 12, which have the same resistance allele but on distinct haplotypes, while the same pattern was also observed in wildtype haplotypes. For instance, the genotype of individual Cx3 (Figure 4.2C) had two completely independent resistant *Kdr* haplotypes with the allele TTT. On the other hand, individual Cx3 shows different haplotype composition but for the susceptible allele. Our results indicate a complex genotype architecture for the *Vgsc* gene in the population studied since distinct haplotypes and individuals with

different haplotype combination were identified, despite studying only a small number (N=12) of mosquitoes and only a partial fragment of the *Vgsc* gene. In terms of insecticide resistance management the complexity of the genomic architecture sets additional challenges for monitoring and understanding the role of target-site mutations linked to pyrethroids and DDT resistance since haplotype variation can be involved in many aspects of gene regulation and expression. For example, analysis of alternative splicing in *C. quinquefasciatus* suggested the presence of four variants in the sodium channel proteins (He et al. 2012). Additionally, characterization of the *Vgsc* haplotype diversity in *Musca domestica* and their implications on fitness cost demonstrate that different resistant alleles and distinct haplotypes of a specific allele can have completely different fitness costs (Rinkevich et al. 2013b).

Another explanation for the high haplotype diversity detected in our study population can be linked to gene duplication of the *Vgsc* gene. This hypothesis was raised through our preliminary analysis, which showed the presence of three distinct *kdr* haplotypes in the individual Cx8 (Figure 4.2C). Furthermore, the results of the *kdr* genotyping by TaqMan also suggested the presence of gene duplication since distinct clusters for heterozygotes were observed (Figure 4.2A), which may result from fluorescence asymmetry due to difference in allele numbers (Kumasaka et al. 2011). The possible *Vgsc* gene duplication observed in our data is also supported by findings of previous studies in *C quinquefasciatus* mosquitoes collected in the US, which suggested the presence of multiple copies of the *Vgsc* gene (Xu et al. 2011). Furthermore, high nucleotide diversity in genes under selection in contrast to the low variability expected due to hitchhiking has been demonstrated for duplicated genes as described for the *Ace-1* gene in *Anopheles* and *Culex* mosquitoes (Djogbénou et al. 2009, Osta et al. 2012b).

To further investigate the hypothesis that gene duplication has occurred in the *Vgsc* gene, we developed and validated a PCR-based method that can be applied through three

commonly used genotyping platforms. The *Vgsc*-CN assay described here in indicated CNP in the *Vgsc* gene in all of the four inference methods applied. We identified mosquitoes with a CN of three or four in all methods, with the exception of the RQPS method that predicted this figure in a much higher frequency (Figure 4.4A). The consistent CN prediction by both qPCR methods and ddPCR using the *Vgsc*-CN assay can provide more flexibility in assay choice for future studies since it is not restricted to a single genotyping platform. Additionally, the *Vgsc*-CN assay validation also indicates that primer sets and probes designed for the qPCR and ddPCR methods perform satisfactorily with different chemistries as well as with different reaction conditions. The optimum performance of each method to accurately determine CN was demonstrated through similar primer efficiencies in the duplexed reactions and separation between positive/negative droplet populations (Bustin et al. 2009, Huggett et al. 2013). This allows not only for the possibility of cross-validation of the results on different platforms without redesigning the assays but also removes the need for different panels of probes.

Evaluation of the assay precision and efficiency to reliably measure CN in field collected mosquitoes was assessed by genotyping *C. quinquefasciatus* mosquitoes from four geographic regions of Uganda. The calculated CN assessed by three methods were compared to the ddPCR calculated CN as this method has been described as the most precise of the real-time methods (Hindson et al. 2013). In our hands the ddPCR and qPCR-Std-curve methods yielded similar results for CN calculation with the lowest standard deviation. This result was expected as both methods rely on absolute quantification, although calculated by different methods. Since the ddPCR platform is not broadly accessible due to high cost, our results indicate that the application of both qPCR methods can provide a good alternative to identify and validate CNV inferences. In contrast, the RQPS methods shows the lowest concordance compared to the other three methods, with three instead of two as the mean predicted CN. Additionally, the RQPS calculated CN was less precise with values widely distributed across a

CN range between 2 and 4 compared to ddPCR. The discrepancy observed in the RQPS results as compared to the other method indeed can be a reflection of the sample preparation. The RQPS method requires mixing of gDNA and RQPS-probe at 1:1 and 1:2 molar ratios, which can increase sample manipulation and consequently can introduce more experimental errors. It is possible that the overestimation of the CN by the RQPS method may be linked to inaccuracy of gDNA and the RQPS-probe ratio mixing since in about 50% of the samples the expected peak ratio of the gDNA and RQPS-probe alleles were not observed.

Comparison of the calculated CN in relation to ddPCR results was also used to infer the assay efficiency and sensitivity. For this, a specific criterion was established. The criterion was that there should be a discordance of calculated CN differing by >1 to indicate a potential false positive CN calling. The lowest efficiency was observed for the RQPS and qPCR-ΔCt method with about 50 and 28.3% of the predicted CN corresponding to probable outliers, respectively. For each sample that fit this criterion the entire workflow was replicated in triplicate to confirm the calculated CN. The re-genotyping of the RQPS outliers does not change significantly the previously predicted CN, indicating low accuracy. On the other hand, the re-genotyping of all outliers observed on the qPCR-ΔCt method results in changes of predicted CN (Appendix 4-E). The significant changes observed in the qPCR results could be linked to assay set-up issues such as heterogeneous distribution of master mix or inaccuracy of gDNA normalization. The first issue was not detected in our reaction conditions because the Ct standard deviation across the parallel replicates was lower than 5%. On the other hand, variation in the concentration of gDNA is the most probable reason since other publications have demonstrated that DNA concentration, purity and integrity can be limitations for CNV accuracy prediction owing to their influence on the ΔCt (Fernandez-Jimenez et al. 2011); nevertheless, this issue is more difficult to resolve.

The workflow applied in this study can minimize the number of false positive CN predictions that occur in natural populations, since the comparison of predicted CN among distinct methods can be used to validate the data as well as to reduce overestimation of CN introduced by experimental variations. In our present data we identified between 8.3 and 9.8% of samples across Uganda with CN=3 or 4 using the qPCR and ddPCR method. However, it is possible that in some populations this figure could be higher, since due to the lack of historical data on insecticide application in this region is not possible to conclude if the populations were collected in the absence of selection, which could reduce the frequency of mosquitoes with gene duplication due to the detrimental fitness cost of CN in insecticide-free environments (Bergthorsson et al. 2007, Djogbénou et al. 2009).

The *Vgsc*-CN assay described here provide a simple and robust workflow for precise measurement of CN in field collected mosquitoes overcoming the limited number of CNV assays available so far for mosquito gene duplication studies. Additionally, by replacing the primer/probe of the gene of interest the approach used here can be easily transferable to investigate the CN frequency of other genes encompassing gene duplication like *Ace*-1 and esterases in field collected *Culex* mosquitoes.

In summary, our data suggests a high level of polymorphism in the *Vgsc* gene in *C. quinquefasciatus* resulting in complex haplotype diversity and heterogeneity of individual genotypes as well as the presence of CNP of the *Vgsc* gene in Ugandan mosquitoes. As insecticidal control methods are rolled out across Uganda these techniques will enable monitoring of CNV, and its association with phenotypic resistance, in these mosquitoes. Additionally, the approach proposed here has greater applicability to provide more precise predictions of CN and data validation. Finally, the developed assay can be an important tool for monitoring and management of variation of gene copy number in insecticide target-site genes.

Chapter V

Molecular Mechanisms Underlying Resistance to Carbamates Insecticides in Ugandan *Culex quinquefasciatus* Mosquitoes

5.1 Abstract

Currently, efforts to interrupt lymphatic filariasis (LF) transmission are mainly based on mass drug administration (MDA) and population control of *Culex quinquefasciatus.* However, widespread and intensive application of insecticides has resulted in an increase of insecticide resistance in *C. quinquefasciatus*. The objective of this study is to identify candidate genes associated with insecticide resistance in *C. quinquefasciatus.* The transcriptome of bendiocarb-selected adult mosquitoes was compared to two unexposed groups of mosquitoes using a whole-transcriptome microarray (Agilent 8 X 60K). Resistance to bendiocarb was marked with high resistance in WHO standard phenotyping assays (2.04 % [95 % confidence intervals (CIs) = 1.93-10.2] mortality to standard 1hr exposure; 58.02 % [CI = 6.23-6.49] mortality following 4hr exposure). Synergist assays using either the P450 inhibitor PBO or the esterase inhibitor TPP resulted in a marked increase in mortality to 83.14% (CI = 6.82-9.73) and 80.51% (CI = 7.83-10.91), respectively suggesting the involvement of metabolic resistance mechanisms. The microarray analysis identified 16 genes significantly overexpressed (false-discovery rate (FDR) adjusted *P* value > 2) in Uganda bendiocarb-resistant samples compared to other two groups (sympatric unexposed mosquitoes from Uganda, and the susceptible TPRI strain). These genes included two P450s (*Cyp-Cx1* and *Cyp6n23*) which showed the highest level of gene expression compared to the TPRI (8.75 and 4.37 fold-change, respectively). The present data set suggests that an upregulated P450 could be associated with bendiocarb resistance in *C. quinquefasciatus* from Uganda. Additionally, these results highlight the necessity of combining genomic and transcriptomic tools for a more effective monitoring of the evolution of resistance as multiple mechanism might underlie the resistant phenotypes.

5.2 Introduction

Lymphatic filariasis (LF), is a major cause of chronic and permanent disability in tropical and subtropical regions as a result of lymphoedema, elephantiasis and hydrocele (Bockarie et al. 2009, de Souza et al. 2012). LF is endemic in 83 countries with more than 1.2 billion people at risk of infection especially in Southeast Asia and African regions (Katiyar and Singh 2011, Rebollo and Bockarie 2013). In sub Saharan Africa the causal agent of LF is the nematode *Wucheraria bancrofti*, which can be transmitted by both Culicine and Anopheline mosquitoes with *Culex quinquefasciatus* being the major vector in urban and semi-urban settings (van den Berg et al. 2013).

In contrast to other vector-borne diseases such as malaria and dengue, for which anti-vector interventions as the major strategy, the Global Program to Eliminate LF (GPELF) launched in 2000 is based on Mass Drug Administration (MDA) of antihelminthics to reduce *Wuchereria bancrofti* transmission. Whilst vector control is not the primary strategy of the GPELF, it is still recommended as an intervention for LF eradication, especially in regions where successful implementation of MDA is challenging; for instance, in very remote areas or where LF is co-endemic with loiasis, which can result in adverse reactions to the drug cocktail used for MDA (Gyapong and Twum-Danso 2006, Rebollo and Bockarie 2013). Additionally, modelling and field studies have shown that integration of vector control into MDA programmes can reduce the required number of chemotherapy rounds and consequently the timeframe to achieve the microfilaria (MF) prevalence threshold necessary for successful interruption of LF transmission (Sunish et al. 2007, Bockarie et al. 2009).

Although vector control has successfully reduced the burden of vector-borne diseases worldwide, the recurrent and extensive application of insecticides in endemic regions has also triggered an increase in the level of insensitivity to those insecticides

approved for public health (Rivero et al. 2010, Nkya et al. 2013). In addition, the limited number of insecticides available and the occurrence of cross-resistance between different classes is especially worrying for the sustainability of vector control (Nauen 2007, Sparks 2013). Consequently, identification and monitoring of resistance patterns, and understanding of the underlying mechanisms is crucial for extending the lifespan of currently available insecticide as well as for planning more effective vector control programmes.

As in many insect species, the insensitivity to insecticides in *C. quinquefasciatus* is thought to result mainly through mutations in target-site genes and/or overproduction of detoxifying enzymes (Rinkevich et al. 2013a). Susceptibility studies in *C. quinquefasciatus* from diverse geographical regions have associated two main target-site mutations to resistant phenotypes. The L1014F mutation in the voltage-gated sodium channel gene, conferring *Kdr* (knockdown resistance), has been associated with pyrethroid and DDT resistance, whilst the G119S mutation in the acetylcholinesterase (*Ace-1*) gene is linked to resistance to carbamates and organophosphates (Jones et al. 2012b, Kioulos et al. 2014, Zhao et al. 2014b). Metabolic resistance, which involves the over-expression or increased catalytic capability of metabolic enzymes, is a less tractable mechanism since members of diverse gene families including esterases, glutathione S-transferases (GST) and cytochrome P450 monooxygenases (P450), have previously been associated with resistance to different classes of insecticide in a range of vector species (reviewed in (Li et al. 2007, David et al. 2013). Over-expression of detoxification genes can be triggered by genomic divergence; for instance, by gene duplication, as observed for the resistance to organophosphates in *C. quinquefasciatus* mediated by esterases (Hemingway et al. 2004) or enhancement of regulatory elements through DNA

sequence variation as shown in *Drosophila melanogaster* DDT resistance associated with transcriptional increase of the *Cyp6g1* gene (Wilding et al. 2012).

Due to the diversity of genes or gene families involved in metabolic resistance, identification of candidate genes requires an agnostic survey of the patterns of gene expression associated with resistant phenotypes. Recently, studies have applied either microarray or RNA-seq platforms to elucidate the relationship between gene expression and insecticide resistance (Bonizzoni et al. 2012, Mitchell et al. 2012, Riveron et al. 2014). But, to date most of these whole-transcriptome studies in vector insects are restricted to mosquitoes of the genus *Anopheles*. Despite the role of *Culex* in transmission of several pathogens such as West Nile virus (WNV) and filarial worms, and reports of high levels of insecticide resistance (Farajollahi et al. 2011), in *C. quinquefasciatus* few studies have addressed the relative impact of metabolic resistance (Komagata et al. 2010, Liu et al. 2011a) and none at a whole-transcriptome scale.

In addition to their role as disease vectors, *C. quinquefasciatus* are nuisance biters and the failure to effectively control this species can lead to the perception of malaria control failure which may ultimately lead to the rejection of controls (e.g. indoor residual spraying -IRS and insecticide treated nets-ITNs). In this study, *C. quinquefasciatus* insecticide susceptibly in mosquitoes from Nagongera, Tororo District, Uganda was inferred using six insecticides (DDT, permethrin, deltamethrin, bendiocarb fenitrothion and lambda-cyhalothrin). A novel 8 x 60K whole-transcriptome microarray design was used to identify candidate genes associated with bendiocarb resistance. Additionally, Taqman allelic discrimination assay was also designed to type individual mosquitoes for the G119S target-site mutation, which

allowed the inclusion exclusively of wild-type (G119) mosquitoes in the transcriptomic profiling analysis.

5.3 Materials and methods

5.3.1 Sample collection

Filed collection of mosquitoes was carried out in Osukuru, Tororo, Uganda (See chapter III, Figure 3.1,) between June and July 2012. Resting adult *C. quinquefasciatus* were collected exclusively inside houses using aspirators and transported to the insectary. From these collections, 64 blood-feed females were maintained in individual Eppendorf tubes lined with moist filter paper to encourage egg laying (Morgan et al. 2010). Emergent larvae were fed on tetramin fish food, with pupae posteriorly transferred to cages and adults allowed to emerge. Eclosion cages were changed every 3 days so that cages contained only 3-5 day-old adults. Adult mosquitoes were fed *ad libitum* on 10% glucose solution, and used for insecticide susceptibility testing.

Genomic DNA from each female from which egg rafts were obtained to found the colony was individually isolated using a DNeasy kit (Qiagen) and then used for identification of *C. quinquefasciatus* using a diagnostic PCR assay (Smith and Fonseca 2004). In addition to these field-collected mosquitoes, a laboratory colony of *C. quinquefasciatus* from the Tropical Pesticides Research Institute (TPRI) Tanzania was used as a susceptible reference strain for the microarray study.

5.3.2 Insecticide susceptibility test

Bioassays were performed using test kits and insecticide-impregnated papers according to standard WHO methods (WHO 2013a). Adult F1 females aged 3-5 day-

old were assayed in four replicates of 25 non-blood fed mosquitoes in laboratory conditions with average of temperature of 26 °C and humidity of 63%. Tests were performed with papers impregnated with diagnostic concentration for six insecticides: DDT (4%), Permethrin (0.75%), Fenitrothion (1%), Lambda-cyhalothrin (0.05%), Bendiocarb (0.1%) and Deltamethrin (0.05%). Mosquitoes were exposed to each insecticide for 1h, with the exception of Bendiocarb and Deltamethrin where four hour exposures were used, to increase discrimination between resistant and unexposed sympatric mosquitoes. Control assays were performed with 25 mosquitoes exposed to non-insecticide treated papers.

Insecticide exposed mosquitoes were then transferred to clean holding tubes and provided with 10% glucose for a 24-hour period after which mortality was recorded and dead (susceptible) mosquitoes were collected and individually stored on silica gel whilst alive (resistant) mosquitoes had a hind leg removed (stored on silica) and the whole body stored in RNAlater (Sigma Aldrich). RNAlater stored mosquitoes were initially held overnight at 4 °C to allow the solution to penetrate the carcass before storage at -20 °C until RNA isolation.

5.3.3 Synergist assays

Synergist tests were carried out following the procedure described above with an additional pre-exposure to three synergist compounds to investigate the potential mechanisms of metabolic resistance for bendiocarb and deltamethrin insecticides. For each synergist assay four batches of 20-25 mosquitoes were either pre-exposed for 1 hour papers (12cm×15 cm, Whatman grade no.1 filter paper) impregnated with a 10% solution of PBO (piperonyl butoxide – CYP450 synergist), TPP (triphenyl phosphate – esterase synergist) or DEM (diethyl maleate – GST synergist). These mosquitoes

were then exposure to Bendiocarb (0.1%) or Deltamethrin (0.05%) for four hours, followed by a 24h recovery period. In addition, synergist controls tubes were also run simultaneously.

5.3.4 *Ace*- 1 genotyping of bendiocarb phenotyped mosquitoes

Pior to microarray analysis all mosquitoes were genotyped for the G119S mutation in *Ace-1*. DNA was isolated from the amputated hind leg of resistant mosquitoes with 50 µl 10% Chelex 100 and 2 µl of proteinase K (10 mg/mL). The homogenate was incubated at 94 °C for 30 min followed by centrifugation at 6,000 rpm for 10 min to collect the supernatant.

A TaqMan assay was designed and applied to detect the G119S mutation in the acetylcholinesterase gene, which was previously associated with resistance to organophosphates and carbamates (Weill et al. 2004). To identify conserved regions for primer/probe design a partial fragment of exon 3 including the position G119S was amplified by PCR, followed by cloning and sequencing. TaqMan assays were carried out using 3 µl of isolated DNA, 1x SensiMix II probe, 400 nM of each primer and 100 nM of each probe in a final volume of 50 µl. Thermocycling was performed on the Stratagene MX3005P and consisted of 95 °C for 10 min and 40 cycles of 92 °C for 15sec and 60 °C for 1 min with endpoint discrimination.

5.3.5 Microarray

5.3.5.1 Construction of the 8 X 60K array and study design

The pattern of gene expression in bendiocarb-resistant mosquitoes was investigated using a newly designed *C. quinquefasciatus* whole genome oligonucleotide microarray. An 8 x 60K microarray format with 60-mer oligonucleotide probes was designed to cover five distinct groups of targets using

eArray (http://earray.chem.agilent.com/earray/). The majority of this array encompassed three probe replicates (56,598 probes) for each of the 19,018 transcripts in the CpipJ1.3 gene build (Arensburger et al. 2010). Additionally, we downloaded 205,396 ESTs from Vectorbase. From these, we identified 1,987 contigs and 4,109 singleton sequences and designed a single probe for each (from these 1,987 contigs, only 1,935 had successfully probes designed and from 4,109 singletons 2,862 had successful probes designed). Additionally, three probe replicates were designed to each of four alternative GST transcripts not annotated in Vectorbase (Kasai et al., 2009), and 250 CV (coefficient of variation) probes split into groups of ten, which are used for correction of the background signal of each dye channel (Figure 5.1A).

From the six insecticides used on Ugandan *C. quinquefasciatus*, we chose bendiocarb selected mosquitoes for the microarray analysis because we observed only moderate mortality for this insecticide following 4h exposure (see Figure5.2) thereby allowing clear discrimination between resistant and unexposed sympatric mosquitoes) and we also detected an increase in mortality in the presence of two synergists (See Figure 5.2; TPP and PBO), indicating a likely involvement of metabolic resistance. The TPRI strain was included in the analysis as a susceptible allopatric control. The TPRI colony was bioassayed with bendiocarb to confirm its complete susceptibility, and the samples used on the microarray were exposed to the same conditions as the field samples including the use of the same control paper that was applied to the Ugandan sympatric control. A comparison between the bendiocarb selected samples and controls (Uganda not exposed and TPRI) was performed using three RNA pools per group in an interwoven loop design (Figure 5.1B) as described by Vinciotti *et al*. (Vinciotti et al. 2005).

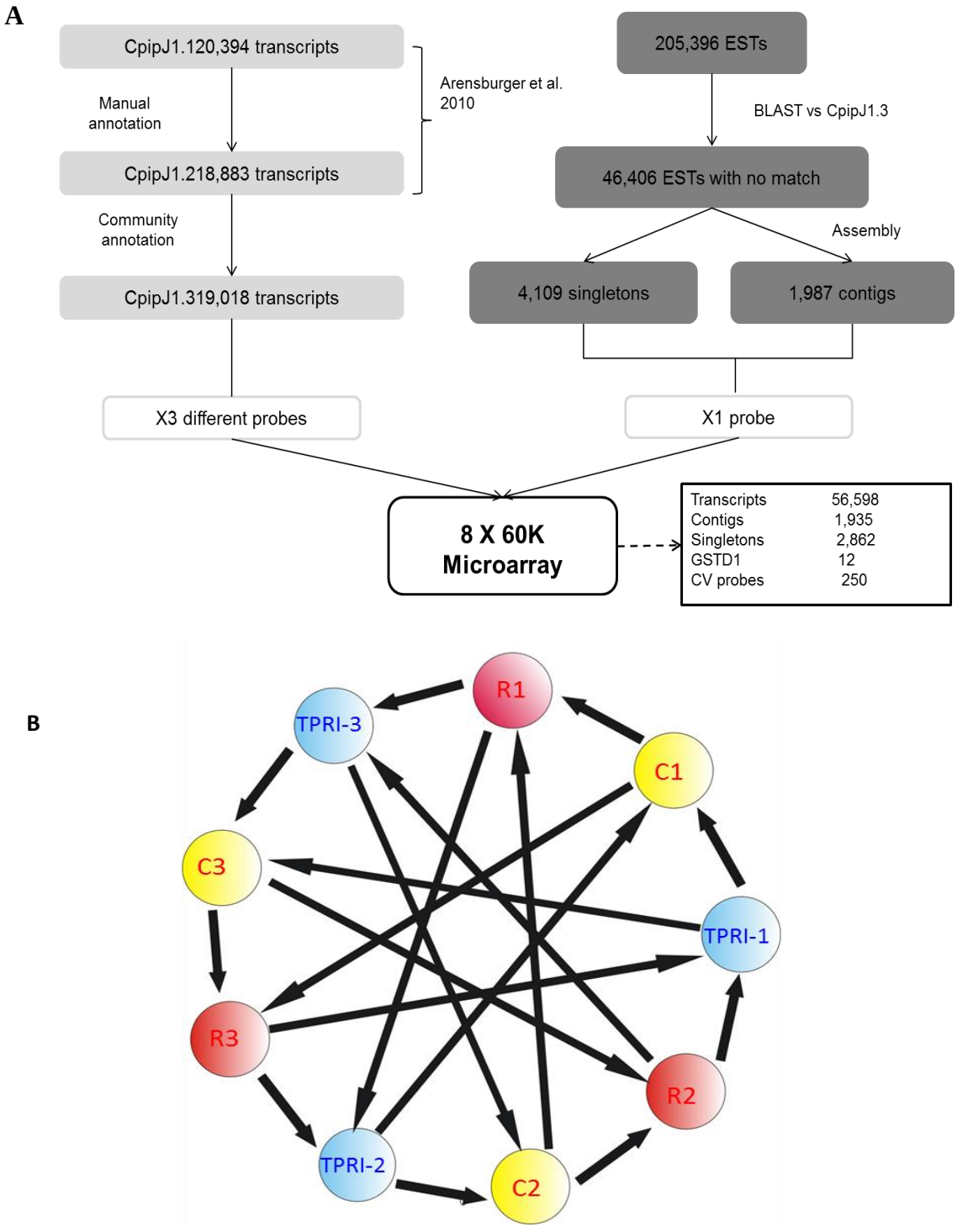


Figure 5.1: Overview of *Culex quinquefasciatus* whole-transcriptome analysis. A) Design of the 8 x 60K Agilent microarray. CpipJ1; consensus gene set of the automated gene prediction from the *C. quinquefasciatus* Johannesburg strain genome sequence. EST; expressed sequence tags. GSTD1, Glutathione S transferase D1. CV probe; coefficient of variation. B) Interwoven hybridization loop design for comparison between bendiocarb-exposed, non-exposed Ugandan field-collected mosquitoes and the TPRI susceptible strain. Circles represents pools of 10 females: C; Uganda non-exposed mosquitoes (sympatric control), R; Uganda Resistant mosquitoes and TPRI (Tropical Pesticides Research Institute); *C. quinquefasciatus* susceptible strain from Tanzania.

5.3.5.2 RNA extraction, labelling and hybridization

Total RNA was isolated from three pools of 10 female mosquitoes for each group using RNAqueous-4PCRkit (Ambion) according to the manufacturer`s instructions. Total RNA quantity was assessed using a nanodrop spectrophotometer and RNA quality was assessed using an Agilent Bioanalyzer. Each pool of RNA was individually labelled with Cyanine-3 and Cyanine-5 (Cy3 and Cy5) using the Low input Quick Amp Labelling Kit (Agilent Technologies) followed by purification through Qiagen RNeasy Columns (Qiagen), with quality and quantity checked using a nanodrop spectrophotometer and bioanalyzer, respectively.

Before hybridization, 300 ng of Cy3- and Cy5-labeled cRNA was fragmented using the gene expression hybridization kit (Agilent) in a total volume of 25 µl including 5 µl of 10x blocking agent and 1 µl of 25x fragmentation buffer. The fragmentation reaction was incubated at 60 °C for 30 min, and then chilled on ice for 2 min before addition of 25 µl of 2X GE hybridization buffer Hi-RPM. Each array was hybridized using 45 µl of the fragmentation solution for 17 hours at 65 °C and 10 rpm. After hybridization arrays were washed with wash buffers one and two for 1 min each, followed by acetonitrile for 10 sec and finally fixation solution for 30 sec. Arrays were scanned using the microarray scanner system (Agilent Technologies) and feature extraction performed using Feature Extraction software (Agilent Technologies) according to the manufacturer's recommendations. All arrays passed the Agilent quality control with QC score ≥10.

5.3.5.3 Data analysis

All microarray data analysis was performed using the R- program (Team 2014). Array normalization was carried out using the Limma 3.2.3 package (Smyth 2005) and data analysis performed using MAANOVA software (Wu et al. 2003) to detect overall differential levels of gene expression across the three treatment groups. The most over-expressed genes were selected after ANOVA *F*-test using a significance level for the FDR-corrected set at $\log_{10}$ (Q value) > 2. Additionally, the pattern of differential transcript level was also characterized through pair-wise comparisons between Ugandan resistant, sympatric control and the TPRI strain using a Student's t-test with genes where $P < 0.05$ considered differentially over-expressed.

Functional characterization of differentially expressed transcripts identified in the ANOVA and pair-wise t-test were then submitted to a Gene Ontology (GO) analysis to classify probes on their GO categories (cellular components, biological process and molecular functions). Two selection criteria were applied to develop the gene list used on for GO analysis. For the ANOVA analysis we applied a FDR threshold of logQ value >2, while for the t-test, probes with significant up / down regulation were selected based on a fold change of two. The list of genes that fit these criteria was submitted to VectorBase for automated functional annotation (GO term identification). The REVIGO web server (Supek et al. 2011) was employed to summarize and visualize the distinct GO terms identified. Relatedness among GO terms was assessed using the uniqueness method, followed by clustering of GO terms with closer semantic similarity, which were visualized by scatterplots for each GO category. The relative frequency of GO terms was assessed through comparison to the frequency in the *Drosophila melanogaster* data-base assuming, that both species have similar frequency of GO terms.

5.3.6 RT-qPCR validation

Reverse-transcription quantification PCR (RT-qPCR) was applied to confirm the expression profile of three out of 16 top candidate gene identified by the microarray (see Table 5.3). Specific primers to amplify PCR fragments with size ranging from 107 to 176 bp (Table 5.1) were designed using primer3 software (Rozen and Skaletsky 2000); however, only for four genes, *Cyp6d3* (here denominated as *Cyp-Cx1*,see Figure 5.6 for further details), *R2d2*, 40S ribosomal protein S3 and β-tubulin) was it possible to design primers spanning exon junctions. Specificity of primer sets was verified by identification of a single symmetrical amplicon peak following melting curve analysis. Additionally, PCR efficiency was verified using a 10-fold serial dilution of standard cDNA, with only primer sets with an efficiency ranging from 90 to 110% taken forward for RT-qPCR reactions.

Three technical replicates of all RT-qPCR reactions were carried out for each gene in a total volume of 20 µl including 1 µl of cDNA (1:4 stock diluted), 10 µl of Brilliant II SYBR® master mix (Agilent Technologies) and 100 nm of each forward and reverse primer. Amplification was conducted under standard qPCR reaction conditions on the Mx3500P qPCR system (Agilent Technologies). Gene expression quantification of the three selected genes was assessed according to the ΔCt method (Livak and Schmittgen 2001) using two housekeeping genes; 40S ribosomal protein S3 and β-tubulin as endogenous controls.

Table 5.1: PCR primers used for qPCR gene expression analysis

Gene name	Sequence (5`-3`)	Fragment size (bp)	Accession number[A]
Cyp-Cx1	F:AATCGCTACCTACCTGAACGA R: AATCGTTCAGGTAGGTAGCG	107	CPIJ020018-RA
Cyp6n23	F: CCGGAAGAGCTCGCCAAA R: GCGTCAATCCGTAGCGGT	176	CPIJ005900-RA
D2r2	F: TTCGAGGTTCCAGAGTTTGAG R: GCATCGCTTGCAGTGTTT	169	CPIJ011746-RA
β-tubulin	F: TCGACCTTCATCGGAAACAC R: GTTCATGTTGCTCTCAGCCT	156	CPIJ003263-RA
40S ribosomal protein S3	F: CCTGACTCGCGAGCTGG R: GAAACCGAATCGCTTCTGGAC	166	CPIJ013941-RA

[A] VectorBase transcript identification

5.3.7 Manual annotation of the CYP6D3 gene

During qPCR primer design for the top candidate gene *Cyp6d3* (CPIJ020018-RA), we concluded that the available, automated gene annotation is unreliable. The genomic sequence of this region in Vectorbase includes a region of approximately 810 bp in supercontig 3.2948 with no nucleotide sequence information, which spanned the automated annotation of *Cyp6d3*. We suspected that additional coding sequence lay within this region. To confirm this we designed primers to span the complete region and amplify a 4.7 kb region covering the full length of the candidate gene genomic sequence. Nine internal sequencing primers were also designed.

PCR reactions to amplify the *Cyp6d3* genomic region were conducted in a final volume of 20 µl including approx. 40 ng of genomic DNA, 1 X Phusion HF buffer, 200 µM of each dNTP, 0.5 µM of each primer forward:Cx-6D3 and reverse: Cx-6D3

(Table 5.2) and 0.02 U/µl of Phusion DNA polymerase. The reaction conditions were 98 °C for 30 sec, 30 cycles of 98 °C for 10 sec, 62 °C for 30 sec and 72 °C for 3 min, with a final extension of 72 °C for 5 min. The PCR product was purified using the GeneJET PCR purification kit (Thermo Scientific) followed by cloning in the pJET1.2 PCR vector (Thermo Scientific). Finally, nine primers (Table 5.2) were used for sequencing the full length of cloned PCR product after plasmid purification using the GeneJET Plasmid Miniprep Kit (Thermo Scientific).

Sequence traces were analyzed using CodonCode Aligner version 4.2.2. Following removal of vector sequences, fragments obtained by sequencing using distinct sequencing primers were assembled to build a single contig. This contig sequence was then used for gene structure prediction and transcript annotation using the Augustus web interface (Stanke and Morgenstern 2005).

Table 5.2: Primer sequences for amplification and sequencing of the *Cyp6d3* genomic region

Primer ID	Sequence (5`-3`)
	PCR primers
Cx-6D3F	AAAGGTGAACTGAGGGCAAA
Cx-6D3R	CTGATAAACAACGTTCGGACA
	Sequencing primers
Cx-6D3seq1	TGGAGGTGAATGCGAAAAGT
Cx-6D3seq2	TTTCATTTCGTGGAGTACATCG
Cx-6D3seq3	ATGGCATCCGTTGAGGTATC
Cx-6D3seq4	TCGAGTACCGATGAGAAGCA
Cx-6D3seq5	CGGCTGATTTCAACCATTTT
Cx-6D3seq6	TTGAAATGTTTTAGGGGAGCA
Cx-6D3seq7	TTTCCGATCTCTTCGCAAAC
Cx-6D3seq8	ATAGTCGTGGGTGCACTTCC

5.4 Results

5.4.1 Insecticide susceptibility status

WHO susceptibility tests using F1 female mosquitoes showed high levels of insecticide resistance to five out of six insecticides tested (Figure 5.2). The lowest mortality (0.97%) was observed with permethrin, while for DDT, lambdacyhalothrin, bendiocarb and deltamethrin the mortality rate ranged from 1.63-3.29% (Figure 5.2). For fenitrothion, we observed the highest mortality among the insecticides tested (69.42%). To investigate the effect of exposure duration on mortality, bioassays with bendiocarb and deltamethrin were also carried out for four hours. For both insecticides, an increase in mortality was detected (Figure 5.2); however, only for bendiocarb did the mortality increase significantly from 2.04% (95% CI = 1.93-10.2) to 58.02% (95% CI = 6.23-6.49).

Adult mosquitoes were also assayed with bendiocarb and deltamethrin for four hours exposure after pre-exposure in parallel to three synergist compounds (TPP, DEM and PBO). Synergism was observed for both insecticides following TPP and PBO pre-exposure whilst no significant effect on mortality was detected for DEM (Figure 5.2). TPP and PBO significantly increased the mortality of bendiocarb by 22.49% and 25.12%, respectively with an increase of mortality from 58.02% (95% CI = 6.23-6.49) to 80.51% (95% CI = 7.83-10.91) for TPP and to 83.14% (95% CI = 6.82-9.73) for PBO. For deltamethrin TPP and PBO synergists increased the mortality by 27.67 and 29.48% respectively, with mortality increasing from 9.09 % (95% CI = 2.97-4.5) to 38.57% (95% CI = 11.16-12.42) for TPP and to 36.76% (95% CI = 11.11-12.62) for PBO.

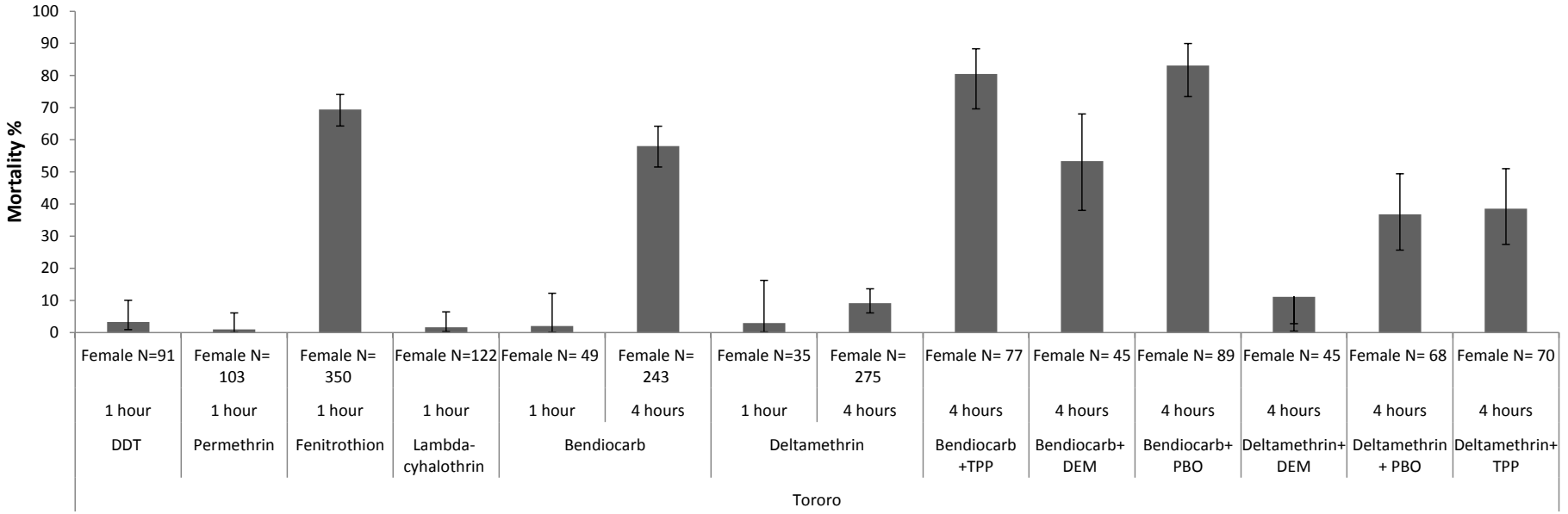
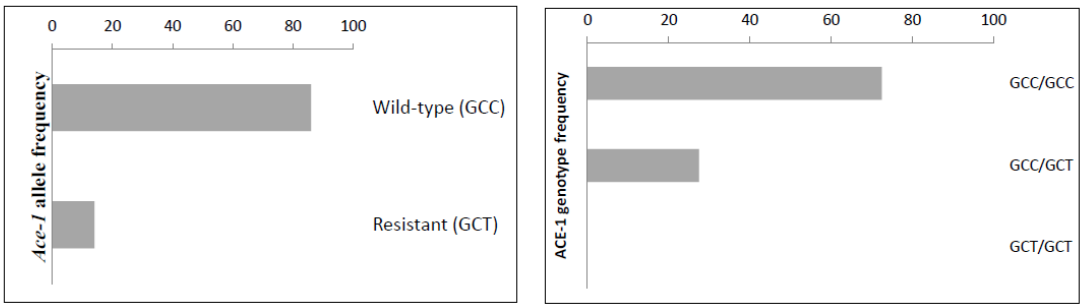


Figure 5.2: Insecticide susceptibility status of *C. quinquefasciatus* from Tororo, Uganda. Bioassay results following exposure to WHO insecticide treated papers at standard conditions and effect of insecticide synergists on the susceptibility status. Error bars represent 95% CI. PBO; piperonyl butoxide, DEM; diethyl maleate, TPP; triphenyl phosphate.

5.4.2 Frequency of an *Ace*-1 resistance allele on bendiocarb selected mosquitoes

Both mosquitoes that did or did not survive exposure to 0.1% bendiocarb (4h) were genotyped for the *Ace1*-119S mutation. The wild-type allele was observed at the highest frequency 86% (95% CI = 0.28-0.39) with homozygous G119 genotypes predominating 72.44% (95% CI = 0.48-0.65) whereas no homozygous resistant genotypes were detected (Figure 5.3A). Association test between the *Ace*-1 resistant allele 119S and bendiocarb resistant phenotype shows a significant genotypic/phenotypic association ($P = 0.001$, Figure 5.3B).

A



B

	Genotype						
	GG	GS	SS	*P*	OR	LCI	UCI
Alive	46	34	0	0.001	25	3.37	186
Dead	46	1	0				

Figure 5.3: *Ace1*-G119S allele genotyping and bendiocarb-selected (4h) mosquitoes association test. A) *Ace*-1 allelic and genotypic frequencies B) Association of the *Ace*-1 genotype and bendiocarb resistant phenotype. GG, GS and SS correspond to homozygous and heterozygous genotypes for the *ace1*-G119S locus.

5.4.3 Gene expression profiling of bendiocarb-selected mosquitoes

To identify candidate genes associated with resistance in bendiocarb-selected mosquitoes, transcriptomic profiles of three groups of *Ace*-1 wild-type (G119) mosquitoes were compared: Ugandan bendiocarb exposure survivors, Ugandan unexposed (sympatric control) and an unexposed fully susceptible TPRI strain. Among the three groups we

identified 32 probes significantly differently expressed in the ANOVA analysis applying a threshold of $-\log_{10}$ false-discovery rate (FDR) adjusted *P* value > 2, 50% of the probes had higher expression in the Ugandan exposed compared to both the sympatric control and TPRI (Table 5.3). The list of top candidate genes included two P450s: *Cyp6d3* (hereafter denominated *Cyp-Cx1* as described later) and *Cyp6n23*, which showed the highest level of gene expression compared to the TPRI strain (8.75 and 4.37 fold-change, respectively).

For functional characterization of the most significantly differentially expressed probes, a list of probes with a -log false-discovery rate (FDR) > 2 obtained from the ANOVA (Figure 5.4A) comparison was submitted to a Gene Ontology (GO) analysis. After removing duplicate probes, 358 unique genes were submitted to VectorBase for the GO term search and 231 terms were obtained. The majority of extracted GO terms were clustered on the molecular function and biological process categories with 28 and 15 distinct terms identified with frequency higher than 1%, respectively (Figure 5.5). Among the top enriched terms, GO linked to metabolic process, biosynthetic process, transport, membrane, integral component of membrane, nucleus, binding, cation binding and metal ion binding were observed with percentage of annotations ranging from 16.80 to 60.61% (Figure 5.4B-D;). Metabolic process was the predominant term across all categories corresponding to 60.61% of enriched GO terms, while in the category of molecular function we observed a large proportion of terms associated with binding functions, with frequencies between 15.70 and 55.14%.

To further explore the transcriptomic profile of the bendiocarb resistance phenotype, pairwise comparisons using Student's t-test were applied to compare exposed and unexposed Ugandan mosquitoes to the TPRI susceptible strain. Significantly up and down-regulated genes with a fold-change > 2 were also investigated by GO analysis. For down-regulated genes we observed similar figures between exposed and unexposed mosquitoes in contrast

to up-regulated genes where we identified 33 genes exclusively in the exposed mosquitoes (Figure 5.6A). The pairwise comparison also identified eight significantly expressed putative genes associated with insecticide detoxification: three exclusively in the pools of exposed mosquitoes: GSTs (CPIJ018629-RA; fold-change 2.79 and CIPJ018632-RA, fold-change 2.0) and esterase (CPIJ013918-RA; fold-change 2.86) whereas for the other five: P450 (CPIJ020018-RA), GSTs (CPIJ010814-RA, CPIJ018624-RA, CPIJ 018626-RA) and esterase (CPIJ013917-RA) were observed in both Ugandan exposed and unexposed mosquitoes.

In general, the GO term enrichment of the up and down-regulated genes for the Ugandan exposed and unexposed mosquitoes shows a similar list of terms (Figure 5.6B, Appendix 5-A) with the exception of three terms: metabolic process, phosphate-containing compound metabolic process and cation biding that were observed exclusively on the exposed mosquitoes with frequency of 60.61, 16.39 and 17.07%, respectively (Figure 5.6C). Nevertheless, this analysis excluded 39 and 18 up and down-regulated genes, respectively, such as esterases (CPIJ013917-RA and CPIJ013918-RA) and Heat shock proteins (CPIJ013880-RA and CPIJ005642) for instance, that were not associated with GO terms from VectorBase.

Table 5.3: Most differentially expressed genes from microarray analysis comparing Uganda resistant, sympatric controls and TPRI susceptible mosquitoes.

Gene	Transcript	GO	log Fold-Change (-log Q value)	
			Ugandan Bendiocarb vs TPRI	**Ugandan Sympatric vs TPRI**
CPIJ020018-RA	cytochrome P450 6D3	monooxygenase activity	3.13 (2.80)	2.59 (2.80)
CPIJ005900-RA	cytochrome P450 6N23	monooxygenase activity	2.13 (2.80)	1.87 (2.80)
CPIJ003654-RA	electron transfer	electron carrier activity	2.36 (2.58)	2.23 (2.58)
CPIJ010823-RA	serine protease 27 precursor	serine-type endopeptidase activity	4.41 (2.53)	3.86 (2.44)
CPIJ013083-RA	brain chitinase and chia	hydrolase activity, hydrolyzing O-glycosyl compounds	1.31 (2.44)	0.93 (2.38)
CPIJ016928-RA	15.4 kda salivary peptide	No information in VectorBase	2.99 (2.44)	2.73 (2.42)
CPIJ004984-RA	serine proteases 1/2 precursor	serine-type endopeptidase activity	4.26 (2.44)	3.60 (2.40)
CPIJ011746-RA	R2D2	double-stranded RNA binding	1.89 (2.41)	1.72 (2.40)

CPIJ004019-RA	saccharopine dehydrogenase	oxidoreductase activity	1.47 (2.44)	1.05 (2.30)
CPIJ004019-RA	saccharopine dehydrogenase domain	oxidoreductase activity	1.24 (2.40)	1.11 (2.40)
CPIJ002104-RA	plasma alpha-L-fucosidase precursor	catalytic activity	1.32 (2.44)	0.90 (2.26)
CPIJ015009-RA	histone H3.3 type 2	DNA binding	1.32 (2.40)	1.13 (2.38)
CPIJ000182-RA	N-acetylneuraminate lyase	catalytic activity	2.44 (2.42)	1.76 (2.26)
CPIJ011590-RA	conserved hypothetical protein	No information in VectorBase	0.87 (2.37)	0.81 (2.38)
CPIJ019290-RA	DNA ligase 4	DNA binding	1.22 (2.34)	1.20 (2.35)
CPIJ016792-RA	hypothetical protein	No information in VectorBase	3.39 (2.38)	2.94 (2.29)

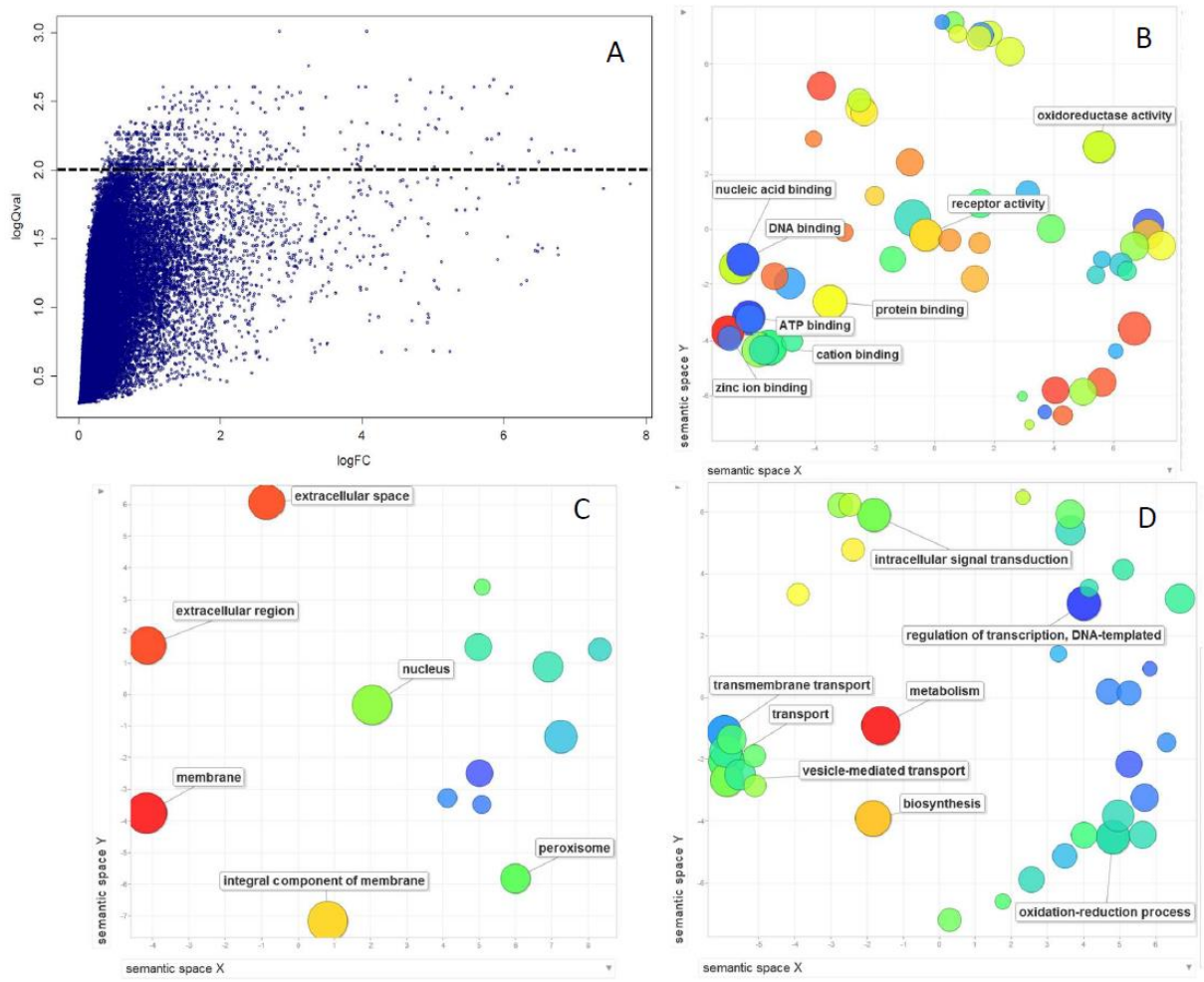


Figure 5.4: Candidate genes differentially transcribed in *C. quinquefasciatus* bendiocarb selected mosquitoes. A) Changes of gene expression between the three groups (Uganda exposed, Uganda un-exposed and TPRI) presented as a volcano plot. B, C and D Scatter plots showing representative GO term clusters (cellular component, biological process and molecular function, respectively) of differentially expressed transcripts with FDR>2.0. labeled circles are GO terms with frequency higher than 5%, while circles size indicates the frequency of the GO term compared to *Drosophila melanogaster* Go term enrichment.

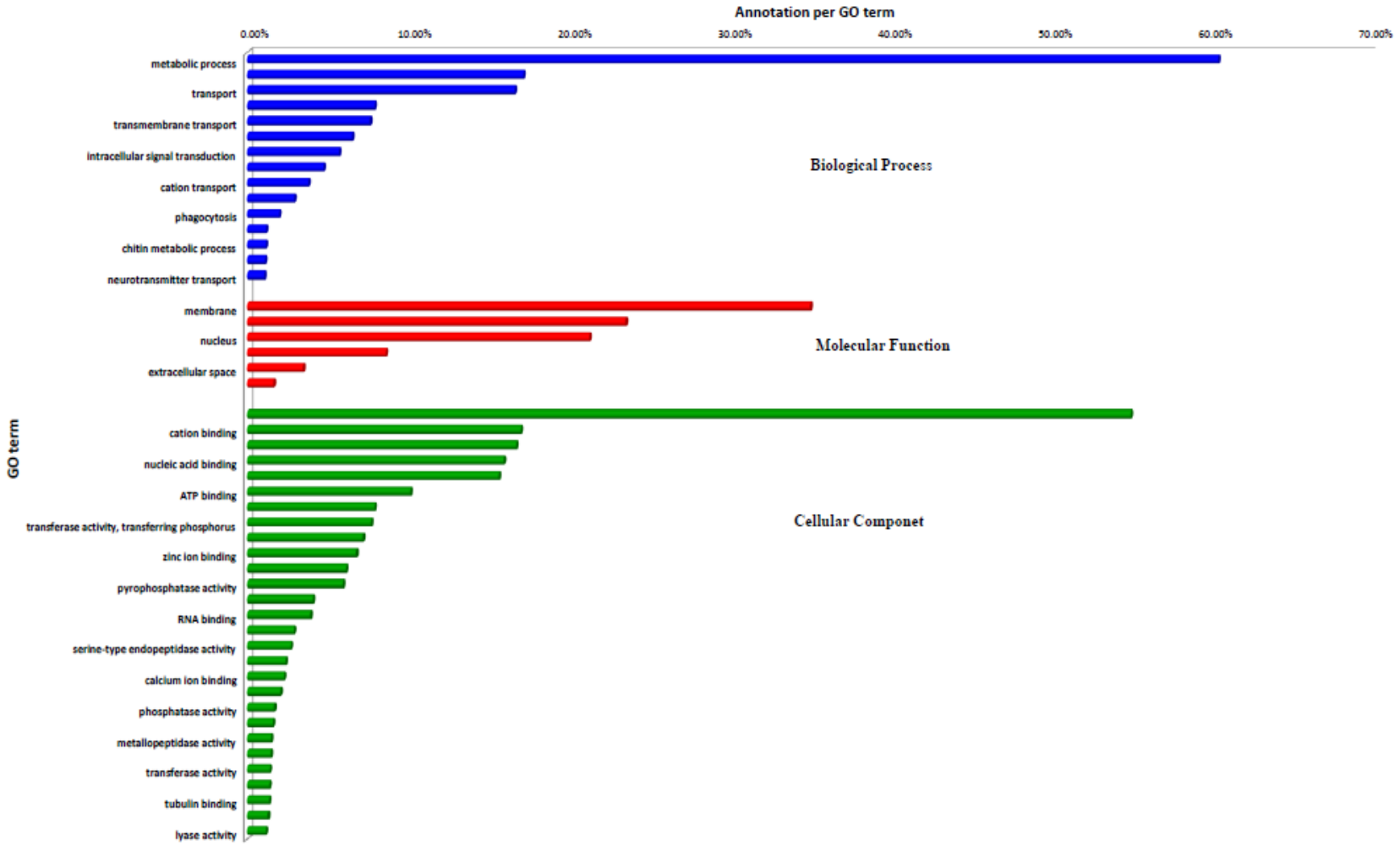
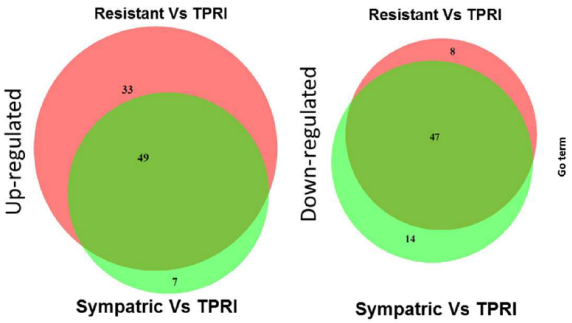
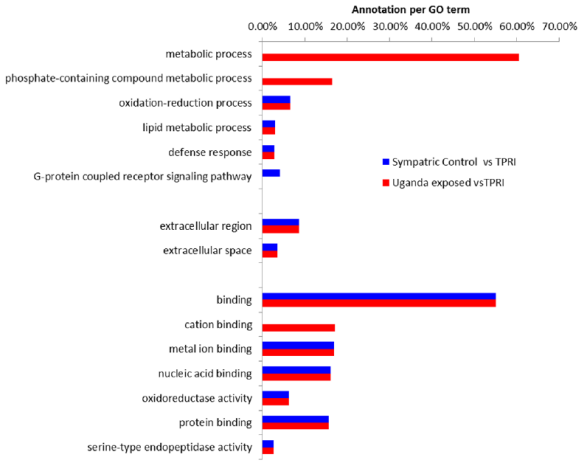


Figure 5.5: Go term enrichment analysis based on significantly differentially expressed probes with a -log false-discovery rate (FDR) > 2 obtained from the ANOVA among three groups (Uganda exposed and non-exposed (sympatric control) and TPRI susceptible strain.

A



C



B

Total number of Genes / GO	Sympatric Control Vs TPRI		Exposed Vs TPRI	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Number of genes with fold-change >2	56	53	82	48
Biological Process	8	3	10	2
Cellular Component	4	3	3	2
Molecular Function	22	24	30	26

Figure 5.6: Transcriptomic profile of differentially expressed genes with fold-change >2 in Uganda exposed and sympatric mosquitoes compared to TPRI. A) Venn diagram showing the overlap of up- and down-regulated transcripts between the three groups. B) Comparison of the number of GO terms identified by each pair-wise comparison. C) GO term enrichment of up-regulated transcripts between the groups with frequency higher than 2%.

5.4.4 Candidate gene validation by qPCR

The expression patterns of both candidate genes from the P450 family (*Cyp-Cx1* and *Cyp6n23*) and the gene *R2d2* (randomly chosen from the list of top candidate) were additionally assessed by qPCR. Satisfactory PCR efficiency ranging from 91.4 to 101.8%, (within the 10% acceptable variation) was obtained for primer pairs designed for candidate genes and endogenous control (Figure 5.7). Additionally, the primer sets were specific, with a single symmetrical amplicon peak in melting curve analyses (Appendix 5-B). For all three candidate genes (*Cyp-Cx1*, *Cyp6n23* and *R2d2*) we observed a good correlation between the microarray and RT-qPCR expression fold-change with ratios of up-transcription level detected by both methods differing by less than 1.5x (Figure 5.8).

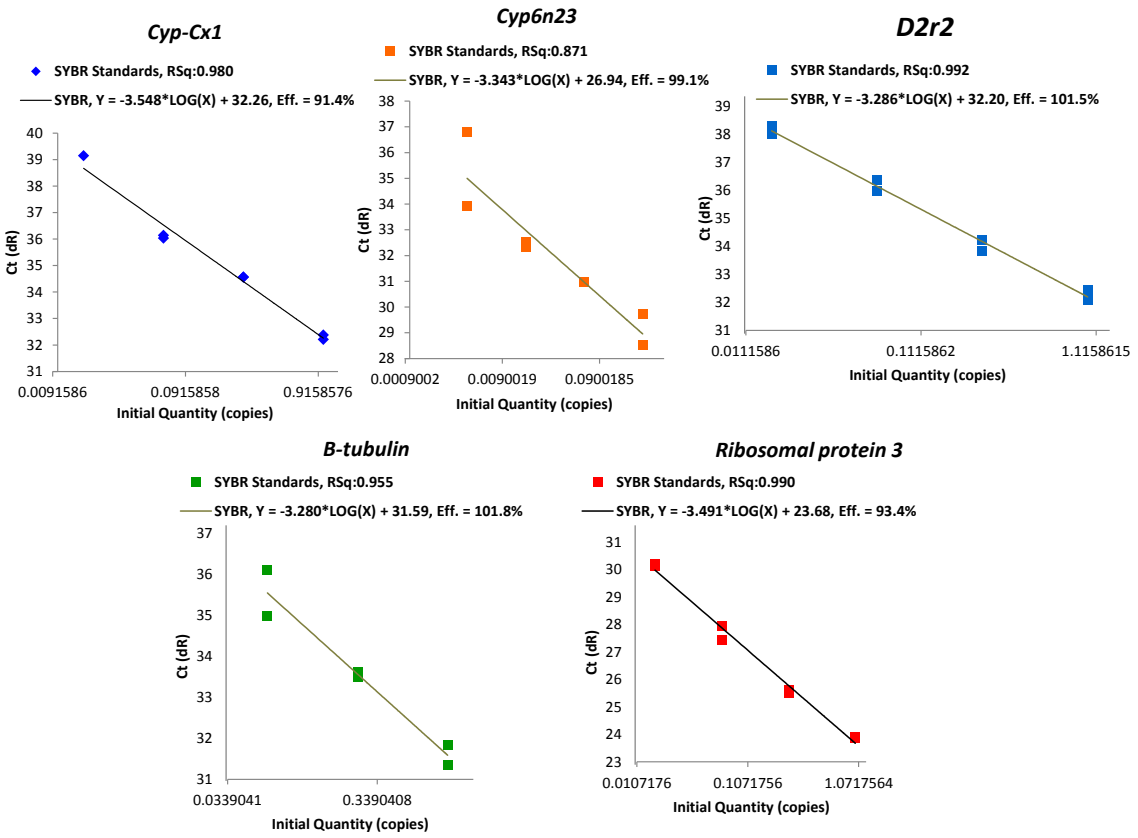


Figure 5.7: Standard curve from primers used on real-time PCR for microarray candidate genes validation. Squares correspond to 1x10 serial dilution of cDNA. qPCR efficiency (Eff) and coefficient of determination (Rsq) were calculated for each prime based on two replicates.

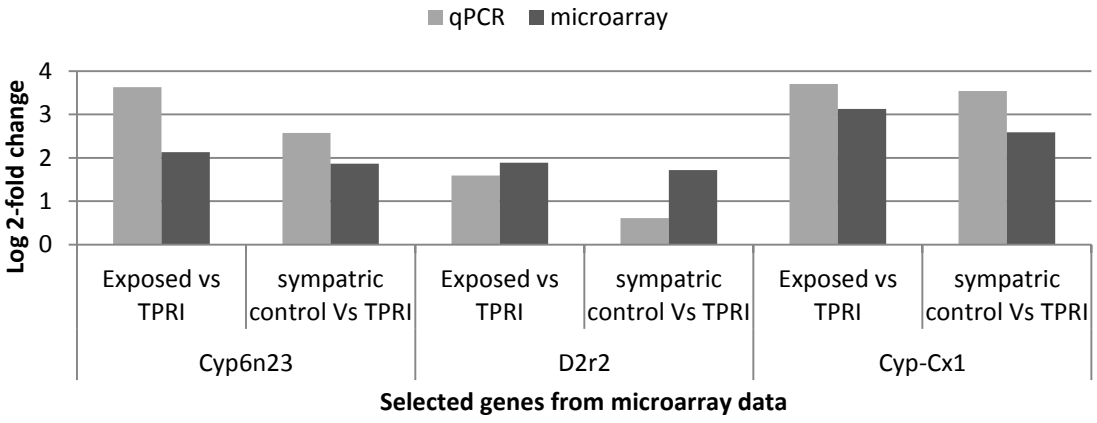


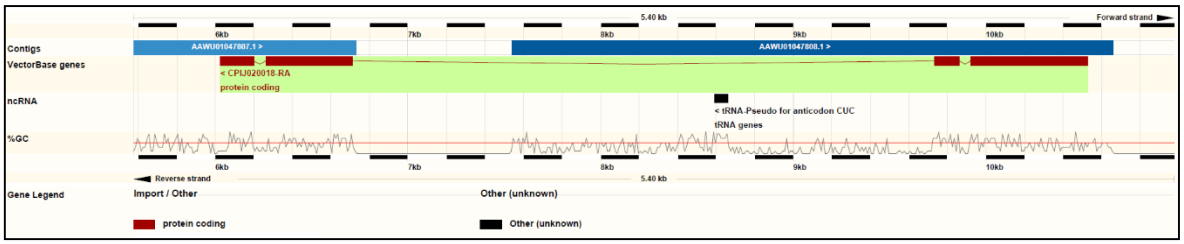
Figure 5.8: Pair wise comparison of microarray and qPCR data based on gene expression profile for three genes found to be significantly different expressed between Uganda exposed, non-exposed mosquitoes (sympatric control) and TPRI susceptible strain

5.4.5 CYP6D3 gene annotation

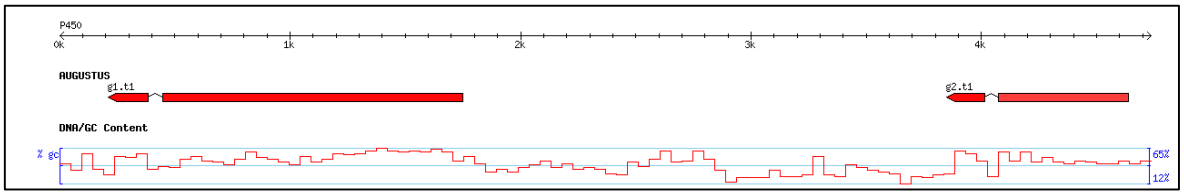
After identification of *Cyp6d3* (CPIJ020018-RA) as the top candidate gene in the microarray results, further analyses on the genomic and cDNA sequences available from VectorBase were conducted. This revealed an atypical gene architecture for a P450 gene with the presence of an intron between exons 2-3 longer than 3Kb (Figure 5.9A). Closer analysis of the genomic sequence indicated a region of 809 bp with no nucleotide information (Ns) internal to the *Cyp6d3* genomic sequence. *In silico* analysis of a contig constructed after PCR amplification and cloning of the complete region encompassing the genomic region of interest, suggested the presence of two distinct P450, here named as *Cyp-Cx1* and *Cyp-Cx2* instead of the single *Cyp6d3* as predicted by the automated annotation in VectorBase. The two genes predicted are each composed of two exons separated by one intron, while the microarray probe (gene: *Cyp6d3*) is based exclusively on the gene *Cyp-Cx1* exon-2 (Figure 5.9B and Figure 5.10).

An homology search in the *Culex* data base in VectorBase for both predicted genes *Cyp-Cx*1 and *Cyp-Cx2* indicated that the *Cyp-Cx1* have 94% similarity to *Cyp6z14* , while the *Cyp-Cx2* is 87% similar to *Cyp6d3*. We note that BLAST analysis of both predicted amino-acid sequences against *A. gambiae* and *A. aegypti* sequences available on VectorBase and the Cytochrome P450 homepage (Nelson 2009) shows no significant similarity to any CYP gene of the family D, while for both predicted genes the top hits belong to CYP genes from the Z family. Together the gene prediction and BLAST analysis carried out indicate that the top candidate gene from the microarray analysis is more close related to P450 genes of the Z family, some members of which have previously been associated with metabolic resistance in both *A. gambiae* and *A. aegypti* mosquitoes (Figure 5.9C).

A



B



C

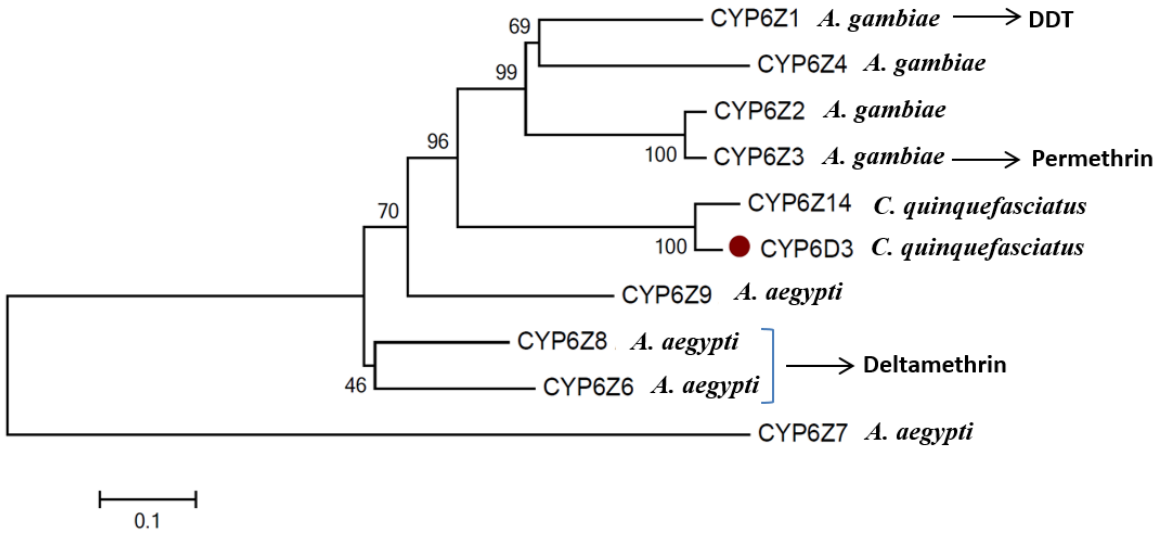


Figure 5.9: *Cyp6d3* predicted gene structure and annotation. A) Output of the VectorBase genome browse suggested a gene architecture with four exons and three introns. B) Schematic representation of the *Cyp6d3* after re-annotation using Augustus software, which indicates two distinct genes here named *Cyp-Cx1* (g1.t1) and *Cyp-Cx2* (g2.t1). C) Unrooted distance neighbour joining tree showing phylogenetic relationship of the predicted gene CYPCx1 from *C. quinquefasciatus* to *A. aegypt* and *A. gambiae* cytochrome P450s from the *CYP6* gene family. The percentage of bootstrap confidence values from 1000 replicates is shown at the nodes.

```
# ----- prediction on sequence number 1 (length = 4735, name = Cyp6d3) -----
#
# Constraints/Hints:
# (none)
# Predicted genes for sequence number 1 on both strands
# start gene g1
Cyp6d3  AUGUSTUS    gene    209 1747    0.94    -   .   g1
Cyp6d3  AUGUSTUS    transcript  209 1747    0.94    -   .   g1.t1
Cyp6d3  AUGUSTUS    stop_codon  209 211 .   -   0   transcript_id "g1.t1"; gene_id "g1";
Cyp6d3  AUGUSTUS    terminal    209 381 0.98    -   2   transcript_id "g1.t1"; gene_id "g1";
Cyp6d3  AUGUSTUS    initial 445 1747    0.96    -   0   transcript_id "g1.t1"; gene_id "g1";
Cyp6d3  AUGUSTUS    intron  382 444 1   -   .   transcript_id "g1.t1"; gene_id "g1";
Cyp6d3  AUGUSTUS    CDS 209 381 0.98    -   2   transcript_id "g1.t1"; gene_id "g1";
Cyp6d3  AUGUSTUS    CDS 445 1747    0.96    -   0   transcript_id "g1.t1"; gene_id "g1";
Cyp6d3  AUGUSTUS    start_codon 1745    1747    .   -   0   transcript_id "g1.t1"; gene_id "g1";
# coding sequence = [atgatcatttactcgctgcttctcatcggtacctcgatctacctgatcctccggtacatctactcgtactgggatcgcc
# atggcctgccgaacctcaaaccggacatcccgttcgggaacatccgtgccgtcgccctcaagcaggaatcgttcggcgtcgccctgaacgctctccac
# gccaaaaccacaggccaactggtcggaatctacctactcttccgcccggcgatcctaatccgggacgcccacctagcccaccgcatcataacgtccga
# cttcaactacttccacgaccgcggtgtgcactgtgacgagagctcggatccctttcggcgcacctgtttgccctgcccgggaagaggtggcgcagcc
# tgaggaacaagctgactccgaccttcaccgctgggcagctgcgcggtatgctgccgacgatcttggccgttgggaggaagtttcagggtttcttggaa
# cccaaggcgaagcgaggggaggtgattgaggccagggatttgatatcgcgctttgtgctggagatcgtggcgtcggtgtttttcggttacgagatcaa
# ctcgattcacgatccgcaggattcgtttcggacggttttacgatcatttcgggaggacaatcacgtcacaaatttgagaacggttggtgcgtttttgt
# gtccgacgttgctgaaggtcagccgggtcaaaacggtacctgaggtggtggacaattttgtaaacaaaatcattagggagcagatcgagtttcgcgag
# aagaacaacgttacgaggaaggatttcatccaactgttgatcgatctccgacgggagaagagtgatttcggactttcgttggaacagtgcgcggccaa
# tgtgtttttgttctacgtagcaggagcggatacctcaacggatgccatcacctacacggtccacgagctgacccatcgaccggatcttatgaagaagg
# ttcaagcagagatcgacgatgcgcttgagaagtccaacggtgaaatcaactacgatgtactccacgaaatgaaacttttagacaactgcgtgaaggaa
# acccttagaaagtacccgttcccaattttaaatcgcgagtgtacccaggactatcaggttccagattcgaagctgatcatcaggaagggaactccggt
# gatcataccgctgcaagcgttcggaatgagtgaggagtacttcccggaacctaatcgctacctacctgaacgatttgattcatccacaaagaattacg
# acgaaaaagcttacattccatttggagatggtccgaggaattgtattggttcccggatgggcagtgccgtttcgaagatcggcatcatcatgctgctt
# tcgaagttcaactttgaggcgactcaaggtgcggagataggctttgctcgggcccaaattgcgctggctccggagaatggcatttcacttaagatttc
# caacaggataagaaactcgatataa]
# protein sequence = [MIIYSLLLIGTSIYLILRYIYSYWDRHGLPNLKPDIPFGNIRAVALKQESFGVALNALHAKTTGQLVGIYLLFRPAIL
# IRDAHLAHRIITSDFNYFHDRGVHCDESSDPFSAHLFALPGKRWRSLRNKLTPTFTAGQLRGMLPTILAVGRKFQGFLEPKAKRGEVIEARDLISRFV
# LEIVASVFFGYEINSIHDPQDSFRTVLRSFREDNHVTNLRTVGAFLCPTLLKVSRVKTVPEVVDNFVNKIIREQIEFREKNNVTRKDFIQLLIDLRRE
# KSDFGLSLEQCAANVFLFYVAGADTSTDAITYTVHELTHRPDLMKKVQAEIDDALEKSNGEINYDVLHEMKLLDNCVKETLRKYPFPILNRECTQDYQ
# VPDSKLIIRKGTPVIIPLQAFGMSEEYFPEPNRYLPERFDSSTKNYDEKAYIPFGDGPRNCIGSRMGSAVSKIGIIMLLSKFNFEATQGAEIGFARAQ
# IALAPENGISLKISNRIRNSI]
# end gene g1
###
# start gene g2
Cyp6d3  AUGUSTUS    gene    3848    4639    0.69    -   .   g2
Cyp6d3  AUGUSTUS    transcript  3848    4639    0.69    -   .   g2.t1
Cyp6d3  AUGUSTUS    stop_codon  3848    3850    .   -   0   transcript_id "g2.t1"; gene_id "g2";
Cyp6d3  AUGUSTUS    terminal    3848    4014    0.96    -   2   transcript_id "g2.t1"; gene_id "g2";
Cyp6d3  AUGUSTUS    initial 4072    4639    0.73    -   0   transcript_id "g2.t1"; gene_id "g2";
Cyp6d3  AUGUSTUS    intron  4015    4071    0.99    -   .   transcript_id "g2.t1"; gene_id "g2";
Cyp6d3  AUGUSTUS    CDS 3848    4014    0.96    -   2   transcript_id "g2.t1"; gene_id "g2";
Cyp6d3  AUGUSTUS    CDS 4072    4639    0.73    -   0   transcript_id "g2.t1"; gene_id "g2";
Cyp6d3  AUGUSTUS    start_codon 4637    4639    .   -   0   transcript_id "g2.t1"; gene_id "g2";
# coding sequence = [atgaccaacctagcgacgcagcagatgaagtatcgtgagaagaatgacttggctagaaaggatttcttgcagttgctga
# atgatcttcaccaagttgatttgtcagctgaagagtgcgcatcgaatgtgaatctgttctacactgcaggttcggaaaccaccaaatctacagtcatc
# tatactcttcacgaactagctcaccatccagaagttatgagacggcttgtggaagaagttgatgaatacgtcaagcaatcaggtggtgagattagcta
# cgatcttgttaagagtatgccatatttggacctgtgcgtgaaggaaaccctgagaaagtatcccggactgtttttcctgaaccggaagtgcacccacg
# actataaggttcccaactctcggctggtcatcaaaaagggtacccaaataattatcccgtcgatggcctacggcatggatgagcggtgtttcccgaat
# ccggagagctacatccccgaacgatttcttgaggagaccaaaaattacgacgaggacgcctacgcaccgtttggagaaggaccgcggaagtgtatcgc
# tcctcgaatgggaattttcgtcgccaaagtgaccttggtgaggctgctgtccaagtttcggttcgaggctacgcaagagctgaaggttgagtttgccc
# cctcggtgattccgctcgtgccgaaggatggagtcaggatgaagattcacaaaagaagtgtttggtaa]
# protein sequence = [MTNLATQQMKYREKNDLARKDFLQLLNDLHQVDLSAEECASNVNLFYTAGSETTKSTVIYTLHELAHHPEVMRRLVEE
# VDEYVKQSGGEISYDLVKSMPYLDLCVKETLRKYPGLFFLNRKCTHDYKVPNSRLVIKKGTQIIIPSMAYGMDERCFPNPESYIPERFLEETKNYDED
# AYAPFGEGPRKCIAPRMGIFVAKVTLVRLLSKFRFEATQELKVEFAPSVIPLVPKDGVRMKIHKRSVW]
# end gene g2
###
```

Figure 5.10: Nucleotide and amino acid sequences of *Cyp-Cx1* (g1.t1) and *Cyp-Cx2* (g2.t1) genes after re-annotation of the *Cyp6d3* gene using Augustus software.

5.5 Discussion

In this study, we investigated the insecticide susceptibility status and possible mechanisms involved with insecticide resistance in *C. quinquefasciatus* mosquitoes collected in a region of Uganda (Tororo), where recently non-official vector control interventions have been conducted against this species, while an ample insecticide-based program has been implemented targeting other important vector species such as *Anopheles gambiae* and *Anopheles funestus* (Morgan et al. 2010, Mawejje et al. 2013). The present study demonstrates that even in the absence of a concerted vector control intervention, a high level of resistance to pyrethroids and DDT is observed. Not surprisingly, the insecticide susceptibility observed in the *C. quinquefasciatus* population studied follows a pattern similar to that detected in *Anopheles* species, where for decades DDT and more lately pyrethroids have been the insecticides applied for malaria control (Pocquet et al. 2014, Fathian et al. 2015).

As reported in monitoring studies of resistance, lower resistance to carbamates insecticides have been observed across African mosquito populations, indicating that insecticides from this class are a possible alternative to tackle the evolution of insecticide resistance. Indeed, Recently, Kigozi et al. (2012) indicated a reduction in malaria prevalence in the Apac district from Uganda following bendiocarb rounds compared to previous rounds of IRS with DDT and alpha-cypermethrin, which strongly suggests that the local population is more susceptible to carbamate insecticides.

Nevertheless, recent evolution of resistance to carbamates insecticides in *Culex* populations possibly in response to malaria vector control or agriculture practices, has already been reported in other African countries such as Tanzania and Zambia (Norris and Norris 2011, Jones et al. 2012b). In these locations, moderate resistance to bendiocarb was observed, in contrast to high levels of resistance detected to both pyrethroids and DDT.

Hence, although carbamate insecticides might be a primary alternative to replace pyrethroids in vector control interventions, monitoring the evolution of resistance for these insecticides is critical to increase the lifespan and sustainability of future action based on this compounds.

Indeed, the genotyping data for the G119S mutation in the *Ace-1* gene suggests that Tororo field-collected mosquitoes have already been exposed to carbamates and/or organophosphates insecticides, the *Ace*-1 resistant allele 119S was detected at a frequency of approximately 20%. However, this frequency was much lower than the frequency observed in other vector mosquitoes where carbamates are routinely used in vector control (Edi et al. 2012). Hence, this figure might reflect the very recent introduction of carbamates insecticide for IRS across Ugandan North region (Indoor Residual Spraying of Insecticide and Malaria Morbidity in a High Transmission Intensity Area of Uganda. Ruth Kigozi, 2012). Nevertheless, is also important consider that the selection for the 119S resistant allele can be driven by insecticide exposure due to agricultural activities as described in *C. quinquefasciatus* populations from Iran (Vatandoost et al. 2004).

Although this study has detected a high frequency of the *Ace*-1 resistant allele in Tororo population, the association between the G119S genotyping and bendiocarb resistant phenotype indicated that around 60% of the resistant mosquitoes were wild-type for the G119S mutation, which suggests that the bendiocarb-resistant phenotype is caused by other mechanism of resistance such as metabolic, behavioural or reduced insecticide penetration as described in other studies (Ramphul et al. 2009, Ranson et al. 2011, David et al. 2013).

The synergist assays conducted in this study indeed indicates a potential involvement of metabolic resistance in the bendiocarb-resistant phenotype in *C. quinquefasciatus* from Tororo, as was observed an increase in mortality to bendiocarb when mosquitoes were pre-exposed to either TPP (a synergist of esterases) or PBO (a synergist of cytochrome P450s). Consequently, together the *Ace-1* genotyping and synergist assay data strongly suggested an

alternative mechanism of resistance to the well already known *Ace1*-119S target-site mutation.

To identify likely candidate genes associated with metabolic resistance a whole-transcriptomic profiling analysis of the resistant phenotype was conducted using a microarray platform. Among the list of up-regulated genes significant differently expressed between the resistant-phenotype and both controls (sympatric mosquitoes non-exposed and TPRI a susceptible strain), remarkably the GO enrichment analysis indicated a large proportion of GO terms related to detoxification and to regulatory transcription factors.

Due to the fitness cost associated with the 119S genotype (Labbe et al. 2007b, Labbé et al. 2014, Weetman et al. 2015), it is possible that alternatively a metabolic resistance have been mostly driven the evolution of resistance to bendiocarb in Tororo mosquitoes. The two most over-expressed genes identified in the whole-transcriptomic profiling of the resistant phenotype, *Cyp-Cx1* and *Cyp6n23* are especially relevant as many genes belonging to this gene family have been associated with insecticide metabolism in a variety of vector species such as *Anopheles*, *Aedes* and *Culex* (Strode et al. 2008, Mitchell et al. 2012). The likely association of this candidate cytochrome P450s with the carbamate resistant phenotype is also supported by the synergism effect of PBO on the mosquitoes susceptibility as PBO has previously been reported as a P450 inhibitor (Feyereisen 2012).

Nevertheless, so far, most CYPs associated with the insecticide resistance phenotype in mosquitoes, for example *Cyp6p3*, *Cyp9j32* and *Cyp6m10* are typically involved with metabolism of pyrethroids and DDT (Djouaka et al. 2008, Müller et al. 2008, Strode et al. 2008), while to date very few examples of bendiocarb metabolism by P450 have been reported. For instance, Edi et al. (2014) demonstrated that *Cyp6p3* is associated with the bendiocarb resistance phenotype in *A. gambiae* from Tiassalé, Cote d'Ivoire and confirmed its capability to metabolise bendiocarb. Additionally, two other *A. gambiae* P450s (*Cyp6z1*

and *Cyp6z2*) have also been demonstrated to be capable of metabolizing the carbamate insecticide carbaryl (Chiu et al. 2008).

Interestingly, both cytochrome P450s identified here belong to the CYP6 family, which includes most of the CYPs genes already described as insecticide metabolizers (Hemingway et al. 2004). Regardless, further analysis of the genomic and transcriptomic sequences of the top candidate gene CYP6D3 suggests annotation inaccuracy. After re-annotation, our analysis indicates that the CYP6D3 gene consists of two distinct CYP genes (here denominated as *Cyp-Cx1* and *Cyp-Cx2*) instead of one as suggested by the automated annotation of VectorBase. Since the annotation problem as observed in *Cyp6d3* might also occurs on other genes (see Neafsey et al. (2015) for annotation deficiencies of recent *Anopheles* sequencing), the design of our whole-transcriptomic microarray may lack some transcript information. A future review of *C. quinquefasciatus* gene annotation, especially of gene families linked to insecticide resistance might be required for effective utility of microarrays for the monitoring of insecticide resistance. Whilst the increased usage of RNASeq for differential gene expression analyses would aid in this, the typical RNA-Seq pipeline maps reads to annotated gene sets and hence a robust annotation of these gene families would still be beneficial.

BLAST analysis of the amino acid sequence of both predicted genes (*Cyp-Cx1* and *Cyp-Cx2*) shows higher similarity to genes of the *Cyp6* family, while the both predicted genes are closer related to the sub-family Z instead of D as previously reported in VectorBase. Remarkably, the predicted gene *Cyp-Cx1*, which includes the microarray probe, is very closely related to four P450s already validated *in vitro* as insecticide metabolizers; two isolated from *A gambiae Cyp6z3* (permethrin) and *Cyp6z1* (DDT and carbamate) and from *A. aegypti Cyp6z8* and *Cyp6z6* (Deltamethrin). Although we identified a high similarity between *Cyp-Cx1* and other P450s already associated with metabolic resistance, it is not

possible infer a direct evidence of the top candidate to bendiocarb metabolism as structural differences and changes in insecticide recognition site between P450s of the same family could result in distinct binding activities and insecticide selectivity (Lertkiatmongkol et al. 2011). Consequently, to confirm the possible role of both candidate genes in bendiocarb resistance, further analysis such as *in vitro* assays to verify their capability to metabolize insecticide as well as characterization of the possible mechanisms involved in their over-expression need to be conducted.

5.6 Conclusion

Our data demonstrate that although Ugandan *C. quinquefasciatus* mosquitoes is not targeted by a specific, local vector control program, a high level of insecticide resistance was identified in the studied population. This indicates that application of insecticide to control other species with public health importance through indoor residual spray (IRS) and insecticide treated nets (ITNs), or through application of insecticides for agricultural purposes, could be driving the evolution of insecticide resistance in this population. This study also provides strong evidence that a metabolic mechanism is associated with the bendiocarb resistant phenotype observed in Tororo. Lastly, by a whole-transcriptomic analysis we identifed two new candidate genes belonging to the cytochrome *CPY6* gene family associated with metabolic resistance to bendiocarb.

Chapter VI

Conclusion

Given the recognized contribution of vector control campaigns in mitigating vector-borne disease transmission, increased resistance to insecticides represents a major challenge for the sustained effectiveness of both current and future actions to reduce the global incidence of vector-borne diseases (Bockarie et al. 2009, Van den Berg et al. 2012, Karunamoorthi and Sabesan 2013). To overcome the threat of insecticide resistance, development of molecular tools to monitor and understand the mechanisms driving resistance are imperative for effective decision-making. In the present study, novel DNA-based and transcriptomic tools were developed and applied for monitoring resistance and enhancing our understanding of the evolutionary basis of insecticide resistance in *C. quinquefasciatus.* Identifying the mechanisms underlying insecticide resistance in *C. quinquefasciatus* is especially important since effective control interventions toward this species assist to decrease the burden of urban transmission of lymphatic filariasis (LF), which is recognized as the second largest cause of global disability and retains a heavy social and economic impact in endemic regions across the tropics and subtropics (Rebollo and Bockarie 2013, WHO 2013b).

This research was designed to address two main questions. Firstly, to infer the likely impact of insecticide selection pressure on the genetic diversity and population structure of Ugandan *C. quinquefasciatus* mosquitoes, which was conducted through genotyping field-collected mosquitoes using neutral microsatellite markers, as well as typing two well-known insecticide resistance associated markers *Vgsc-1014F* and *Ace1*-119S (Casida and Durkin

2013, Scott et al. 2015). The second aim was to investigate the basis of the molecular mechanisms driving the evolution of insecticide resistance to four classes of insecticide (carbamates, organophosphates, pyrethroids and organochlorides), which are recommended for mosquito vector control (WHO 2012b). This was conducted by combining phenotypic (insecticide bioassays) and genotypic (resistance associated-markers *Vgsc-1014F* and *Ace1*-119S) assays. In addition, adult mosquitoes were also genotyped to characterize the *Vgsc* gene copy number. A whole-transcriptome profiling of bendiocarb resistant mosquitoes was also utilized to identify candidate genes related to metabolic resistance.

The population genetic study carried out used a five-six microsatellite multiplex reactions (N loci = 30) demonstrated similar levels of genetic diversity in the four studied *C. quinquefasciatus* populations from Uganda's Eastern-Southwest regions. These results strongly suggest that demographic effects such as genetic drift, effective population size and bottleneck effects have not differentially impacted the genetic composition of these populations.

Despite the observed intense gene flow with a strong pattern of isolation-by-distance, a low genetic structure among Ugandan *C. quinquefasciatus* populations was detected by the microsatellite markers. On the other hand, for both resistant-associated markers (*Vgsc-1014F* and *Ace1*-119S) it was not possible to identify a clear pattern of geographic distribution, which indicates that across the studied location's mosquitoes have been exposed to a heterogeneous insecticide selection pressure imposed by insecticides from distinct classes with variation in the level of exposure creating a pattern of local adaptation. Nevertheless, is also important to consider that the non-structured distribution of insecticide-resistant alleles in the studied populations might also reflect distinct environmental features across the mosquito collection locations, such as from rural to urban dwellings with distinct levels of human populous, which could be reflected in mosquito population density.

The heterogeneous distribution of both resistant-associated alleles described herein across the Ugandan populations is especially worrying with regard to future vector control strategies since resistance can impose additional challenges for developing a nationwide control program. For instance, the non-structured distribution of both associated-resistant markers (*Vgsc-1014F* and *Ace1*-119S) with distinct frequency across all populations can limit the use of strategies such as rotation of insecticides (insecticides with different modes of action are rotated annually). On the other hand, the results of the present study emphasize the importance of population genetics studies to understand the local pattern of insecticide resistance evolution, and thus provide valuable information to develop more effective control action for each local population. Based on the present data, the local government could, for instance, apply a mosaic IRS approach (insecticides from different classes are used in distinct local populations despite nearby geographic location) to increase the effectiveness of control actions, with decision-making for the most suitable class of insecticide for each location based on frequency and geographic distribution of resistant-allele markers.

Surprisingly, after genotyping field-collected Ugandan mosquitoes for the 1014 variant in the *Vgsc* gene using two newly-developed assays: a TaqMan allelic discrimination assay and a pyrosequencing genotyping assay, both methods indicate that some mosquitoes assessed have likely undergone duplication of the *Vgsc* gene. This figure was also suggested from Sanger sequencing and haplotype analysis, which detected one individual containing at least three *Vgsc* haplotypes simultaneously. Indeed, the results of the *Vgsc*-CN assay corroborate the *Vgsc* gene copy number suggested by the other genotyping methods, with variation in copy number (CNV = 3 or 4) detected in around 10% of the mosquitoes investigated. This result strongly indicates the need to investigate whether the CNV observed in the *Vgsc* in *C. quinquefasciatus* is associated with changes to the level of resistant phenotypes, as already reported for example for the *Ace-1* gene in *C. quinquefasciatus*

(Labbe et al. 2007a), as well as in other insects like *T. urticae* (Kwon et al. 2010) and *A. gossypii* (Shang et al. 2014). Hence, the *Vgsc*-assay described in this study is an important tool for inferring CNV in natural populations, which will facilitate future studies to elucidate the possible impact of the *Vgsc* copy number to the level of insecticide resistance to pyrethroids and organochlorines in *C. quinquefasciatus*.

In addition to the enquiry of the likely evolution of insecticide resistance mediated by target-site mutations, the possible impact of metabolic resistance was also verified. After genotypic/ phenotypic association tests were conducted between the 119S resistant allele and bendiocarb-selected mosquitoes, it was detected that around 60% of resistant mosquitoes were wild-type (G119), strongly indicating that in Ugandan mosquitoes the carbamate insecticide resistant-phenotype is likely driven by an independent mechanism of resistance. The likely involvement of an alternative mechanism to the already well known *Ace*-1 119S target-site mutation was also supported by synergist bioassays, which showed an increase in mortality to bendiocarb in mosquitoes pre-exposed to either TPP (a synergist of esterases) or PBO (a synergist of cytochrome P450s), indicating a probable resistance mediated by detoxification enzymes (Moores et al. 2009, Edi et al. 2014, Pereira et al. 2014).

To further investigate the apparent role of metabolic resistance in the Ugandan bendiocarb resistant-phenotype, this work as far as I know is the first to conduct whole-transcriptomic analysis using a microarray platform on field-collected *C. quinquefasciatus.* Remarkably, among the top candidate genes of the bendiocarb-resistant transcriptome profiling two P450s (*Cyp-Cx1* [see details in chapter V] and *Cyp6n23*) were identified with the highest over-expression level in the resistant samples, with both candidate genes belong to the *Cyp6* family, which includes most of the *Cyp* genes already described as insecticide metabolizers (Hemingway et al. 2004). Interestingly, genomic sequence of the top candidate gene *Cyp-Cx1* is very closely related to two P450s already validated *in vitro* as insecticide

metabolizers; CYP6Z1 (DDT and carbamate metaboliser) from *A. gambiae* (David et al. 2005) and CYP6Z6 (Deltamethrin metaboliser) from *A. aegypti* (Chandor-Proust et al. 2013). Nevertheless, it is important to bear in mind that although a high similarity between *Cyp-Cx1* and other P450s already associated with metabolic resistance was identified, it is not possible to infer directly that this top candidate gene can metabolise bendiocarb, as structural differences and changes in insecticide recognition site between P450s of the same family could result in distinct binding activities and insecticide selectivity (Lertkiatmongkol et al. 2011).

Although this work applied a custom-designed whole-transcriptomic microarray to identify candidate genes involved in insecticide metabolic resistance, it is important to note that the microarray designed may lack some transcript information triggered by inaccuracy of gene annotation as was described for the candidate gene *Cyp6d3* (*Cyp-Cx*1); herein annotated as two distinct *Cyp* genes. This aspect emphasizes the necessity for a future review of *C. quinquefasciatus* gene annotation, especially of gene families linked to insecticide resistance (as was necessary for the recently sequenced 16 *Anopheles* genomes, see (Neafsey et al. 2015)) to more effectively apply microarrays and other transcriptomic tools (which map against known transcripts) for the identification of candidate insecticide resistance genes.

Whilst the DNA-based and transcriptomic tools developed in this study permitted for the first time quantification of *Vgsc* gene copy number in field-collected mosquitoes, as well as resulting in the identification of new metabolic resistance candidate gene (*Cyp-Cx1*), complementary work is required to improve the field-applicability of these markers for monitoring of insecticide resistance. Firstly, for the *Vgsc* copy number variation detected in the investigated populations, additional studies including genotypic/phenotypic association tests are required before further conclusions about the relationship between the *Vgsc* copy

number and insecticide resistance level can be made. Additionally, future research might also be conducted to investigate the impact of the *Vgsc* copy number on mosquito fitness.

In the context of the candidate gene (*Cyp-Cx1*) future work is also necessary for functional enzymatic characterization for insecticide metabolism. Thus, additional investigation on this can be done through *in vitro* metabolism assay (Mitchell et al. 2012), or alternatively through *in vivo* characterization by transgenic gene expression (Riveron et al. 2013). For both the *Vgsc* assay and *Cyp-Cx1* candidate gene, if this additional research confirms their role in the resistant phenotype, these studies can be complemented by including *C. quinquefasciatus* mosquitoes from distinct geographic backgrounds to verify a possible broad field-applicability of these markers.

The present study demonstrated that the evolution of insecticide resistance in *C. quinquefasciatus* is driven by multiple mechanism of resistance, which together may pose a severe threat to the effectiveness of the ongoing vector control interventions.

Appendices

Appendix3-A

Table 3-A: Microsatellite locus characterization across all *C. quinquefasciatus* populations.

Locus	**Allele number**	***Rs***	***PIC***	H_E	***P***	F_{IS}	F_{IT}	F_{ST}	R_{ST}
MCQ 1	4	4.000	0.418	0.490	0.000	0.485	0.487	0.024	0.010
MCQ 2	3	2.884	0.332	0.405	0.000	0.294	0.293	0.025	0.007
MCQ 3	7	6.910	0.454	0.461	0.000	0.252	0.247	0.049	0.034
MCQ 4	4	3.642	0.544	0.621	0.3184	0.065	0.065	0.011	-0.005
MCQ 5	6	5.640	0.641	0.692	0.000	0.199	0.191	0.016	0.030
MCQ 8	5	4.642	0.604	0.664	0.000	0.288	0.294	0.016	-0.015
MCQ 9	6	5.650	0.665	0.709	0.0339	0.141	0.138	0.026	0.029
MCQ 10	8	7.642	0.652	0.694	0.000	0.404	0.407	0.017	-0.006
MCQ 11	7	6.581	0.493	0.545	0.0535	0.094	0.096	0.045	-0.001
MCQ 13	6	5.910	0.602	0.647	0.1393	0.154	0.153	0.036	0.073
MCQ 16	7	7.000	0.696	0.724	0.0281	0.106	0.103	0.014	-0.003
MCQ 19	9	8.596	0.563	0.594	0.0014	0.046	0.044	0.016	0.038
MCQ 20	3	3.000	0.554	0.623	0.000	0.292	0.290	0.018	0.009
MCQ 21	12	11.14	0.617	0.607	0.000	0.303	0.301	0.072	0.113
MCQ 22	9	8.873	0.688	0.723	0.000	0.214	0.214	0.029	0.080
MCQ 23	5	4.600	0.436	0.513	0.000	0.439	0.436	0.009	0.019
MCQ 24	6	5.983	0.653	0.691	0.1059	0.035	0.035	0.017	-0.004
MCQ 25	5	4.985	0.463	0.546	0.000	0.313	0.310	0.012	-0.005
MCQ 26	10	9.873	0.825	0.830	0.6902	0.086	0.084	0.027	0.030
MCQ 28	4	4.000	0.553	0.606	0.000	0.594	0.596	0.022	0.004

MCQ 29	5	4.909	0.61	0.662	0.0163	0.07	0.069	0.029	-0.009
MCQ 31	6	5.642	0.717	0.741	0.2309	-0.021	-0.023	0.030	-0.004
MCQ 33	4	4.000	0.368	0.424	0.000	0.649	0.662	0.032	-0.010
MCQ 34	8	7.541	0.625	0.680	0.000	0.264	0.271	0.016	0.008
MCQ 36	4	3.642	0.355	0.429	0.2056	0.012	0.013	0.004	-0.003
MCQ 37	4	4.000	0.361	0.376	0.693	0.052	0.054	0.039	0.037
MCQ 39	7	6.634	0.527	0.567	0.8118	-0.035	-0.040	0.012	0.007
MCQ 41	6	5.989	0.651	0.703	0.1518	-0.018	-0.022	0.020	0.018
MCQ 42	8	7.636	0.548	0.560	0.8428	-0.028	-0.030	0.053	-0.003
MCQ 45	8	7.7	0.589	0.630	0.0158	0.025	0.021	0.019	0.001

(*R*s), allelic richness. (PIC), polymorphic Information content. (H_E), expected heterozygosity. (F_{IS}, F_{IT}, F_{ST}) Weir and Cockerham's (1984) F-Statistics. (*P*), probability value using Fisher`s method for Hardy-Weinberg departures.

Appendix 3-B

Table3-B: Summary of microsatellite variation in different Ugandan populations of *C. quinquefasciatus.*

	Location															
	Jinja				Kampala				Kanungu				Tororo			
Locus	***A***	***H*E**	***P***	***F*IS**	***A***	***H*E**	***P***	***F*IS**	***A***	***H*E**	***P***	***F*IS**	***A***	***H*E**	***P***	***F*IS**
MCQ 1	4	0.532	0.000*	0.504	3	0.459	0.002^{NS}	0.415	2	0.347	0.007^{NS}	0.479	4	0.586	0.000*	0.523
MCQ 2	2	0.420	0.717	0.060	3	0.489	0.002^{NS}	0.328	2	0.284	0.052	0.362	2	0.400	0.015^{NS}	0.418
MCQ 3	5	0.537	0.015^{NS}	0.240	6	0.403	0.002^{NS}	0.334	5	0.617	0.000*	0.181	5	0.257	0.545	0.091
MCQ 4	3	0.630	0.647	0.083	3	0.596	0.677	0.020	4	0.635	1.000	-0.036	3	0.589	0.195	0.208
MCQ 5	5	0.655	0.045^{NS}	0.230	6	0.675	0.001*	0.370	5	0.724	0.101	-0.049	5	0.671	0.005^{NS}	0.228
MCQ 8	4	0.679	0.218	0.221	5	0.674	0.132	0.066	4	0.661	0.000*	0.582	4	0.600	0.016^{NS}	0.310
MCQ 9	5	0.716	0.759	0.058	6	0.693	0.046^{NS}	0.190	5	0.661	0.241	-0.061	5	0.728	0.012^{NS}	0.306
MCQ 10	7	0.717	0.000*	0.398	7	0.576	0.134	0.221	7	0.734	0.000*	0.451	8	0.702	0.000*	0.522
MCQ 11	5	0.566	0.614	0.022	5	0.552	0.399	-0.017	6	0.530	0.158	0.093	6	0.502	0.014^{NS}	0.226
MCQ 13	5	0.554	0.398	0.175	5	0.691	0.523	0.123	5	0.719	0.689	0.086	5	0.588	0.241	0.162
MCQ 16	7	0.744	0.457	0.094	6	0.643	0.138	0.127	6	0.706	0.213	0.032	7	0.765	0.176	0.152
MCQ 19	7	0.481	0.890	-0.050	8	0.586	0.003^{NS}	0.119	6	0.660	0.096	-0.230	7	0.616	0.004^{NS}	0.328
MCQ 20	3	0.636	0.513	0.129	3	0.625	0.006	0.355	3	0.566	0.000*	0.499	3	0.626	0.125	0.193
MCQ 21	10	0.461	0.000*	0.414	10	0.741	0.079	0.208	7	0.712	0.023^{NS}	0.163	5	0.474	0.012^{NS}	0.330

MCQ 22	5	0.637	0.013^{NS}	0.359	4	0.611	0.424	0.060	9	0.811	0.062	0.095	8	0.790	0.007*	0.294
MCQ 23	4	0.500	0.000*	0.598	4	0.531	0.000*	0.367	3	0.494	0.000*	0.475	3	0.488	0.064	0.329
MCQ 24	6	0.702	0.004^{NS}	0.108	6	0.715	0.445	-0.069	5	0.702	0.445	-0.010	6	0.609	0.357	0.105
MCQ 25	4	0.508	0.001*	0.386	5	0.554	0.055	0.272	3	0.565	0.365	0.200	4	0.521	0.002^{NS}	0.405
MCQ 26	10	0.839	0.465	-0.033	9	0.826	0.236	0.103	9	0.820	0.315	0.168	10	0.789	0.251	0.046
MCQ 28	4	0.635	0.000*	0.658	4	0.639	0.000*	0.488	4	0.605	0.000*	0.698	4	0.491	0.000*	0.531
MCQ 29	4	0.676	0.645	-0.127	4	0.600	0.090	0.158	5	0.637	0.007^{NS}	0.145	4	0.698	0.910	0.060
MCQ 31	5	0.697	0.972	-0.107	5	0.730	0.259	-0.015	6	0.723	0.613	-0.015	5	0.778	0.647	-0.035
MCQ 33	3	0.316	0.000*	0.795	3	0.377	0.001*	0.465	3	0.501	0.000*	0.713	4	0.446	0.000*	0.678
MCQ 34	6	0.708	0.001*	0.409	6	0.588	0.000*	0.271	5	0.662	0.000*	0.115	6	0.708	0.003^{NS}	0.285
MCQ 36	3	0.421	0.860	-0.087	2	0.397	1.000	-0.019	3	0.388	0.747	0.086	4	0.488	0.279	0.096
MCQ 37	4	0.483	0.694	0.102	4	0.451	0.249	-0.097	3	0.145	1.000	-0.048	3	0.406	0.631	0.106
MCQ 39	6	0.556	0.245	0.090	5	0.485	1.000	-0.067	5	0.601	0.724	-0.096	7	0.601	0.763	-0.081
MCQ 41	5	0.677	0.707	-0.068	5	0.668	0.288	0.024	4	0.743	0.094	-0.057	4	0.690	0.914	-0.016
MCQ 42	6	0.535	0.869	-0.036	8	0.534	0.558	-0.053	4	0.407	0.676	-0.059	6	0.738	0.655	-0.127
MCQ 45	6	0.658	0.003^{NS}	-0.062	7	0.500	0.181	0.056	5	0.682	0.321	0.110	7	0.649	0.006^{NS}	-0.041

(*A*), number of alleles. (H_E), expected heterozygosity. (*P*), probability value using Fisher`s method for Hardy-Weinberg departures. (FIS), Weir and Cockerham's (1984) inbreeding coefficient.

Appendix3-C

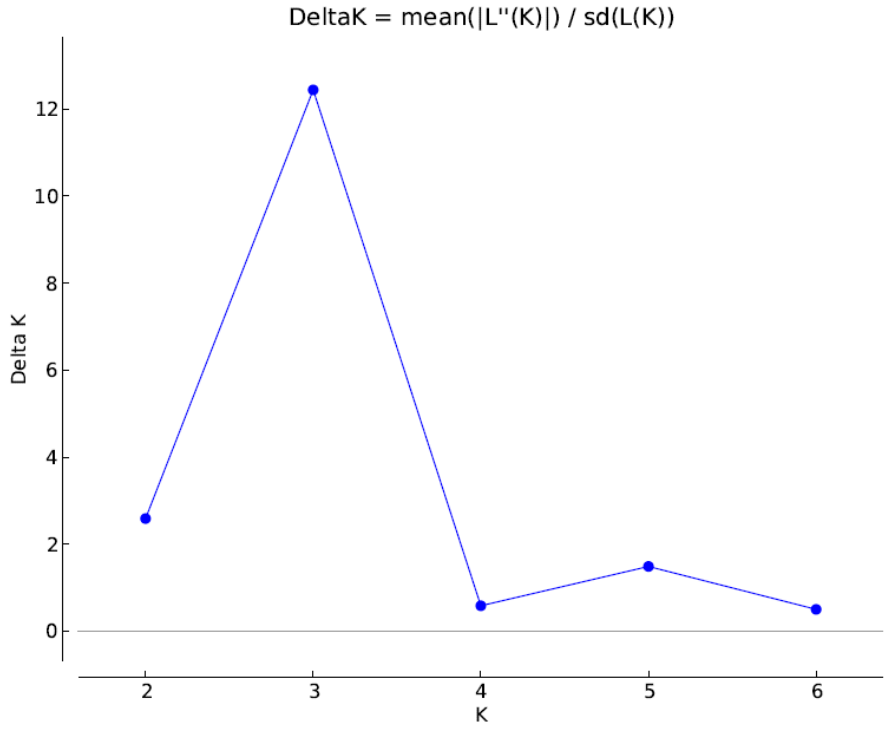


Figure 3-C: delta K results for the optimal value of K for structure analysis. Mean posterior probabilities of twenty runs for each K, K = 1 to K = 7

Appendix 3-D

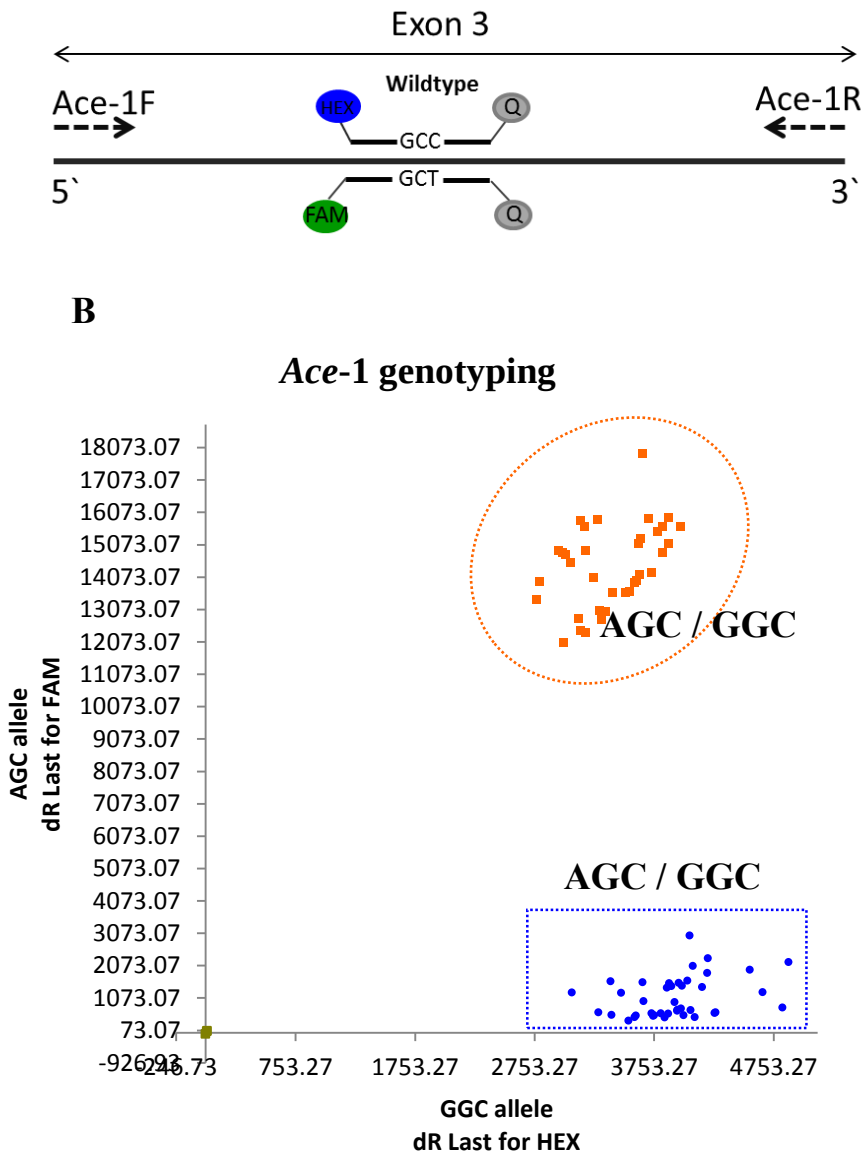


Figure 3-D: *Ace*-1 target-site mutation (G119S) genotyping in *C. quinquefasciatus*. A) Scheme of the TaqMan probes for 119S allelic discrimination. Susceptible allele-specific probe labelled with HEX and resistant allele-specific probe labelled with 6-FAM. B) Allelic discrimination for the 119 codon position. X-axis designates the susceptible allele and the y-axis designates the resistant allele. Blue dots indicate individuals homozygous for the wild-type and orange squares indicates individuals heterozygous for the mutation.

Appendix 4-A

Construction of the *Vgsc-Pka* plasmid standard for qPCR absolute quantification

PCR conditions

The plasmid used as standard DNA for construction of the standard curve for qPCR absolute quantification was developed by the fusion of partial fragments of the *Vgsc* and *Pka* gene including the PCR and probe region of the *Vgsc*-CN assay. The fusion PCR reaction was carried out on two steps. The first round was conducted in a final volume of 50 µl reaction including: approx 40 ng of gDNA, 1 X of 5X phusion HF buffer, 200 µM of each dNTP, 0.4 µM of each primer (Cx:*Vgsc-Pka*-P1: 5`-GCAAAGGATATAACAAAGCAGT-3` and Cx: *Vgsc-Pka* -P2: 5`-AAACCATCTATGCCCTTTGATCTAAGTAGATCCTTTA GTTCTGAC-3`) and 0.02 U/µl of Phusion DNA polymerase. On the second step, was used the same condition of the first with the first PCR product diluted 1:10 as template and the external primers (Cx: *Vgsc-Pka*-P3: 5`-TCAAAGGGCATAGATGGTTTACAACGTGGA CCGCTTC-3`and Cx: *Vgsc-Pka*-P4: 5`-TGCAGTCCCACATGGATTC-3`). Both steps were amplified with the following conditions: 98 °C for 30 sec, 25 cycles of 98 °C for 10 sec, 57 °C for 30 sec and 72 °C for 15 sec, with a final extension of 72 °C for 5 min. Lastly, the plasmid containing the *Vgsc-Pka* fragment of 488 bp (Figure 4A) was linearized with the *Alu* I restrict enzyme follow by purification of the digested product extracted from a 2% agarose gel.

5`–CTATGTGGAACTCCAGAATATTTGGCACCAGAAATAATTTTAAGCAAAG
GATATAACAAAGCAGTTGACTGGTGGGCATTAGGTGTTCTTGTGTACGAGA
TGGCAGCCGGATATCCACCTTTTTTTGCTGATCAGCCAATACAAATTTATGA
AAAAATTGTTTCAGGAAAGGTACGATTTCCATCTCATTTCGGGTCAGAACTA
AAGGATCTACTTAGAAATCTTCTACAAGTTGATTTAACAAAACGTTACGGA
AATCTAAAAGCAGGAGTTAACGACATCAAAGGGCATAGATGGTTTACAACG
TGGACCGCTTCCCGGACAAGGACCTGCCACGGTGGAACTTCACCGACTTCA
TGCACTCATTCATGATCGTGTTCCGGGTGCTGTGCGGCGAGTGGATCGAATC
CATGTGGGACTGCATGCTGGTGGGCGACGTGTCCTGCATTCCGTTCTTCTTG
GCCACCGTAGTGATAGGAAATTTAGTC-3’

Figure 4-A: Partial sequence of the *Vgsc-Pka* plasmid, which correspond to the linked partial fragments of the *Vgsc* and *Pka* gene obtained by fusion PCR

Appendix 4-B

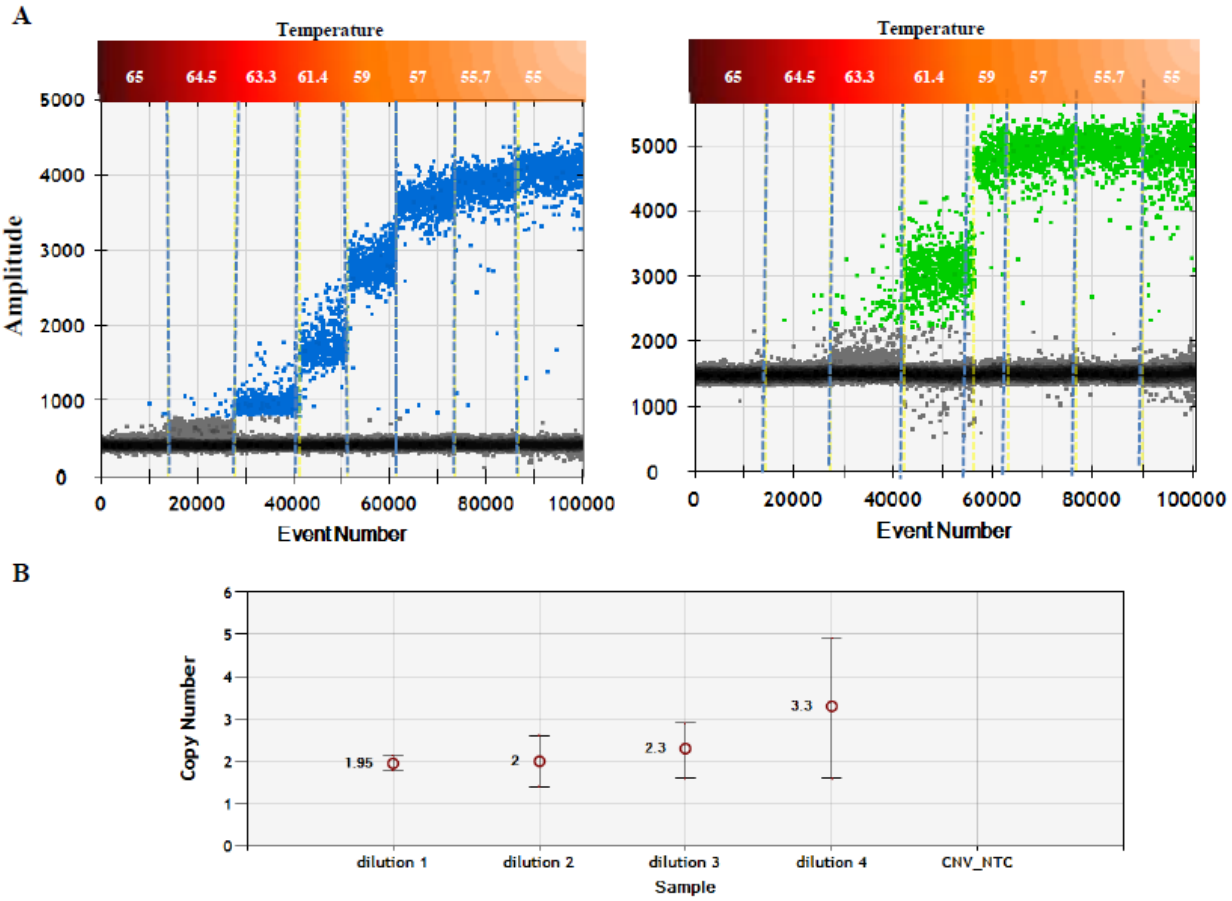


Figure 4-B: Optimization of annealing temperature and concentration of gDNA for application of the *Vgsc*-CN assay on ddPCR analysis. A) Thermal gradient to identify appropriated fluorescent droplet amplitudes for the *Vgsc* (blue dots, FAM channel) and *Pka* (green dots, VIC channel) genes. B) Two-fold *Vgsc-Pka* plasmid dilution to identify DNA concentration to precise CN quantification. Dilution 1 to 4, correspond to concentration ranging from 17 x 10^3 copies/µl to 2x10^3 copies/µl.

Appendix 4-C

RQPS Reference-query probe design and construction

The RQ-probe design for *C. quinquefasciatus Vgsc* CNV analysis was constructed by the fusion of a partial fragment of the exon 1 of the *Pka* gene (reference gene with single copy in *Culex* genome) and of the exon 20 of the *Vgsc* gene to a fragment of 386 bp of the actin gene (CPIJ012573) used as stuffer DNA (A fragment of any gene used to linked the fragments of both genes of interest) (Figure 4C-1). On the selected fragment of each gene was introduced a SNP that differ the RQ-probe allele construction from the gDNA allele as shown in (Figure 4C-1).

The RQ plasmid construction was executed in two rounds of PCR. The first step was conducted in 50 µl reaction which contained: approx 40 ng of genomic DNA, 1 X of 5X phusion HF buffer, 200 µM of each dNTP, 0.4 µM of each primer Cx.ref-FI and Cx.quer-RI (Table 1- 4C) and 0.02 U/µl of Phusion DNA polymerase. The reaction conditions were 98 °C for 30 sec, 25 cycles of 98 °C for 10 sec, 58 °C for 30 sec and 72 °C for 15 sec, with a final extension of 72 °C for 5 min. The second reaction of the fusion PCR was carried out using the same concentration and condition of the first, with the exception for the genomic DNA and primers, which were replaced by the product of the first reaction diluted 1:10 and the primers Cx.ref-FII and Cx.quer-RII (Table 4C-1).

The PCR product yield on the second round of the fusion PCR was loud on 2% agarose gel, and then the 551 bp of the fused fragment (Figure 4C-2) was extracted and purified followed by cloning in the pJet 1.2 PCR vector (Thermo Scientific). Finally, after the plasmid has been purified and sequenced to confirm the correct insertion of the chemeric QR-probe in the vector, it was linearized with the *Alu I* restrict enzyme.

Table 4C-1: Primer for RQ plasmid construction by fusion PCR**.**

Primer	Sequence 5`-3`
	Fusion PCR Step I
Cx.ref-FI	GATGCCGCAGAAAGTGTAAAACAATTCCTGGATCAAGCTAAAG AAGATTTGGTATCCTCACCCTGAAGT
Cx.quer-RI	AGGTCACGTCGCCCACCAGCATGCAGTCCCACATGGATTCGATC TCATCAGGTAGTCGGTCAGAT
	Fusion PCR Step II
Cx.ref-FII	ATGGGAAACAACGCAACTTCAACAAATAAAAAAGTAGATGCCG CAGAAAGTGTAAAACA
Cx.quer-RII	GAATTCTTCCTATCACTACGGTGGCCAAGAAGAACGGAATGCAG GtCACGTCGCCCACCAG

A)

5`–ATGGGAAACAACGCAACTTCAACAAATAAAAAAGTAGATGCCGCAGAAAGTGTAA AA**S**AATTCCTGGATCAAGCTAAAGAAGATT–3`

B)

5`–GATCGAATCCATGTGGGACTGCATGCTGGTGGGCGACGTG**W**CCTGCATTCCGTTCT TCTTGGCCACCGTAGTGATAGGAA–3`

C)

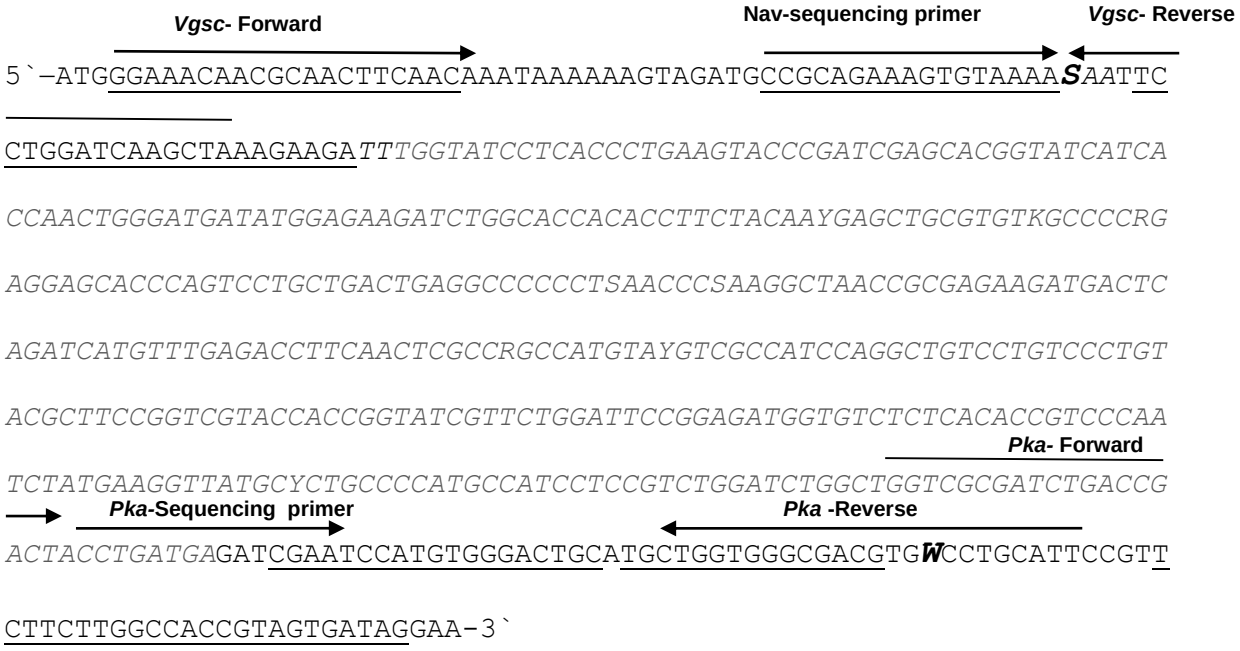
5`–TGGTATCCTCACCCTGAAGTACCCGATCGAGCACGGTATCATCACCAACTGGGATG ATATGGAGAAGATCTGGCATCACACCTTCTACAATGAGCTGCGTGTTGCCCCAGAGGA GCACCCAGTCCTGCTGACTGAGGCCCCCCTGAACCCCAAGGCTAACCGCGAGAAGATG ACTCAGATCATGTTTGAGACCTTCAACTCGCCAGCCATGTATGTTGCCATCCAGGCTGT CCTGTCCCTGTACGCTTCCGGTCGTACCACCGGTATCGTTCTGGATTCCGGAGATGGTG TCTCTCACACCGTCCCAATCTATGAAGGTTATGCTCTGCCCCATGCCATCCTCCGTCTG GATCTGGCTGGTCGCGATCTGACCGACTACCTGATGA–3`

Figure 4C-1: Sequences of genomic DNA used for construction of the RQPS probe. A and B) partial fragment of the *Pka*KA and *Vgsc* gene, respectively with bold and underlined bases correspond to a SNP introduced to differ RQ-alleles from gDNA sequence. C) Partial fragment of the Actin gene (CPIJ012573) used as stuffer region. S; G→C. W, T→A.

A)

5`–ATGGGAAACAACGCAACTTCAACAAATAAAAAAGTAGATGCCGCAGAAAGTGTAAAACAAT
TCCTGGATCAAGCTAAAGAAGATTTGGTATCCTCACCCTGAAGTACCCGATCGAGCACGGTATCAT
CACCAACTGGGATGATATGGAGAAGATCTGGCATCACACCTTCTACAATGAGCTGCGTGTTGCCCCAG
AGGAGCACCCAGTCCTGCTGACTGAGGCCCCCCTGAACCCCAAGGCTAACCGCGAGAAGATGACTCA
GATCATGTTTGAGACCTTCAACTCGCCAGCCATGTATGTTGCCATCCAGGCTGTCCTGTCCCTGTACG
CTTCCGGTCGTACCACCGGTATCGTTCTGGATTCCGGAGATGGTGTCTCTCACACCGTCCCAATCTAT
GAAGGTTATGCTCTGCCCCATGCCATCCTCCGTCTGGATCTGGCTGGTCGCGATCTGACCGACTACCT
CATGAGATCGAATCCATGTGGGACTGCATGCTGGTGGGCGACGTGACCTGCATTCCGTTCTTCTT
GGCCACCGTAGTGATAGGAA–3`

B)



5`–ATGGGAAACAACGCAACTTCAACAAATAAAAAAGTAGATGCCGCAGAAAGTGTAAAASAATTC

CTGGATCAAGCTAAAGAAGATTTGGTATCCTCACCCTGAAGTACCCGATCGAGCACGGTATCATCA

CCAACTGGGATGATATGGAGAAGATCTGGCACCACACCTTCTACAAYGAGCTGCGTGTKGCCCCRG

AGGAGCACCCAGTCCTGCTGACTGAGGCCCCCCTSAACCCSAAGGCTAACCGCGAGAAGATGACTC

AGATCATGTTTGAGACCTTCAACTCGCCRGCCATGTAYGTCGCCATCCAGGCTGTCCTGTCCCTGT

ACGCTTCCGGTCGTACCACCGGTATCGTTCTGGATTCCGGAGATGGTGTCTCTCACACCGTCCCAA

TCTATGAAGGTTATGCYCTGCCCCATGCCATCCTCCGTCTGGATCTGGCTGGTCGCGATCTGACCG

ACTACCTGATGAGATCGAATCCATGTGGGACTGCATGCTGGTGGGCGACGTGWCCTGCATTCCGTT

CTTCTTGGCCACCGTAGTGATAGGAA-3`

Figure 4C-2: Fusion PCR and pyrosequencing primer locations on the RQ-plasmid. A) Position of both primer sets used on the fusion PCR. Sequences in red and blue font colour correspond to partial sequences of the *Vgsc* and *Pka*, respectively. Sequence in Black correspond to the partial fragment of the action gene used to link both *Vgsc* and *Pka* B) Sequence of the RQ-plasmid probe showing the location of the SNP introduced in both *Vgsc* (S; G to C) and *Pka* (W, T to A) and PCR and sequencing primers annealing positions used for the RQPS method.

Appendix 4-D

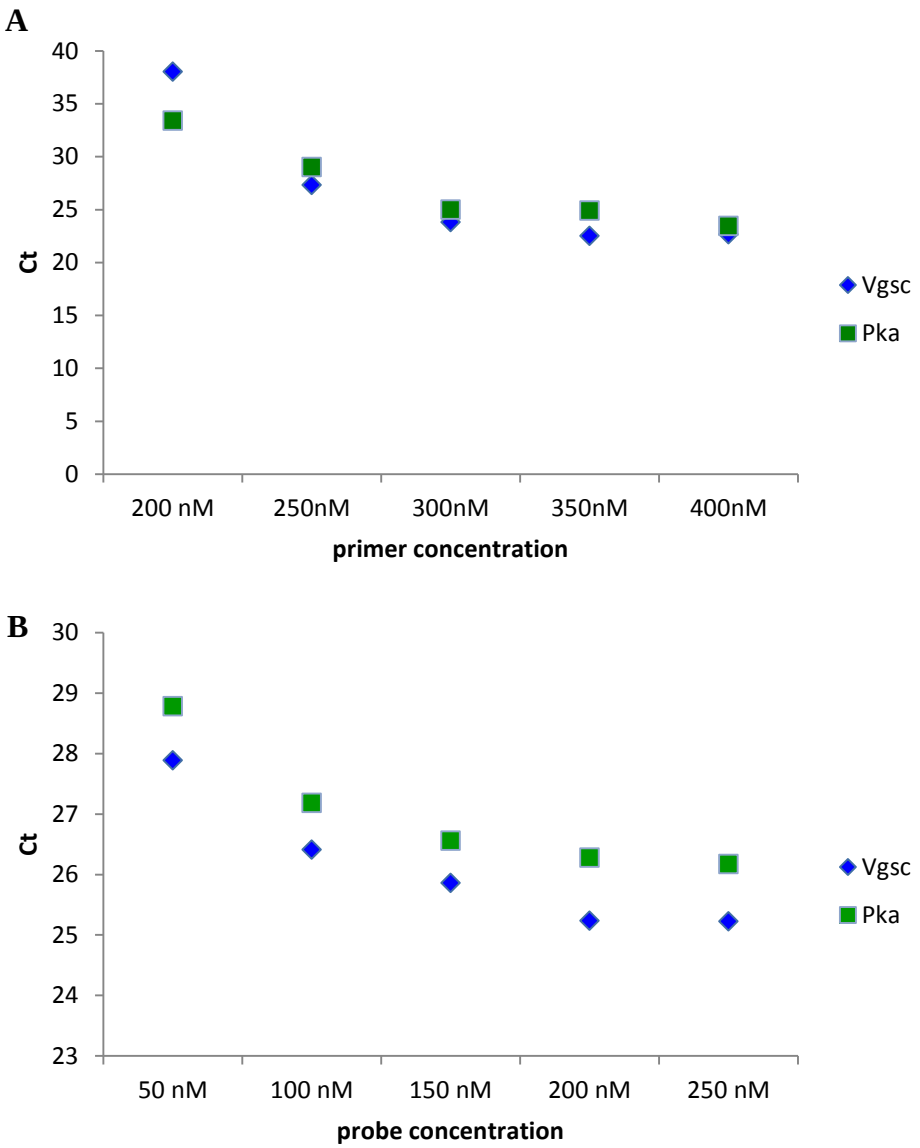


Figure 4-D: Primer and probe validation. A) Primer set titration to identify concentration with similar pattern of amplification based on SYBR-GREEN detection. B) Probe titration to identify early and constant Ct values.

Appendix 4-E

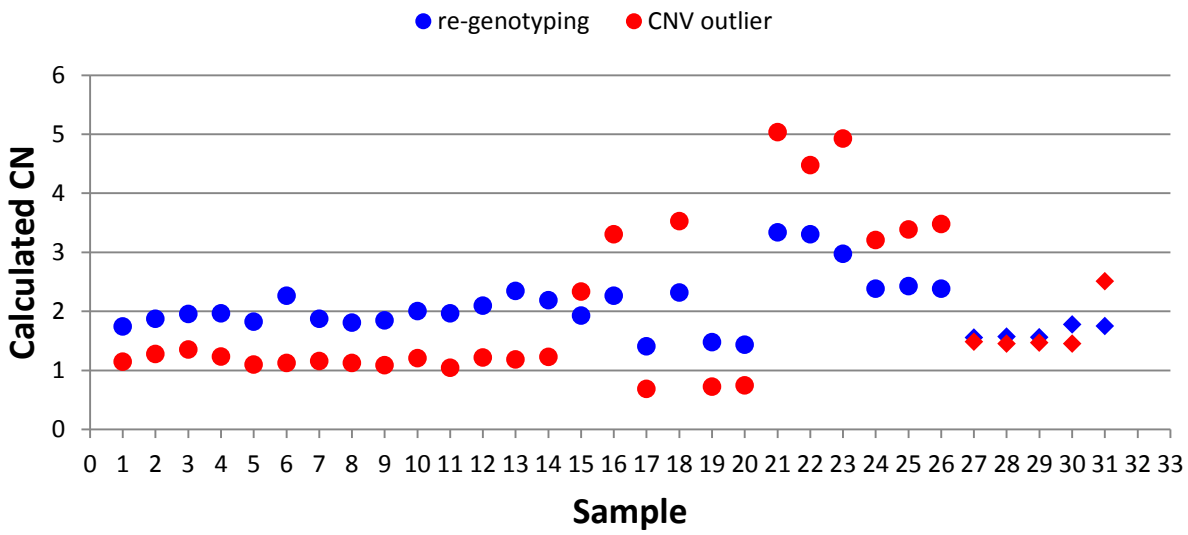


Figure 4-E: Re-genotyping of likely qPCR CN outliers. Dots and lozenge correspond to samples genotyped by qPCR-ΔCt and qPCR-Std, respectively.

Appendix 5-A

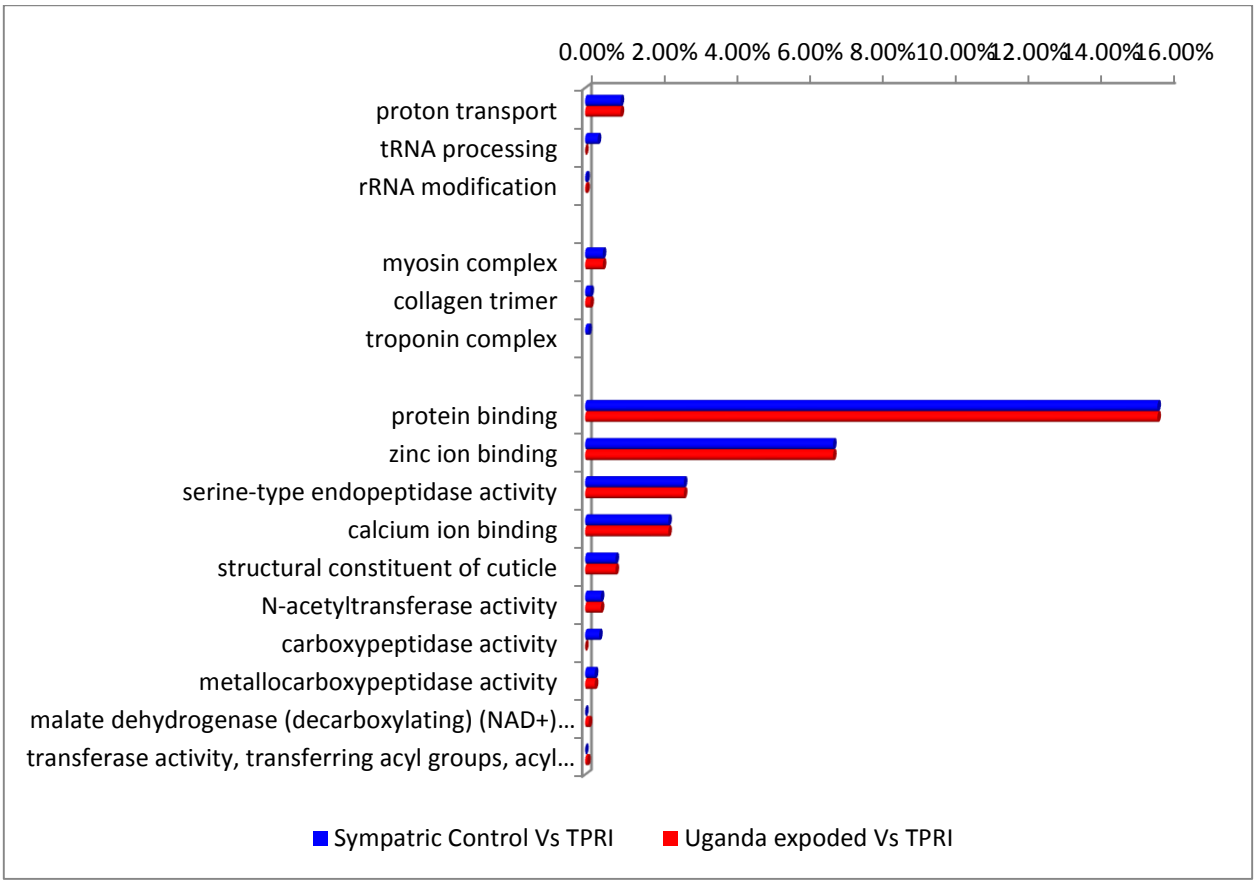


Figure 5-A: Transcriptomic profile of differentially expressed genes with fold-change >2 in Uganda exposed and sympatric mosquitoes compared to TPRI. GO term enrichment correspond to Down-regulated transcripts between the groups with frequency higher than 2%.

Appendix 5-B

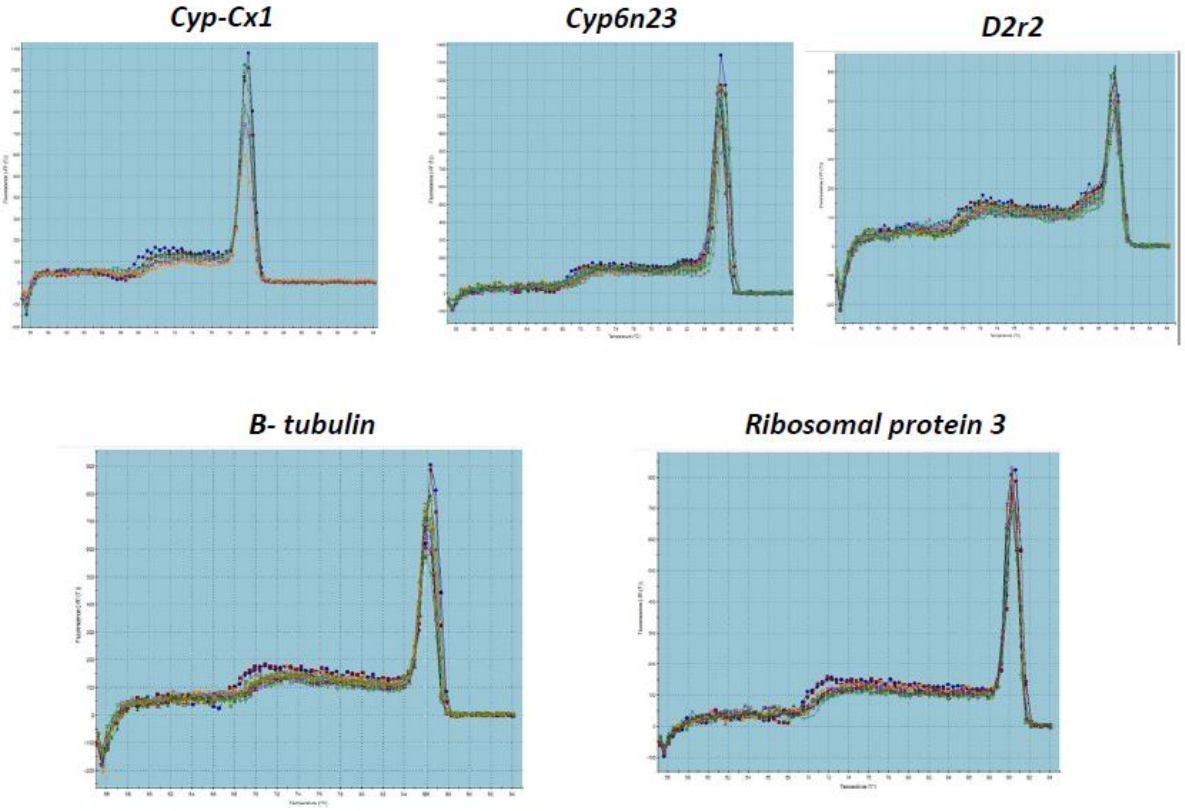


Figure 5-B: Validation of qPCR primers. Dissociation curves of real-time PCR amplification of microarray top candidate genes and endogenous control.

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