The evolution and genetics of vector competence in mosquito disease vectors

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2012

This dissertation is submitted for the degree

of

Doctor of Philosophy

Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in text. Where the work is a collaboration or submitted as a manuscript for publication, the word 'we' is used (Chapter 2, 3, 5). 'I' is used in Chapters 4 and 6 which are written purposely for the thesis.

Jewelna Osei-Poku

Thesis abstract

Vector competence is a complex characteristic which governs an insect's ability to acquire, support the development and transmit a parasite from one host to another. It influences variation in disease transmission among mosquito populations, hence affecting disease epidemiology. In this project, I have studied some aspects of ecological interactions and genetic factors in a step towards understanding how these affect variation in disease transmission and exploiting these in future disease control programmes.

Mosquito gut bacteria affect the development of parasites ingested by mosquitoes. As different bacterial species have different effects, dissimilarities in gut composition could be an important cause of variation in vector populations. The first study investigates the gut microbiome of mosquitoes collected from Kenya. Using 454 pyrosequencing of 16S rRNA, I provide a comprehensive catalogue of the gut composition of 8 species of mosquitoes (Chapter 2). I show that while there is greater variation within host species (fixation index= 0.64), different mosquito species tend to have rather similar gut bacteria. An individual mosquito gut has a low diversity of bacteria with, the microbiota being dominated by a single Operational Taxonomic Unit. This suggests that gut bacteria may be one factor influencing within-species variation in disease transmission, and a minor factor in between-species variation.

Wolbachia endosymbionts are able to reduce the intensity and development of RNA viruses and metazoan parasites in their insect hosts, blocking the transmission of such parasites. This makes *Wolbachia* a likely candidate for control programmes. I extend the investigation of naturally-occurring bacteria to *Wolbachia* (Chapter 3). Using the gut samples used in Chapter 2, I amplify the *Wolbachia* surface protein gene to identify *Wolbachia* infections. I identify *Wolbachia* in *Aedes bromeliae*, a vector of yellow fever, and its close relative *Aedes metallicus* and in *Mansonia uniformis* and *Mansonia africana*, which are competent vectors of human bancroftian filariasis. *Aedes bromeliae* showed the highest prevalence (75%) suggesting that this strain of *Wolbachia* may be manipulating the host reproduction by cytoplasmic incompatibility. Using a multi locus typing system and accounting for effects of recombination in the construction of bacterial phylogeny, I show that these mosquito *Wolbachia* strains cluster into supergroups A and B of *Wolbachia*. The phylogeny also shows significant recombination events indicating horizontal transfer events between taxa. These *Wolbachia* strains, isolated from the disease vectors, may be reducing parasite intensity and transmission, and could be a better choice for transinfecting other mosquito vectors rather than distantly related strains.

Previous studies show that high frequency of susceptibility to *Brugia pahangi* exists among populations of *Aedes aegypti* from East Africa, providing an excellent resource for investigating variation in a natural population. I test the frequency of susceptibility of peri-domestic subpopulations of *Aedes aegypti* collected from Kenya to *Brugia malayi* (Chapter 4). The results are consistent with previous data with up to 30% of individuals being susceptible.

The number of susceptible individuals varied significantly between populations (Fisher's exact: $p= 0.03$). These populations now provide the resource to identify polymorphisms associated with susceptibility to *Brugia* and also enable comparison with results obtained from laboratory strains.

In Chapter 5, I continue with efforts to identify and map quantitative trait loci (QTL) associated with *Brugia* susceptibility in *Aedes aegypti*. However, with the *Aedes* genome still highly fragmented with many supercontigs having no chromosomal assignments, mapping the gene to a definitive locus is almost impossible. Using an improved DNA-based mapping technology, Restricted-site Associated DNA tags (RADtags), I make novel assignments of 79 supercontigs to the 3 chromosomes of *Aedes aegypti*. These new assignments account for 122Mb of the genome, increasing the percentage genome mapped to ≈40%. The technique also identifies potential scaffold misassemblies and misassignments of supercontigs to chromosomes. I also use the same method to prepare libraries for sequencing which will provide more markers and allow mapping and identification of candidate genes which can be evaluated for involvement in susceptibility to *Brugia* infections.

Aedes aegypti and *Anopheles gambiae* share similarities in their immune proteins, but little is known about the functions of immune proteins in *Aedes aegypti*. To be able to make functional comparisons between mosquito vectors, I inoculate Sephadex beads into a laboratory strain of *Aedes aegypti* to investigate the expression of pathogen recognition genes (Chapter 6). Thioester-containing proteins (TEPs) show significant up-regulation ($p= 0.03$-

0.0002) with up to 7-fold increase in gene expression of TEP20 in immune-challenged individuals compared to non-challenged controls. TEP20 is an orthologue of *Anopheles gambiae* TEP1, emphasising the evolutionary function of TEPs in immune activation. As TEP1 is an important determinant of vectorial capacity in *Anopheles gambiae*, this indicates that TEPs may also be an important factor influencing variation in susceptibility to pathogens in *Aedes aegypti*.

Generally, this project has contributed to three broad areas of factors that influence variability in diseases transmission by mosquitoes: ecological interactions with bacteria, host genetic background and immune system. The results, resources and techniques used in this thesis can be widely used in further studies in these areas and extended to other mosquito vectors and natural populations.

Acknowledgements

It has been a great experience undertaking this research and from start to finish, I have had great support from many people. I have made mention of the contribution made by specific persons at the end of each chapter.

I am very grateful to my supervisor, Francis Jiggins for being very supportive and guiding my understanding through his project. From that first discussion about the project to the very last, he has been patient and always has his door open for a quick chat. I could not have asked for a better supervisor. I also wish to thank my advisors through time: Remy Ware and John Welch.

I also thank the rest of the Jiggins group for being such great lab colleagues, making me feel welcome among 'Drosophilians': Lena Wilfert, Mike Magwire, Claire Webster, Jenny Bangham, Ben Longdon, Fergal Waldron, Chuan Cao. I thank Fergal Waldron for frequently asking me for update on my work (that kept me on my toes), Lena Wilfert and Mike Magwire for advice and contribution to my research understanding, Ben Longdon for keeping me posted on publications connected to my work and for being quick to answer my questions on phylogenetic trees, and to Florian Bayer for keeping the lab running. Thanks to Emma Rhule for also being so kind and offering lifts to the field station. To my associates in mosquito affairs- Cristina Ariani, Punita Juneja, Katherine Short and William Palmer- I wish you had joined the group earlier than you did. Your immense help and contributions during the last two years of my PhD did make a massive difference and are deeply appreciated.

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1. INTRODUCTION

1.1. Mosquitoes as disease vectors

Mosquitoes belong to the insect family Culicidae and are further grouped into three sub-families: Anophelinae, Culicinae and Toxorynchitinae (Edwards, 1932). The most important mosquitoes known to transmit disease to humans belong to Anophelinae and Culicinae as Toxorynchitinae are only predaceous on other mosquito larvae and do not bite humans (Steffan and Evenhuis, 1981). For more than a century, mosquitoes have been implicated and found to transmit some of the most medically important diseases that affect man (Bastianelii and Bignami, 1900; Low, 1900). There are about 41 genera and over 3,000 species of mosquitoes known (CDC, 2010a) and few of these known species transmit disease. The ability of mosquitoes to carry and support the development of protozoa, helminthes and viruses probably makes mosquitoes the most diverse vectors of human disease in that respect.

Among the mosquito species that are known to transmit these parasites are members of the genera *Anopheles*, *Aedes*, *Culex* and *Mansonia*. Within these genera of mosquitoes there are designated complexes which comprise polytypic subspecies. Members of a complex show variation in their ability to transmit different pathogens. For instance, *Anopheles* consists of the *Anopheles gambiae* Giles *sensu lato* complex with members which are known vectors of malaria and filariasis (Gillies and de Meillon, 1968; White, 1974). *Aedes scutellaris* and *Aedes simpsoni* also represent two complexes within the *Aedes* genus that are well-known for

transmission of arboviruses (Christophers, 1960; Huang, 1986). Table 1.1 outlines some important disease vectors and the diseases they transmit.

Table 1.1: List of important mosquito vectors and the disease they transmit to human.

Genera	Complex	Species	Disease transmitted	References
Anopheles	*gambiae* Giles *s.l*	*An. gambiae s.s*	malaria, bancroftian filariasis	Coetzee et al., 2000; Gillies and de Meillon, 1968; White, 1985
		An. arabiensis	malaria, bancroftian filariasis	
		An. melas	malaria, bancroftian filariasis	
		An. merus	malaria, bancroftian filariasis	
		An. bwambae	malaria	
	-	*An. stephensi*	malaria	Christophers, 1933
	funestus s.l.	*An. funestus Giles*	malaria, bancroftian filariasis	Gillies and Coetzee, 1987
Aedes	*simpsoni*	*Ae. bromeliae*	yellow fever	Huang, 1986
	-	*Ae. aegypti*	dengue, yellow fever, chikungunya	Christophers, 1960; Ligon, 2006
	scutellaris	*Ae. albopictus*	dengue, chikungunya	Chan et al., 1971; Gould et al., 1968
		Ae. polynesiensis	bancroftian filariasis	Belkin, 1962; Rosen et al., 1954
Culex	*pipiens*	*C. pipiens*	bancroftian filariasis, West Nile and Rift Valley fever	Harbach, 1988; McMahon et al., 1981
		C. quinquefasciatus	bancroftian filariasis	
Mansonia	-	*M. uniformis*	brugian filariasis	Wharton, 1962

1.2. The *Aedes aegypti* mosquito

Aedes aegypti (Linnaeus) is a mosquito species belonging to group A of the subgenus Stegomyia (Theobald) within the *Aedes* genus (Edwards, 1932). Like all members of the Stegomyia subgroup, *Ae. aegypti* is characteristically black with white markings on its thorax, abdomen and legs. *Aedes* (*Stegomyia*) species can, however, be morphologically distinguished from each other by the pattern of white marking on the sternum and in part by the white bands on the legs. For example, *Aedes aegypti* has a white lyre-shaped pattern on the dorsal side of its thorax while *Ae. bromeliae* has two broad white patches of scales one on either side of the upper corners of the dorsal thorax (images available on WRBU website wrbu.si.edu).

Aedes aegypti (Linnaeus) has two morphological forms. The two forms differ in their morphology (McClelland, 1960), behaviour (McClelland and Weitz, 1963; Petersen, 1977) and physiology (Machado-Allison and Craig, 1972). One form is darker with no white scales on its first abdominal tergite (Mattingly, 1957, 1967) while the other is brownish with wide variation in the number of white scales on the abdominal tergite (McClelland, 1974). Mattingly (1957) first proposed the concept of designating the two forms as polytypic based on their morphological variations. After observation in East Africa, the species was designated as a complex, *Aedes aegypti sensu lato* (McClelland, 1960). Classification of the two forms as a subspecies, however, is debatable as the two forms have no genetically distinguishable traits (Failloux et al., 2002) and the morphological traits previously used to differentiate them are not clearly reliable (Brown et al., 2011). The darker and lighter forms of *Ae. aegypti* may be less misleading if referred to as *Ae. aegypti f. aegypti* and *Ae. aegypti f. formosus* where *f.*

stands for 'form'. For the purpose of keeping consistency with literature, the darker and lighter

forms will be referred to as *Ae. aegypti formosus* and *Ae. aegypti aegypti*, respectively.

1.2.1. Bionomics

Aedes aegypti is one of the few species within the subgenus Stegomyia that has adapted to

living in close proximity to man, feeding on man (anthropophily) and breeding in their

drinking water (Trpis and Hausermann, 1978). Other species such as *Ae. simpsoni* (Theobald)

and *Ae. metallicus* (Edwards) belonging to the same group are also anthropophilic, but do not

breed in drinking water (Trpis and Hausermann, 1978). Anthropophily and preference to breed

in drinking water are more profoundly observed in *Ae. aegypti aegypti*. The darker, sylvan *Ae.*

aegypti formosus is mostly found in forest areas away from human dwelling and prefer to feed

on animals (zoophily) (McClelland and Weitz, 1963; Petersen, 1977). Sylvan forms have

strong preference for breeding in tree holes and axils in forest areas (Mattingly, 1967).

Aedes aegypti are day biting mosquitoes, laying between 38-60 eggs about 3-4 days after

every blood meal (Bacot, 1916; Mathis, 1935). Autogeny— production of mature eggs

without blood-meals— is commonly observed in sylvan *Ae. aegypti* (Trpis, 1977). *Aedes*

aegypti females lay their eggs in singles as compared to rafts with other Culicines, and usually

on a wet substrate in their chosen breeding site. By laying their eggs attached to a wet surface,

the egg stage is able to survive several months of desiccation (Clements, 1963). Once water is

available the eggs hatch into larvae and become adults after about a week. Adults live for

several weeks or months depending on environmental conditions such as moisture (Beeuwkes et al., 1933; Shannon and Putnam, 1934) and temperature (Bacot, 1916; Davis, 1932; Johnson, 1919). Females are able to survive over 2 months, living longer than males (Christophers, 1960).

1.2.2. Distribution

Aedes aegypti has a widespread distribution and can be found in most parts of the world. The spread of the mosquito species, originating from Africa, seemed to have occurred through trading between continents (Failloux et al., 2002) causing a split that resulted in African and non-African populations (Brown et al., 2011). Through isozyme and polymorphic microsatellite analyses it has become evident that many domestication events of the ancestral *Ae. aegypti formosus* resulted in new global populations of *Ae. aegypti aegypti* (Ayres et al., 2003; Brown et al., 2011; Failloux et al., 2002; Mousson et al., 2005; Powell et al., 1980; Trpis and Hausermann, 1975; Wallis et al., 1983).

Within Africa, both polymorphic forms occur and exist in sympatry in East Africa (Petersen, 1977). The ancestral form is the only form found in West Africa except along the coastal areas (Brown et al., 2011; Mattingly, 1957). The occurrence of the sympatric populations in the East African region is believed to have been a reintroduction of *Ae. aegypti aegypti* into the region (Trpis and Hausermann, 1975) resulting in allopatric species. Observations made in West Africa show *Ae. aegypti formosus* occurring even in human dwellings which suggests

domestication of the sylvatic populations in this region (Brown et al., 2011) This raises epidemiological concerns with respect to the variability in the susceptibility of the two subpopulations to disease transmission (Lorenz et al., 1984; Paige and Craig, 1975; Tabachnick et al., 1985). Domestication may result in higher competence to disease transmission among *Ae. aegypti* populations.

1.2.3. *Medical importance*

Aedes aegypti is widely known as a primary vector of yellow fever and dengue (Christophers, 1960). The discovery of *Ae. aegypti* as a vector of the yellow fever virus (Reed et al., 1900) earned the mosquito species its common name the 'Yellow Fever' mosquito. Besides yellow and dengue fevers, *Ae. aegypti* also transmits chikungunya virus (Ligon, 2006). These viral diseases transmitted by *Ae. aegypti* place a medical and economic burden on endemic countries and territories, especially in the tropics and sub-tropics (Table 1.2). There is an estimated 200,000 cases of yellow fever with 30,000 people dying from the disease each year (WHO, 2011c). Dengue and dengue haemorrhagic fever, which was first reported in the Philippines and Thailand, has now spread to most parts of Asia and the Latin Americas (WHO, 2012). Now, over 40% of the world's population, including countries in Africa, is at risk of dengue infection (WHO, 2012). There is no available treatment for these arboviral diseases. Early detection in the case of dengue and vaccination for yellow fever are the only effective ways of preventing deaths from these diseases.

Table 1.2: Diseases transmitted by *Ae. aegypti* with WHO estimations of affected regions and population at risk. Information was obtained from the WHO website (www. who.int).

Disease	Regions affected	Estimated population at risk	Treatment
Yellow fever	Africa, Americas	900 million	None; preventive by vaccination
Dengue fever	Tropics, sub-tropics	3.5 billion	none
Chikungunya	Africa, Asia, Italy	No estimations made	none

1.2.4. Genomics

The increasing importance of *Ae. aegypti* as a disease vector and its use as a laboratory model for understanding host-parasite interactions required a better understanding of the genome and gene functions. *Aedes aegypti* is the most characterised mosquito species, attributable to the ease with which it adapts to laboratory conditions (Christophers, 1960). It has provided a lot of information on mosquito biology (Clements, 1992), physiology (Clements, 1963) and genomics (Severson et al., 2001). The species provided tools for the first genetic map for any mosquito species (Munstermann and Craig, 1979; Craig and Hickey, 1967). Subsequently, DNA-based technology for improving the coverage of the genome was developed to replace the classic mapping methods such as use of morphological mutants and isozymes (Antolin et al., 1996; Severson et al., 1993).

Size and organization

Based on improved DNA-based technology the draft genome of *Ae. aegypti* was sequenced (Nene et al., 2007), making it the second mosquito species to be fully sequenced after *An. gambiae* (Holt et al., 2002). *Aedes aegypti* has a large genome and by far the largest sequenced among the Culicidae family of insects. The genome is approximately 1.3 Gigabases (Nene et al., 2007), making it one of the biggest insect genomes sequenced. Large genome size is characteristic of Culicines (Rao and Rai, 1990); the *C. quinquefasciatus* genome is 579 Megabases (Arensburger et al., 2010). In the *Ae. aegypti* genome, the large genome size is attributed to the high abundance of transposable elements (TE), repetitive sequences and tandem repeats (Nene et al., 2007). TEs make up 47% of the genome while repetitive sequences and simple tandem repeats constitute 15% and 6%, respectively (Nene et al., 2007). Such genome organization puts limitations on the use of some molecular methods for genome analyses For instance, due to the repetitive nature of the genome, microsatellites have been shown not be ideal for single copy genetic markers in *Ae. aegypti* (Severson et al., 2004).

Comparative genomics

The *Ae. aegypti* genome shares similarities with *An. gambiae* and *Drosophila melanogaster* genomes. The similarities shared with *An. gambiae* is especially important in vector biology research and the proposed manipulation of mosquito vectors as a mechanism for controlling diseases (Beerntsen et al., 2000; Carlson et al., 1995; Cockburn and Seawright, 1985; Crampton et al., 1990, 1994). The phylogeny of the three insect species supports the observed similarities: Culicidae and *D. melanogaster* diverged ≈250MYA (Gaunt and Miles, 2002)

while the Anophelines diverged from the Culicines ≈150MYA (Krzywinski et al., 2006). All three insect groups have three main pairs of chromosomes. *Anopheles* and *Drosophila* have heterogametic sex chromosomes with the X chromosome being larger than the Y (Heckel, 1993). *Aedes aegypti*, like all Culicines, lacks heterogametic sex chromosomes (Craig and Hickey, 1967). Sex in Culicines is controlled by a single locus on chromosome-1 and chromosomes have the same size in both males and females (Craig and Hickey, 1967).

Conservation among chromosomes is evident among the three insect species, especially between the two mosquito species (Bolshakov et al., 2002; Severson et al., 2004; Zdobnov et al., 2002). There is a 1:1 mapping on almost all the chromosomes between *An. gambiae* and *Ae. aegypti* except on chromosome arms 2p and 3q (Nene et al., 2007). High orthology is also observed in the protein coding genes between the two mosquito species. About 67% of the proteins in *Ae. aegypti* are orthologous in *An. gambiae*. Due to the infiltration of TEs in the *Aedes* genome, coding genes in *Aedes* usually have a 4-6 fold average length increase compared to those in *Anopheles* (Nene et al., 2007). Sequencing of the *C. quinquefasciatus* genome has revealed higher conserved genome organization with *Ae. aegypti* (Arensburger et al., 2010) than with *An. gambiae* (Nene et al., 2007).

1.3. The filarial parasite: *Brugia malayi*

Brugia malayi is one of the three species of filarial worms that cause lymphatic filariasis. Human lymphatic filariasis, also known as elephantiasis, is a debilitating infectious disease endemic in some countries in the tropical region. It is a disease that has been with human

population for centuries (reviewed in Routh and Bhowmik, 1993), but probably because of its chronic nature and low mortality rates, it has not received much attention until fairly recently. Lymphatic filariasis caused by *Brugia* sp. is sometimes referred to as brugian filariasis. Brugian filariasis caused by *B. malayi* and *B. timori* accounts for about 10% of lymphatic filariasis cases; *Wuchereria bancrofti* causes approximately 90% of human filariasis cases worldwide (Melrose, 2002) and is the commonest human filarial parasite. The distribution of *Brugia* is restricted to South and South East Asia.

1.3.1. Morphology

Microfilariae of *B. malayi* are slender, transparent and 177-230μm long (CDC, 2010b). Each microfilaria possesses a sheath which is a protective egg-shell for the parasite (Rogers et al., 1976). A *B. malayi* microfilaria possesses two nuclei at its tip, making it morphologically distinguishable from *W. bancrofti*. *B. malayi* and *B. timori* are distinguishable by Giemsa staining. The sheath of *B. malayi* stains pink with Giemsa while *B. timori* does not stain. Adult worms of *B. malayi* (4-6cm long, 130-170 μm) are smaller than *W. bancrofti* (4-10cm long, 100-300 μm wide) (CDC, 2010b). Female worms are larger than males.

1.3.2. Binomics

The transmission of lymphatic filariasis involves a simple cycle of pathogen, vector and vertebrate host. The cycle is described as cyclodevelopmental because the parasite develops in the vector to become infective to the vertebrate host without multiplying (Erickson et al.,

2009). A mosquito ingests microfilariae by biting an infected person. The microfilariae invade the midgut epithelium of the mosquito in about 2 hours and enter the haemocoel. The sheaths of the microfilariae are shed by the time they reach the haemocoel and they move through the haemocoel to the thoracic muscles where they moult twice. Within 2 weeks after ingestion parasite develop into infective larvae (L3). These migrate through the haemocoel and into the head and proboscis of the mosquito where they are transmitted to human when the mosquito obtains a blood meal again. In the human host, the parasites migrate to the lymphatic tissues where they develop into adults, increasing both in size and length over a period of 6-12 months. The adults mate and produce many sheathed microfilariae.

1.3.3. Genomics

The need to identify novel drug targets for getting rid of infection from filarial nematodes instigated the sequencing of the *B. malayi* genome. The sequencing of the filarial nematode genome was done with *B. malayi* because it is the only human-infecting filarial nematode that can be maintained in the laboratory (Ghedin et al., 2007). The nuclear genome of *B.malayi* consists of 5 pairs of chromosomes (Sakaguchi et al., 1983) and sex is determined by a dimorphic pair of sex chromosomes XY. The size of the genome was initially estimated to be between 80-100 Megabases (McReynolds et al., 1986; Sim et al., 1987). Organizing sequences obtained from whole genome shotgun sequencing of the TRS strain of *B.malayi*, Ghedin et al. (2007) estimated the genome size to be 90-95 Megabases.

Protein-coding regions make up about 32% of the genome with 11,515 coding genes present. Operons, a characteristic of bacteria and nematode genomes (Guiliano and Blaxter, 2006) were found in *B. malayi*. About 16% of genes were contained in operons (Ghedin et al., 2007). One striking thing about the *B. malayi* genome is the absence of essential enzymes required for de novo synthesis of purine, riboflavin and heme (Ghedin et al., 2007). The bacteria endosymbiont, *Wolbachia*, may be providing these essential products for the survival of the filarial nematode (Foster et al., 2005) as depletion of bacteria endosymbionts of *B. malayi* by antibiotics results in death of the nematode (Landmann et al., 2011). This symbiotic relationship between the nematode and bacteria makes antibiotics a promising drug therapy for control of filariasis.

1.4. Vector competence

Vector competence and vectorial capacity are most often misconstrued to mean similar things and are sometimes used interchangeably. Vector competence is a component of vectorial capacity. It is the quantitative measure of the ability to be a disease vector. Vector capability includes the behavioural, environmental, cellular and biochemical factors that influence the relationship between the vector and the parasite (Beerntsen et al., 2000). Vector competence, as an element of vectorial capacity, is governed by intrinsic factors (Black et al., 1996; Hardy et al., 1983; Woodring et al., 1996). Genetic factors largely contribute to the success of insects as disease vectors, influencing characteristics such as susceptibility and insecticide resistance (Ayres et al., 2003; Beerntsen et al., 2000). Vector competence varies within and between

species (Black IV et al., 2002; Severson et al., 2001) and therefore has a direct effect on the epidemiology of vector-borne diseases.

Susceptibility of an insect to a parasite refers to the ability of the vector to support the development of the parasite to its infective stage. Susceptibility to pathogens is a trait largely controlled by the genetic makeup of the vector. It is a quantitative trait that ranges from complete receptiveness, where all individuals support infection, to the opposite end of the spectrum, total refractoriness, where no individuals support infection. The majority of mosquito vectors are positioned somewhere between the two extremes depending on geographical origin of both the parasite and the mosquito (James et al., 1932; Nace et al., 2004).

'Facilitation' and 'limitation' are two concepts especially used in describing vector competence of mosquitoes to filarial parasites. In 'facilitation', vectors are only able to support the development of ingested parasites when the number of ingested parasites have gone above a certain threshold (Bain, 1971; Brengues and Bain, 1972). It is a density dependent trait. On the other hand, 'limitation' describes when a vector is more efficient in supporting the development of the parasites when it ingests fewer parasites; a negative dependent trait (Bain, 1971; Brengues and Bain, 1972). For example, studies on the ability of *Anopheles*, *Culex* and *Aedes* to support the development of filarial parasites showed that Anophelines and *Aedes* exhibit 'facilitation' while 'limitation' occurs in Culex (Southgate and Bryan, 1992; Snow et al., 2006). However, members of the *Anopheles gambiae s.l.*

demonstrate differences with regards to these two concepts of vector capabilities to transmission of *Wuchereria bancrofti*. *Anopheles melas* transmits *W. bancrofti* effectively where parasitemia is low in the human population and *An. gambiae sensu strict* (*s.s*) does not (Amuzu et al., 2010). A better understanding of the factors governing such observations is important for control programmes such as the Global Programme for Elimination of Lymphatic Filariasis (GPELF).

1.4.1. Cibarial teeth

Ingested parasites enter the body of the mosquito through the proboscis and enter the gut. In the foregut, parasites are presented with the first line of defence from the mosquito host. Several teeth-like structures, also referred to as cibarial teeth, present in the foregut may reduce the number of parasites that make it to the midgut (McGreevy et al., 1978). This feature has been detected as an important factor in the vector competence of a mosquito, especially in the transmission of filarial parasites (Snow et al., 2006). Cibarial teeth lacerate microfilariae while they pass through the fore gut reducing the number of ingested parasites that enter the midgut (Bryan and Southgate, 1988; McGreevy et al., 1978). Despite this, there is no clear correlation between the possession of cibarial teeth and vector competence because, although *C. quinquefasciatus* has cibarial teeth, only about 6% of parasites are lacerated— most of the ingested parasites make it through to the midgut and thorax where they are later killed (McGreevy et al., 1978, 1982). *Anopheles gambiae* and *An.funestus* are two other mosquito species that posses cibarial teeth (Southgate and Bryan, 1992). In their investigation of vector capabilities of *Anopheles* to transmission of filariasis after rounds of mass drug

administration, Amuzu et al. (2010) showed that *An. gambiae s.s* has more cibarial teeth than *An. melas* which may be influencing the exhibition of 'facilitation' by *An. gambiae s.s* and 'limitation' by *An melas*.

1.4.2. Midgut

The midgut is an important passage way for blood-borne parasites since it forms a barrier between the ingested parasites and the haemocoel of the insect host. Parasites need to traverse the midgut and pass through the haemocoel to their developmental site in a mosquito host. The environmental and genetic background of the midgut influences the penetration of pathogens into the haemocoel (Beerntsen et al., 1995; Gordon and Lumsden, 1939; Obiamiwe, 1977; Sutherland et al., 1986). The midgut has been shown to confer a selective barrier to parasites and a reduction in the number of parasites occur here (Al-Olayan et al., 2002; Michalski et al., 2010; Nayar and Knight, 1995). With the movement across the midgut, *Plasmodium* develops from ookinetes to oocysts and microfilariae often shed their sheaths.

With regards to microfilariae, exsheathment depends on the host and the nematode species. Microfilariae may shed their sheaths in the midgut lumen (Esslinger, 1965; Ewert, 1965b; Denham and McGreevy, 1977; Nayar and Knight, 1995) or while crossing the midgut epithelium (Yamamoto et al., 1983). For example, comparative studies demonstrated that more *B. malayi* microfilariae lose their sheaths in the midgut lumen of *Ae. aegypti* than in *An. quadrimaculatus* which led to differences in the encapsulation and melanisation of the parasite

(Nayar and Knight, 1995). Studies with *B. malayi* and *B. pahangi* in both *Ae. aegypti* and *Armigeres subalbatus* showed the importance of the midgut to the vector host in eliciting an immune response— parasites escape encapsulation and melanisation when they are inoculated into the host without passing through the midgut (Beerntsen et al., 1989; LaFond et al., 1985). The midgut is the site where immune responses begin (Osta et al., 2004).

Immune activation

Immune responses begin once a 'foreign' body has been detected in the mosquito body. Often, ingested parasites make their first contact with the host tissues when they try to move across the midgut epithelium. Various anti-parasite genes show up-regulation in the midgut during the early stages of infection correlating with the period following ingestion when the parasites are moving across the midgut epithelium (Aliota et al., 2007; Blandin et al., 2004; Erickson et al., 2009). Increased levels of antimicrobial peptides (AMP) (eg. defensin) and pathogen recognition proteins (PRRs) such as thioester-containing proteins (TEPs) have been observed in *Anopheles gambiae* 20-48 hours following *Plasmodium* infection (Blandin et al., 2004; Dong et al., 2006; Richman et al., 1997). A few hours after filarial nematode infections in *Ae. aegypti*, there is up-regulation of AMPs with correlating decrease in parasite development (Bartholomay et al., 2004; Magalhaes et al., 2008). Other enzymes such as phenylalanine hydroxylase (PAH) which is important in the synthesis of melanin also peak after filarial parasite infection in *Armigeres subalbatus* (Aliota et al., 2007).

In filarial parasite infections, different host species may use different strategies to avoid infection. Transcriptional profiling studies in *Ae. aegypti* and *Ar. subalbatus* indicate differences in gene regulation during *B. malayi* infection (Aliota et al., 2007; Erickson et al., 2009), suggesting that the two species are responding differently to infection. Conversely, the parasite species is important in immune activation by the vector host. *Brugia malayi* and *B. pahangi* differ in their ability to elicit an immune response in *Ar. subalbatus* and the host effectively melanises *B. malayi* and not *B. pahangi* (Beerntsen et al., 1989; Yamamoto et al., 1985). Another example is observed with *Plasmodium* infections; silencing of *Caspar* in the *Imd* immune-signalling pathway allows killing of *Plasmodium falciparum* and not *P. berghei* in three Anopheline species (reviwed in Cirimotich et al., 2010; Garver et al., 2009). Differences in expression of thioester-containing protein-1 (TEP 1) between refractory and susceptible strains of *An. gambiae* against *Plasmodium* is an important determinant of vectorial capacity (Blandin et al., 2004).

Gut bacteria

The gut of insects is inhabited by bacteria which are involved in various aspects of the insect's physiology, nutrition and protection against pathogens (Azambuja et al., 2005; Broderick et al., 2006; Hosokawa et al., 2006; Kaufman and Klug, 1991; Janson et al., 2008). These bacteria are either obtained horizontally through feeding, vertically from mother to offspring or trans-stadially through developmental stages (Lindh et al., 2008). The presence of certain bacteria in the midgut of insect vectors has been shown to affect the development of ingested parasites (reviewed in Azambuja et al., 2005). For example, *Serratia marcescens* in the gut of

the triatomine bug, *Rhodnius prolixus*, reduces the intensity of *Trypanosoma cruzi* infection (Azambuja et al., 2004), and *Enterobacter* is effective against the development of *Plasmodium* ookinetes in *An. arabiensis* (Cirimotich et al., 2011).

Such observations have stimulated lots of interest in using gut microbiota as a potential mechanism for control of vector-borne diseases. The mechanisms by which some gut bacteria prevent parasite development have recently become evident. Dong et al. (2009) showed that the presence of bacteria in the gut of *An. gambiae* led to the up-regulation of basal immune genes which were cross-reactive with *Plasmodium*, so that aseptic mosquitoes were more susceptible to infection. This is an example of indirect effect of bacteria on the development of a parasite. Bacteria can also affect parasite directly as has been shown with an *Enterobacter* sp. isolated from *An. arabiensis*, which produces reactive oxygen species that interfere with development of *P. falciparum* (Cirimotich et al., 2011). The evolutionary relationship between microbiota and their hosts (Dale and Moran, 2006) is a factor that could influence variability in vector competence between species, both locally and, within species on a wider geographic scale.

1.4.3. *Genetic variation in vector populations*

The various physical and chemical barriers presented against parasites by vectors alone do not explain the variation in disease transmission observed in vector populations. The genetic makeup of both the host and parasites are important in determining the interaction between

both entities (Collins et al., 1986). The linkage maps produced for mosquito vectors have provided the tools for mapping genetic bases for vector competence in both *An. gambiae* (Gorman et al., 1997; Zheng, 1997; Zheng et al., 2003) and *Ae. aegypti* (Beerntsen et al., 1995; Gomez-Machorro et al., 2004). Phenotypic traits such as the amount of parasite ingested, melanotic encapsulation and lysis of parasites, which determine vector competence, are controlled by single or multiple genes (Beerntsen et al., 1995; Collins et al., 1986; Crews-Oyen et al., 1993; Feldmann et al., 1998; Severson et al., 1995; Vernick et al., 1989; Zheng, 1997). Identification of loci, such as ones that are involved in conferring refractoriness to a vector against the parasites they carry, is essential if novel genome-based approaches to vector control is sought.

1.5. Vector control

The transmission of mosquito-borne diseases is highly dependent on the availability of competent vectors, thus previous control strategies for eliminating diseases such as malaria have been largely targeted at the vector. Previous control methods employed the use of pesticides such as malathion and DDT (reviewed in Phillips, 2001) to kill mosquitoes. Excessive use of such pesticides, both in controlling malaria and in agriculture, caused the evolution of resistance to these chemicals in mosquitoes (Bruce-Chwatt, 1985) and other agricultural pests (Georghiou, 1986). Pyrethroids are now the accepted insecticides used in the control of mosquitoes through spraying and impregnation into bed nets. However, there is growing interests in finding a suitable replacement for pyrethroids because, resistance to these

insecticides have already been reported, especially in the malaria vector *An. gambiae* (reviewed in Ranson et al., 2011).

Although many control strategies aimed at eliminating the mosquito vector have proven futile, especially in areas where the burden of the disease is greater, vector control still remains the most effective way by which diseases associated with mosquitoes can be prevented. Genetic factors are responsible for most of the characteristics that contribute to the success of insect vectors, including insecticide resistance (Ayres et al., 2003), and this has drawn attention to extensive research on the genetics of mosquitoes. Obviously, previous vector control mechanisms have focused on the physiology of the mosquito with chemicals affecting either development or neurological system. The genetic control of mosquitoes only gained consideration when resistance to the chemicals used became evident.

1.5.1. *Genetic manipulation*

Genetic manipulation of insect disease vectors is a growing concept and a probable future mechanism for controlling the transmission of diseases (Carlson et al., 1995; Crampton et al., 1990, 1994). The concept proposes the genetic transformation of disease vectors to enable a previously characterised susceptible strain to disrupt the development of a parasite, and prevent transmission. Before this can be achieved, extensive studies on the inter-relationship between parasites and insect host— using naturally occurring host and parasite systems and/or model systems— are required. Identification of immune genes and trait loci that are involved

in preventing the complete development of disease pathogens in mosquito vectors provide candidate genes for genetic manipulation. Examples are TEPs and leucine-rich immune genes (LRIM) found to be effective against *Plasmodium* in *Anopheles* sp. (Blandin et al., 2004; Christophides et al., 2002; Osta et al., 2004).

Linkage mapping analyses have mapped genomic regions conferring refractoriness of natural *An. gambiae* populations to *Plasmodium* (Riehle et al., 2006). Comprehensive studies using various parasite species and hosts is also essential if a synergistic benefit of controlling most mosquito-borne diseases can be achieved. For instance, the laboratory infections done by Macdonald and Ramachandran (1965) using *Ae. aegypti* revealed that susceptibility to *Brugia* sp. and *Wuchereria bancrofti* is controlled by the same gene.

1.5.2. *Paratransgenesis*

Paratransgenesis is a term that describes the engineering of natural endosymbionts of insect vectors to express anti-parasitic factors which will prevent the development of disease pathogens in the insect host (Beard et al., 2002). The approach is preferred for a number of reasons: (1) It uses naturally-occurring symbionts of insects hence; it is not a novel introduction and unlikely to affect the fitness and behaviour of the host; (2) It will not affect the insect genome since host genome itself is not altered (Beard et al., 1992); (3) Since the bacteria are important symbionts they can spread more rapidly through the insect host population. Symbiont transformation has been shown to be feasible in the Chagas vector,

Rhodinus proxilus, where a symbiotic bacterium was engineered to express cecropin A, an anti-parasitic factor against *Trypanosoma cruzi* (Durvasula et al., 1997).

Bacteria, such as ones that live in the midgut lumen of insect host, are important for the physiological functions of the host (Buchner, 1965; Ishikawa, 1989), including nutrition (Dasch et al., 1984; Nogge, 1978) and development (Baines, 1956). The host and bacteria usually share evolutionary relationships, which have involved adaptation and co-evolution to exist in mutualism. Transformation of mutualistic bacteria like those that inhabit the midgut is preferred because, there will not be a selective pressure on the insect genome in response to the introduced bacteria. The midgut lumen also allows the transformed bacteria to gain close proximity with the parasite which increases rate of response and expression of the anti-parasitic factor by the bacteria. It is also important that the transformed midgut symbiont is able to compete with its natural counterparts and be able to maintain its functions in the host (Beard et al., 1993a).

1.5.3.   *Wolbachia as a tool for disease control*

Wolbachia is an intracellular Rickettsia-like bacterium that has the potential of establishing itself in the germ-line of insect hosts because it is maternally transmitted. Some strains of *Wolbachia* induce cytoplasmic incompatibility in their hosts (Yen and Barr, 1971) increasing their chances of being transmitted to subsequent generations. It has been considered as a potential mechanism for driving genes of interest for genetic manipulation of disease vectors

(Beard et al., 1993b; Sinkins et al., 1997). One aspect of using transgenesis as a vector control strategy that needs in-depth consideration is the spread of the engineered bacteria through the insect host population. That has been one of the draw-backs of using the *P*-element in other insect vectors (Handler and O'Brochta, 1991; Kidwell and Ribeiro, 1992) even though it is successful in the transformation of germ-line in *Drosophila* (Spradling, 1986). Cytoplasmic incompatibility is one mechanism that can be useful in establishing engineered symbionts in the germ-line of the host and allow a rapid spread through the insect population (Laven, 1959; Turelli and Hoffmann, 1991).

Protective characteristics have recently been discovered in some *Wolbachia* strains. *Wolbachia* confers anti-viral resistance in *Drosophila* (Hedges et al., 2008; Osborne et al., 2009). When the *Drosophila* strain of *Wolbachia*, *w*Mel*,* is transinfected into *Ae. aegypti*, a stable germ-line infection is established, and strong cyptoplasmic incompatibility is induced (McMeniman et al., 2009). Transinfected lines of *Ae. aegypti* show resistance to dengue (Hoffmann et al., 2011; Walker et al., 2011) and chikungunya (Moreira et al., 2009) viruses, hence reducing virus transmission. *Wolbachia* is also involved in stimulating immune gene expression against *Plasmodium* in *Ae. aegypti* (Moreira et al., 2009) and *An. gambiae* (Kambris et al., 2010). *w*Mel also affects filarial parasite development in *Ae. aegypti* (Kambris et al., 2009).

A virulent *D. melanogaster* strain of *Wolbachia*, *w*MelPop, induces life-shortening in infected hosts (McMeniman et al., 2009; Min and Benzer, 1997). It may seem to be a phenotype that

will prevent the complete development of disease pathogens and reduce transmission, but the evolutionary implication will need to be critically considered. Using a *Wolbachia* strain that reduces fitness will not only force a selective pressure against *Wolbachia* infections, it will also imply that infected females will die before they are able to produce offspring and reduce the spread of the bacteria through the population. Recent advances have been made in testing how effectively transinfected *Wolbachia* spreads through the population and reduces disease transmission (Walker et al., 2011; Yeap et al., 2011).

1.6. The *Aedes-Brugia* model system

Model systems are used extensively in disease research and have provided tools for understanding complex processes in advanced systems. The use of laboratory models offers a parsimonious advantage to using natural systems. Through the use of models, advances have been made in gaining a better understanding of the relationships and dynamics that exist between the mosquito vector, parasite and vertebrate host for diseases such as malaria and lymphatic filariasis. These findings have brought to light novel ways of eliminating the burden that such diseases impose on the human population.

Aedes aegypti and *Brugia* sp., especially *B. malayi*, have been extensively used as a model system in filariasis research. The combination is attributable to the easy transition of *Ae. aegypti* from the field to the laboratory and *B. malayi* being the only human filarial parasite that can be maintained in laboratory mammals (Ghedin et al., 2007). Although they do not

occur naturally as a vector-parasite system, the use of this model has provided answers to interesting questions from the point of parasite ingestion to transmission of the parasite to the human host.

1.6.1. *Aedes immune responses to filarial parasites*

Mosquitoes, like all other insects, lack the adaptive immune system which involves immune memory and development of specificity towards infection. Insects use innate immune responses– a more primitive form of immunity (Vilmos and Kurucz, 1998)– against pathogens they encounter. Innate immunity in insects has largely been studied in *D. melanogaster* (reviewed in Lemaitre and Hoffmann, 2007). Although bacterial challenge has been the most common way of studying immune responses in mosquitoes (Hillyer et al., 2003a; Lowenberger, 2001), the importance of understanding immunity against metazoans such the *Plasmodium* and filarial parasites have recently gained attention due to the public health importance of these latter parasites. Metazoans are more complex organisms and may be eliciting different immune responses from bacteria, fungi or viruses.

Immune peptides

When *Ae. aegypti* is challenged with bacteria via intrathoracic injection (Lowenberger et al., 1995, 1999b) or when mosquito lines are exposed to bacteria (Gao et al., 1999; Hernandez et al., 1994), an arsenal of antimicrobial proteins (AMPs) is produced by the immune system. Cecropins and defensins are common AMPs expressed in insects following bacterial and

fungal infections (Brey et al., 1993; Dimarcq et al., 1994; Ekengren and Hultmark, 1999; Kylsten et al., 1990). Mosquitoes respond similarly to bacterial and fungal exposure (Lowenberger et al., 1995, 1999b) with high concentrations of defensins and cecropins detectable in the fat body (Lowenberger et al., 1999a) and haemolymph (Lowenberger et al., 1999b), respectively. Transferrins are another family of AMPs expressed in *D. melanogaster* (Yoshiga et al., 1999) and *Ae. aegypti* (Yoshiga et al., 1997) in response to bacteria and filarial worm inoculations (Beerntsen et al., 1994)

There is evidence that cecropins and defensins are also effective against metazoan parasites (Lowenberger et al., 1999a; Richman et al., 1997). Microinjection of cecropins and defensins prior to *B. pahangi* infection reduces parasite development (Albuquerque and Ham, 1996; Chalk et al., 1995a, 1995b). Increased levels of cecropins and defensins are observed when bacteria are injected prior to *B. malayi* infection in *Ae. aegypti* (Lowenberger et al., 1996). These observations emphasize the non-specificity of the innate immune system. It also suggests there is a link between bacterial infection and the initiation of immune responses against other parasites. *Brugia malayi* contains a *Wolbachia* symbiont, *wBm*, (Bandi et al., 2001) and it is not known if this bacterium may be involved in triggering the expression of AMPs by the mosquito host. In the vertebrate host, however, the protective functions of *wBm* are clearer as antibacterial treatment causes arrest in worm development which is a secondary effect of *wBm* killing (Bandi et al., 1999; Landmann et al., 2011).

Melanotic encapsulation

Melanotic encapsulation is a haemocyte-mediated immune mechanism (Christensen et al., 2005) in arthropods and in other invertebrates. It is generally thought to be a defense mechanism against parasites that are too large to be phagocytosed. In adult mosquitoes, melanotic encapsulation occurs with less involvement of haemocytes (reviewed in Beerntsen et al., 2000). Humoral responses play a larger role in sequestering the pathogen and activating the phenoloxidase cascade for the production and deposition of melanin (reviewed in Beerntsen et al., 2000). Melanotic encapsulation is known to be effective against some bacteria species (Hillyer et al., 2003a), *Plasmodium* (Collins et al., 1986) and filarial parasites (Beerntsen et al., 2000).

Susceptible and refractory strains of mosquitoes exhibit melanotic encapsulation in response to filarial parasites (Christensen, 1986). The strength of the response is dependent on both the genotype of the vector and the parasite. For example, *Ae. aegypti* has been shown to elicit stronger melanization response and is more resistant to *B. malayi* than to *B. pahangi* (Beerntsen et al., 1989). Comparison of melanotic encapsulation of *B. malayi* between *An. quadrimaculatus* and the susceptible strain of *Ae. aegypti* showed that more worms were melanized in both the resistant and susceptible strains of the *An. quadrimaculatus* (Nayar and Knight, 1995). The authors suggested that *Ae. aegypti* was more susceptible because, the rate of migration across the midgut was slower in *Ae. aegypti* and more exsheathment (shedding of the sheath in microfilariae) occurred in the midgut (Nayar and Knight, 1995). This is an indication that initiation of immune response against filarial parasites does occur during

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migration across the midgut epithelium and worms probably escape the immune response when they exsheath in the midgut lumen.

1.6.2. *Genetic control of filarial infection*

The concept of genetic control of filarial infection was first suggested by observations of variations in susceptibility among vector populations (Kartman, 1953). The selection of a convenient susceptible laboratory strain for the study of filarial infection in mosquitoes showed that, susceptibility to *B.malayi* is inherited and genetically controlled (Macdonald, 1962b, 1962a). In *Ae. aegypti*, the trait is Mendelian and previously assumed to be a single sex-linked gene (Macdonald, 1962a). The Mendelian gene, designated *fm*, (Macdonald, 1962a) gained more interest as it was found to be the same gene conferring susceptibility to other strains of *Brugia* and the pan-tropic *W. bancrofti* (Macdonald and Ramachandran, 1965). With the linkage map for *Ae. aegypti* available (Severson et al., 1993; Munstermann and Craig, 1979), the genetic control for *B. malayi* was mapped to two chromosomal regions (Severson et al., 1994). Chromosome-1 contained *fm* in the *fsb*[1, LF178] marker region and chromosome-2 had a minor effect gene, *fsb* [2, LF98].

Linkage map organization in *Ae. aegypti* mapped *fsb*[1, LF178] within an 8.3 centiMorgan (cM) region between two genes with easily detectable phenotypes. The white-eye colour gene, (*AeW*) and the sex determining gene (*Sex*) of *Ae. aegypti* lie on either side of the susceptibility marker region (Severson et al., 2002). These two phenotypes provide convenient visual

markers for performing crosses between susceptible and refractory strains of *Ae. aegypti*. For instance, eye colour was useful in selecting a homozygous refractory strain of *Ae. aegypti* (McClelland, 1962).

1.6.3. Frequency of resistance in vector populations

Variations in responses to parasite infection exist between populations of a strain of vector due to genetic differences and/or other factors (Kartman, 1953). Although *Ae. aegypti* is not a natural vector of *Brugia* sp., there are observed variation in susceptibility to the parasite among populations of the vector (Hawking and Worms, 1961; Ramachandran et al., 1960). As an experimental vector, the variation in susceptibility was investigated further in wild populations of *Ae. aegypti* (Rodriguez and Craig, 1973). The study further emphasised the genetic variations in *Ae. aegypti* and showed geographic populations with as high as 53% susceptibility (Rodriguez and Craig, 1973). Generally, worldwide strains of *Ae. aegypti* were mostly refractory to *B. pahangi* with susceptibility frequencies between 0-2%. *Aedes aegypti* collected from East Africa showed high levels of susceptibility (0-53%). Categorizing the strains according to the habitat from which they were collected, the high susceptibility in African strains were particularly observed in those collected from tree holes and other natural breeding containers (Rodriguez and Craig, 1973), suggesting they were *Ae. aegypti formosus* (Mattingly, 1957, 1967).

Genetic variation is thought to be an important determinant of disease prevalence in the human population (Coetzee et al., 2000). The two forms of *Ae. aegypti* existing only in sympatry in East Africa (Petersen, 1977) may be exposed to different selection pressures due to the differences in habitat and behaviour. Heterozygosity has been hypothesised to be correlated with disease resistance and homozygosity with susceptibility (Allendorf and Leary, 1986). Townson (1971) hypothesised that filarial susceptibility, which is a homozygous recessive trait, might be maintained in populations at high frequencies due to heterozygous advantage. This implies that homozygous recessives will be common and a balanced polymorphism is established. We can only ascertain the evolutionary, epidemiological and public health significance of *Brugia* susceptibility in *A. aegypti* populations when the gene and allele frequencies are known.

1.7. Research aims

Clearly, vector competence is a complex trait with many interlinking factors. *Aedes aegypti*, as an experimental vector for filariasis research, has provided tools and initiated our understanding of the genetic, biochemical and evolutionary relationships between vector and filarial parasites. Although there have been many advances in understanding mosquito-parasite relationships, some aspects of the interactions are yet to be understood.

1.7.1. Determine the bacteria fauna of field-caught *Aedes aegypti* and other co-existing mosquito species.

Bacteria in mosquitoes have largely been identified and characterized using culture-dependent and -independent methods which often underestimated the species richness. These methods were also unable to allow the relative abundance of bacteria to be estimated, a measure that can be implicated in the observed variations in disease transmission by different species and individuals within species. Using modern and sensitive molecular techniques will help re-evaluate the differences in bacteria diversity among natural populations of disease vectors, characterize and speculate on their involvement in disease transmission.

1.7.2. Identify *Wolbachia* strains in mosquitoes collected from a wild population.

Wolbachia endosymbionts have increasingly gained a lot of research interest. Their ability to reduce the virulence of RNA viruses in their insect hosts makes them potential vehicles for reducing disease transmission in mosquitoes. However, not many mosquito disease vectors have been found to naturally habour *Wolbachia*. Screening wild populations of mosquitoes will help discover prevalence of these bacteria in mosquito disease vectors and potentially identify a strain that could be technically easier to use in the intended disease control strategy.

1.7.3. Determine the frequency of susceptibility among Aedes aegypti populations to Brugia malayi.

As a previous study investigated susceptibility of geographic strains of *Ae. aegypti* to *B. pahangi* and found East African strains to have high susceptibility, the aim is to investigate the susceptibility of East African populations to *B. malayi*. Although susceptibility to both parasites is controlled by the same loci, penetrance and expressivity may vary for both infections. Since *Aedes-B. malayi* model system is more widely used, this lack of information presents a gap in the information provided by the model system.

1.7.4. Improving the Aedes aegypti genetic map and mapping the gene for susceptibility

Genetic control of *B. malayi* susceptibility in *Ae. aegypti* is still only known as a QTL. Isolation of the gene is important for further characterization of the gene and how it functions in conferring susceptibility. Mapping the gene is also important if synteny is to be identified in other mosquito species such as *An. gambiae* which is a major vector of *W. bancrofti*, However, the current state of the *Aedes* genome makes it impossible to fine-map the gene. Using advanced DNA-based genome sequencing techniques can contribute to the improvement of the genetic maps.

1.7.5. Determine the recognition genes important in eliciting immune responses in Aedes aegypti.

Recognition genes such as TEPs are important in immunity against *Plasmodium* in the malaria vector, *An. gambiae*. The direct functions of such immune genes in immunity against parasites in *Ae. aegypti* has been largely ignored. As a model organism, this needs to be known and compared against infections with different parasites. This will allow common recognition genes to be identified that can be potential targets for genetic manipulation.

2. VARIATION IN GUT BACTERIA OF FIELD-CAUGHT MOSQUITOES

This chapter has been published as:

2.1. Introduction

In order to be transmitted, mosquito-borne parasites must penetrate the insect midgut before completing their development in the tissues of the insect. However, the midgut is a hostile environment in which many parasites perish — for example, in *Anopheles* mosquitoes only a small minority of *Plasmodium* parasites survive the midgut (Al-Olayan et al., 2002). Therefore, understanding the factors that are affecting the survival of parasites in the mosquito midgut has the potential to allow us to reduce or even block disease transmission. One important factor is the innate immune system of the insect, which can be activated when parasites such as *Plasmodium* and filarial parasites invade the midgut (Osta et al., 2004; Michel and Kafatos, 2005; Erickson et al., 2009; Michalski et al., 2010), resulting in the up-regulation of immune genes such as those encoding antimicrobial peptides (AMPs) and thioester-containing proteins (TEPs) that have anti-parasite effects (Blandin et al., 2004; Richman et al., 1997; Vlachou et al., 2005). However, it has recently become clear that bacteria living in the insect gut can also have an important role.

The mosquito gut is naturally inhabited by a community of bacteria that can reduce the intensity and development of human parasites such as *Plasmodium* (Pumpuni et al. 1993; Pumpuni et al. 1996; Straif et al. 1998;Gonzalez-Ceron et al. 2003; Dong et al. 2009) that are ingested by mosquitoes. One way in which gut bacteria can interfere with parasite development is by exerting direct anti-parasite effects (reviewed in Azambuja et al., 2005). This has been shown in the case of an *Enterobacter* bacterium isolated from African populations of *Anopheles arabiensis,* which generates reactive oxygen species that make the mosquitoes resistant to *Plasmodium* infection (Cirimotich et al., 2011). The presence of gut microbiota has also been shown to activate the immune response of mosquitoes, causing the release of immune proteins that are cross-reactive with the parasites (Dong et al., 2009), and this may indirectly block the development of parasites such as *Plasmodium*.

The different bacteria that have been isolated from mosquito guts can have dramatically different effects on the development of human parasites. For example, Cirimotich et al. (2011) isolated four bacterial species from wild *An. arabiensis* mosquitoes, and found that when fed to mosquitoes in the laboratory an *Enterobacter* sp. almost completely inhibited *Plasmodium* development while the bacterium *Bacillus pumilus* had no effect. As these are natural gut bacteria, it is therefore possible that the composition of the gut microbiota might have an important impact on rates of disease transmission in the wild, and cause differences in the rate that different species or populations of vectors transmit disease. Furthermore, if the composition of the gut microbiota could be manipulated, then this could be a method of

disease control. For example, sugar-bait methods that are used for killing mosquitoes (Müller et al., 2010) could be adapted for infecting adult mosquitoes with specific bacteria.

To predict how gut bacteria will affect disease transmission, it is important to investigate how the community of gut bacteria varies across different mosquito species, populations and individuals. One approach is to culture the bacteria isolated from the gut and characterise the different isolates. It is common, however, to find that the majority of bacteria in environmental samples cannot be cultured, so this approach may give a false representation of the bacterial species present and their abundance. A less biased approach is to amplify the bacterial 16S rRNA gene by PCR, and then clone and sequence the PCR product. This approach has led to the identification of numerous gut bacteria from a range of different mosquito species (Gusmão et al., 2010, 2007; Lindh et al., 2005; Pidiyar et al., 2004; Rani et al., 2009), but it is a slow and expensive process so the number of sequences are usually relatively small. The advent of new sequencing technologies has both removed the need for cloning the PCR product and cut the cost of sequencing. This has led to 454 pyrosequencing being increasingly used to investigate microbial communities in other fields (Sogin et al., 2006; Roesch et al., 2007; Chandler et al., 2011; Huse et al., 2008; Bishop-Lilly et al., 2010; Wang et al., 2011).

In this study, we use 454 pyrosequencing to investigate the bacterial diversity in the guts of eight species of mosquitoes collected from the coastal region of Kenya. This allowed us to comprehensively catalogue the bacterial taxa present, and examine how the bacterial community varies in different mosquitoes. We found that mosquito gut typically has a very

simple gut microbiota that is dominated by a single bacterial taxon. Although different mosquito species share remarkably similar gut bacteria, individuals in a population are extremely variable.

2.2. Materials and methods

Mosquito collection and identification

All mosquito samples were collected from towns and villages near Kilifi and Malindi on the Kenyan coast (Figure 2.1). Collections were made in different localities in each area; Mbogolo in the Malindi district, and KEMRI, Mkwanjuni, Mnarani, Matsangoni and Jaribuni in the Kilifi district. BG-Sentinel traps (Biogents AG, Germany) or CDC light traps (Center for Disease Control, U.S.A.) were set to collect the mosquitoes. In general *Anopheles* and *Mansonia* were collected from Mbogolo and Jaribuni, while *Aedes* and *Culex* were captured from the remaining sites. The mosquitoes were morphologically identified with the aid of taxonomic keys (Edwards, 1941; Gillies and de Meillon, 1968) and images from Walter Reed Biosystematics Unit (available from http://wrbu.si.edu/genera_mq.html). We later verified the mosquito identifications by amplifying the insect ribosomal internal transcribed spacer region-1 (ITS1) as described in von der Schulenburg et al. (2001), as different mosquito species produce different length of PCR products. PCR products were cleaned and sequenced with the BigDye Terminator Kit (Perkin-Elmer Corporation, U.S.A). Sequencing was done at the Source BioScience Center, Cambridge, UK. Sequences were aligned and visually inspected in Sequencher v4.5 (Gene Codes Corporation). Resulting consensus sequences were searched against existing sequences in NCBI BLAST to confirm mosquito identification.

In total, 86 female mosquitoes distributed across 4 genera were analysed for their gut microflora. The number of amplified individuals for each species and location is shown on Table 2.1. We classified the sample sites into peri-urban and rural based on the type of buildings (concrete or mud) and infrastructure such as state of roads (tarred or untarred). Most of the Culicines were collected from Kilifi and its environs, while all the Anophelines were collected from rural Mbogolo.
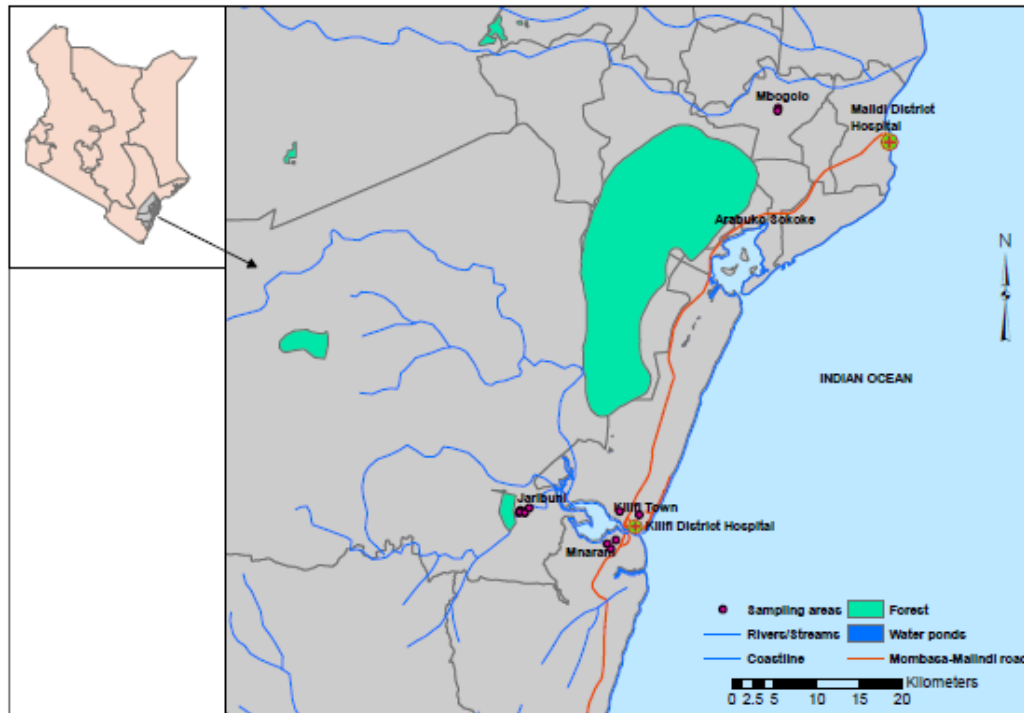
Figure 2.1: Map of Kenya showing Kilifi and Malindi: the two districts from which mosquitoes were collected. Jaribuni and Mbogolo are inland villages in both districts, respectively, while the other towns (shown only as pink circles) are coastal areas in and around the main town Kilifi.

Table 2.1: Mosquito samples collected in towns along the coast of Kenya.

Mosquito species	District	Location	Habitat type	Number
Ae. aegypti	Kilifi	KEMRI	Peri-urban	3
C. quinquefasciatus	Kilifi	KEMRI	Peri-urban	4
Ae. aegypti	Kilifi	Mnarani	Peri-urban	3
Ae. bromeliae	Kilifi	Mnarani	Peri-urban	10
Ae. aegypti	Kilifi	Mkwanjuni	Peri-urban	5
Ae. bromeliae	Kilifi	Mkwanjuni	Peri-urban	4
Ae. aegypti	Kilifi	Matsangoni	Rural	2
An. gambiae s.l.	Kilifi	Jaribuni	Rural	1
C. quinquefasciatus	Kilifi	Jaribuni	Rural	1
An. gambiae s.l.	Malindi	Mbogolo	Rural	11
An. funestus	Malindi	Mbogolo	Rural	11
An. coustani	Malindi	Mbogolo	Rural	1
M, africana	Malindi	Mbogolo	Rural	10
M. uniformis	Malindi	Mbogolo	Rural	13
C. quinquefasciatus	Malindi	Mbogolo	Rural	7

Dissection of mosquitoes

Females without blood-engorged abdomens were selected for dissection. Mosquitoes were surface sterilised prior to dissection; 10mins in dilute sodium hypochlorite, 1min in 1X sterile phosphate buffered saline (PBS), 1min in 70% ethanol and then a final wash in sterile 1X

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PBS. Dissections were performed under a stereomicroscope in a contained environment which was sterilised with 70% ethanol frequently to eliminate as much contamination as possible. Each mosquito gut was pulled out into a drop of sterile 1X PBS on a sterilised microscopic slide. Extracted guts were returned to a sterile 1.5ml microcentrifuge tube containing about 500μl absolute ethanol and stored till extraction.

DNA extraction

DNA from guts was extracted with QiAamp DNA Micro kit (Qiagen) according to manufacturer's manual. All extractions were done under aseptic conditions; surfaces were cleaned with dilute sodium hypochlorite solution and 70% ethanol. Extractions were done in localized aseptic microenvironment provided by flame from a Bunsen burner to prevent contamination from bacteria in the surrounding air. Microcentrifuge tubes for final DNA elution were irradiated with 200mJ of ultraviolet light for 1min in a UV Stratalinker 2400 (Stratagene Ltd. La Jolla, Ca., USA) prior to use. A negative control, in which the extraction procedure was performed without adding any tissue, was included to check for contamination.

Primer design

We chose primers that amplified the V3 variable region of the 16S rRNA in Eubacteria, as this region is known to be informative in distinguishing bacterial species (Huse et al., 2008). The basic 16S primers, 338-358 F (5´ ACT CCT ACG GGA GGC AGC AGT 3´) and 683-700 R (5´ CGM ATT TCA CCK CTA CAC 3´) are highly conserved across the Eubacteria and

amplify a region from position 359-682 (excluding primers) in the 16S rRNA of *E. coli* (Wang and Qian, 2009). To obtain a set of Fusion Primers (Roche) we added additional sequences required for Roche 454 Titanium Amplicon sequencing to the 5´ end of the primers. This also allowed multiplexing of the samples. Each complete HPLC-purified Fusion Primer consisted of a 21-mer Primer A (5´ CGTATCGCCTCCCTCGCGCCA 3´) or Primer B (5´ CTATGCGCCTTGCCAGCCCGC 3´) followed by a 4-mer Key sequence (5´ TCAG 3´), a 10-mer Multiplex IDentifier (MID), and finally the 16S primer. In total we used 12 different MIDs for the forward primer and 12 different MIDs for the reverse primer (Table 2.2), which allowed us to multiplex up to 144 different samples in a single sequencing lane.

PCR and 454 parallel sequencing of gut bacteria

PCR amplification was performed with Phusion High-Fidelity DNA Polymerase (NEB, UK), following manufacturer's recommendation for the reaction mix and cycle. Briefly, each 20µl PCR reaction contained 4µl of 5x buffer HF, 0.4µl of 10mM dNTP mix (Fermentas, UK), 0.4µl of 20µM forward and reverse Fusion Primer mix, 0.2µl of 2U/µl Phusion HF Polymerase, 1µl of sample DNA and 14µl of sterile water. All reactions were prepared under sterile conditions as described for DNA extraction above. Roughly equimolar concentrations of all positive samples were pooled into a single tube. The pooled sample was run on a 2% agarose gel and the resulting band excised and extracted from the gel using QIAquick gel extraction kit (Qiagen). The sample was then sequenced in both directions on an eighth of a Roche 454 FLX Genome Sequencer plate using Titanium Series reagents at the Department of Biochemistry Sequencing Facility, University of Cambridge.

Table 2.2: Multiplier Identifier (MID) sequences.

ID	MID Sequence
MID1	ACGAGTGCGT
MID2	ACGCTCGACA
MID3	AGACGCACTC
MID4	AGCACTGTAG
MID5	ATCAGACACG
MID6	ATATCGCGAG
MID7	CGTGTCTCTA
MID8	CTCGCGTGTC
MID9	TAGTATCAGC
MID10	TCTCTATGCG
MID11	TGATACGTCT
MID12	TACTGAGCTA
MID13	CATAGTAGTG
MID14	CGAGAGATAC

Pre-processing of sequences

Sequencing on the GS FLX Automated Sequencer produced 49,576 sequences that had passed the machine's filter criteria. The average and median length of these sequences was 365.18 and 378.0 respectively, with a standard deviation of 48.79. The mean base quality score of the sequences was 35.94. GS FLX reports base quality in Phred equivalent where a maximum score of 40 indicates a base calling accuracy of 99.99% i.e. a probability of 1 in 1000 that a base is incorrect (Margulies *et al*. 2005; Roche Applied Science, 2009). As a quality control we included in further analyses only sequences that were 340-400bp long, had < 20% ambiguous bases and an average quality score > 25. The resulting sequences were grouped by their barcodes using the Geneious software (Drummond et al., 2001). During the barcode assignment, 4,594 sequences did not have an exact match to our barcodes and so were not included. To remove chimeric sequences that arise during PCR, the remaining 42,951 sequences were run through the chimera-slayer (Haas et al., 2011) program on Mothur (Schloss et al., 2009). To ensure we were only analysing bacterial sequences, a further 362 sequences with less than 75% similarity to any sequence in the SILVA–bacteria dataset (Pruesse et al., 2007) were removed and, 14 sequences classified as chloroplast rDNA by Mothur (Pruesse et al., 2007) were also removed.

We proceeded to use the QIIME pipeline (Caporaso et al., 2010a) to organize the libraries by barcodes and align them. We aligned our sequences with the Python Nearest Alignment Space Termination Tool (PyNAST) (Caporaso et al., 2010b) using the Greengenes Core Set alignment as a template (DeSantis et al., 2006). We removed alignment columns where 95%

of the positions were gaps. The aligned sequences were then assigned to Operational

Taxonomic Units (OTUs), each with sequences sharing at least 97% similarity using the

furthest-neighbour algorithm implemented in Mothur (Schloss et al., 2009). To reduce the

number of sequences and enable faster analyses to be performed, representative sequences

from each OTU were then selected and used in most analyses. Beta diversity estimates were

made using UniFrac distances (Lozupone and Knight, 2005), and are based on weighted

Unifrac distances that have not been normalized unless otherwise stated. Other analyses such a

rarefaction curves, heatmaps and statistical tests were done with custom scripts in R (R

Development Core Team, 2008).

2.3. Results

The mosquito gut has a low bacterial diversity

As bacterial species cannot be directly identified from our data, we classified the 33,757

sequences that passed our quality criteria into Operational Taxonomic Units (OTUs). Each

OTU was defined by sequences with at least 97% nucleotide identity between them. In total

there were 789 OTUs, but only 53 of these ever exceeded a frequency of 1% in any of the guts

we sampled. To estimate the species richness of the mosquito gut microbiota — the total

number of OTUs present in a single gut — we used the Chao1 method (Chao and Lee, 1992)

to correct for our finite sample sizes. To assess the performance of this approach, we

recalculated this statistic from different sized subsamples of the sequences from each gut, and

used these estimates to plot a rarefaction curve. As shown by the asymptotic curves in Figure

2.2-A, this analysis suggests that our sequencing depth was sufficient to obtain good estimates

of the total species richness. Apart from two individuals with exceptionally high numbers of OTUs, we estimate that a mosquito gut contains ≈5-71 OTUs (Figure 2.2-A). The median number of OTUs in a mosquito gut is ≈42. The different species of mosquitoes had similar numbers of OTUs in their guts (Figure 2.2-B; $F_{7,69}$= 1.50, p= 0.18).

Despite a typical gut containing roughly 42 different OTUs (Figure 2.2-A), most of these are rare, and the bacterial community is nearly always dominated by a small number of taxa. On average, the commonest OTU within a gut constituted 67% of all the bacteria sequenced from each sample, and the four most abundant OTUs together represented 90% of bacteria. This pattern of a few dominant OTUs within each gut can be clearly seen in the heatmap shown in Figure 2.3-A (see Appendix Table S1 for OTU information). Therefore, the bacterial diversity — which reflects both the number and abundance of OTUs — is very low.

A

B
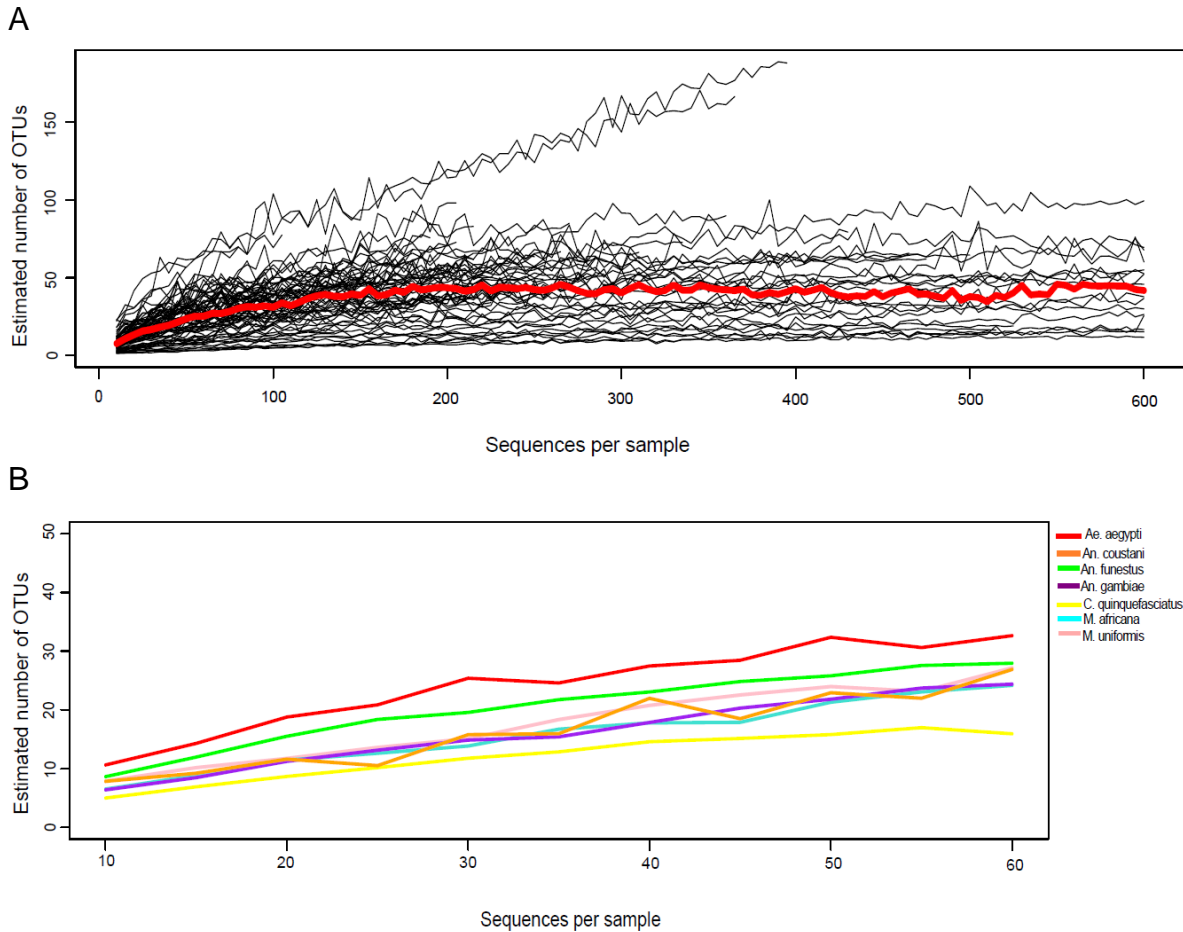
Figure 2.2: The estimated number of bacterial OTUs in mosquito guts. The number of OTUs in each gut was estimated using the Chao1 method (Chao and Lee, 1992). The rarefaction curve was produced by randomly re-sampling different numbers of sequences from each individual (20 replicate samples/individual gut/sample size), and then calculating the mean of the 20 replicates. Panel A shows the estimated number of OTUs of the 86 mosquito guts re-sampled to a maximum depth of 600 sequences. The red line is the median number of OTUs at each level of sub-sampling. Panel B shows the mean of OTU estimates for the guts from each mosquito species. Note that there was only a single individual of *An. coustani*.

High variation within host species

There was extensive variation between individuals of the same host species in the composition of their gut microbiota. This is clear in the heatmaps shown in Figure 2.3, which illustrate that it is common to find that an OTU or bacterial genus that makes up over 90% of the microbiota in one individual may be absent from the gut of another individual of the same species (Figure 2.3). Certain OTUs were also exclusively found in a single individual within a host species (Figure 2.3). Examples were observed in *C. quinquefasciatus* (OTU 160), *Ae. aegypti* (OTU 500), *M. africana* (OTU 215) and *M. uniformis* (OTU 426).

To summarise the similarity of the gut microbiota in different individuals of the same species (β diversity), we calculated the weighted Unifrac distance between every pair of guts (Lozupone and Knight, 2005). This statistic measures the distance between two communities by calculating the fraction of the branch length in a phylogenetic tree that leads to descendants in either, but not both, of the two communities. The weighted Unifrac distance which we used also accounts for the abundance of each bacterial taxa, and is closely analogous to the fixation index F_{st}. In this analysis the distances were normalized, so a value of zero indicates that two guts have identical communities and a value of one that they have non-overlapping communities (i.e. when all the taxa are plotted on a phylogeny, no branches on the tree are shared). The average normalized Unifrac distances within species was 0.64, indicating that there is usually very little overlap in the composition of the gut microbiota of two individuals of the same species (Table 2.3).

Figure 2.3: The frequency of different bacterial OTUs (panel A) and genera (panel B) in the guts of individual mosquitoes. Each column is a different individual, and each row is a different OTU or genus. The colour represents the proportion of sequence reads from a given OTU or genus in that mosquito. White spaces are OTUs found at a frequency of less than 1%. The OTUs are arranged so the most frequently occurring are at the top of the figure, and only individuals with at least 20 sequence reads are included. In panel B, the absence of a genus name indicates that the sequences could only be classified to the level of Class.

Table 2.3: Variation in the gut microbiota of individuals of the same species. The distances between individuals of the same species are normalised weighted Unifrac distances. A value of 0 indicates identical bacterial communities, and a value of 1 indicates no phylogenetic overlap between the communities.

Host species	Mean Distance
C. quinquefasciatus	0.63
M.uniformis	0.64
M.africana	0.68
An.gambiae	0.74
An.funestus	0.72
Ae.aegypti	0.43
Ae.bromeliae	0.66

Different host species have similar bacteria

Different species of mosquitoes have widely varying abilities to vector human parasites, so we were interested in whether each species had a unique gut microbiota that could be influencing their vector competence. To do this, we examined how the total bacterial diversity was partitioned among individuals within each species and between species using the weighted Unifrac statistic described above. We found that while different individuals did have significantly different gut microbiota (Mantel test on matrix of weighted Unifrac distances: $r=$ -0.07, $p< 0.001$), only 7% of the variation was explained by between-species differences.

Therefore, two individuals from the same mosquito species will typically have almost as great a difference in their gut microbiota as two individuals from different species. After taking mosquito species into account, sampling location had no significant correlation with the species composition of bacteria found in guts (Partial Mantel test on matrix of weighted Unifrac distances: r= -0.01, p= 0.33).

To visualize these differences between species, we used the matrix of weighted UniFrac distances to construct a UPGMA tree (Lozupone et al., 2007; Lozupone and Knight, 2005). From this analysis it is clear that it is normal for individual mosquitoes to have gut microbiota that are more similar to individuals in other species than individuals from the same species (Figure 2.4). The same pattern is evident if the same data is used to create principal components plots (Figure 2.5). Despite this, there is clearly a tendency for certain species to cluster together. For example, *Culex* and *Mansonia* species tend to have similar gut bacteria, as does *A. aegypti* and the *Anopheles* species (Figures 2.4 and 2.5).
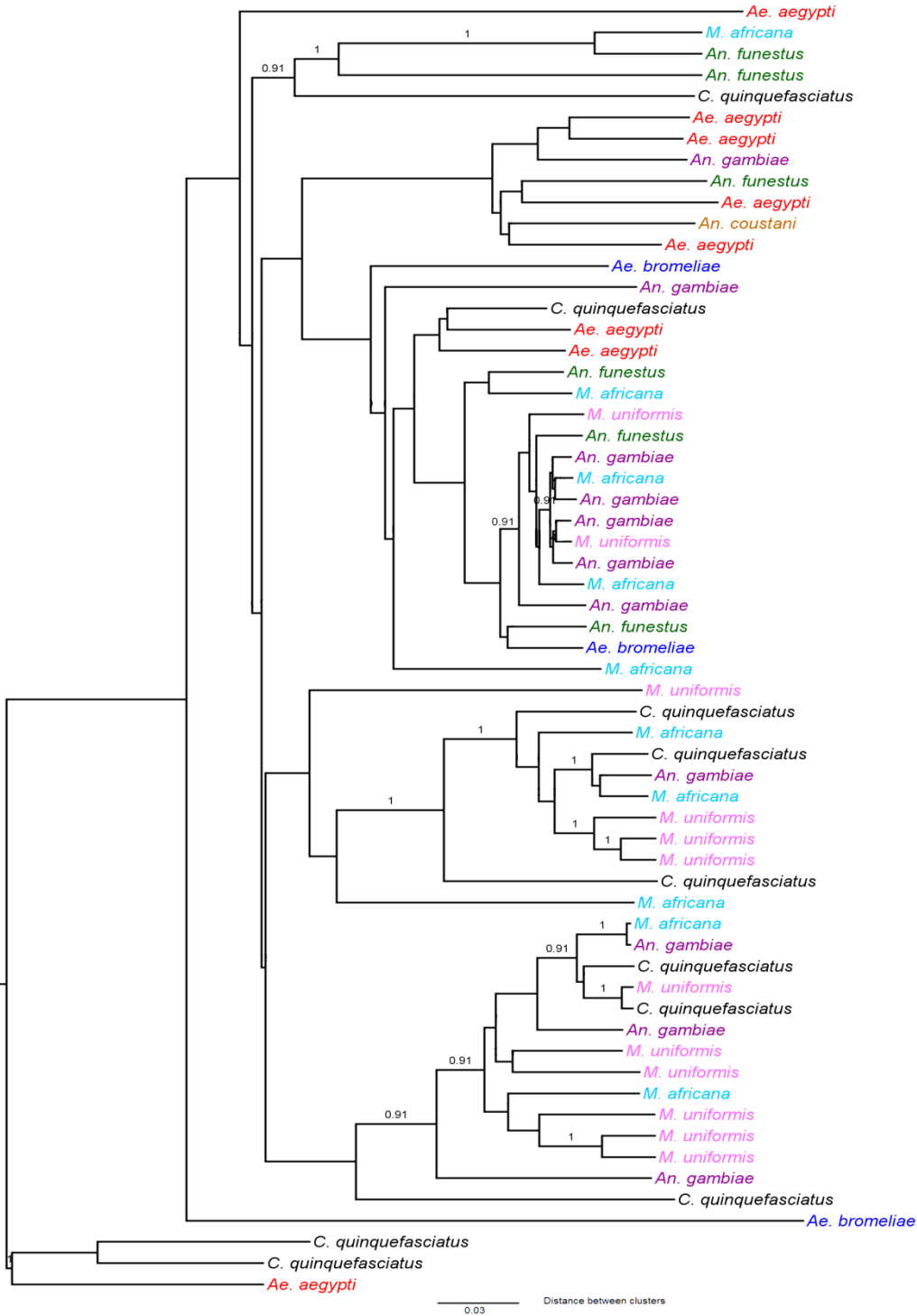
Figure 2.4: UPGMA tree showing clustering of the bacterial communities in the mosquito guts. Weighted (quantitative) classification was used. Jackknifed support values above 0.90 are shown.
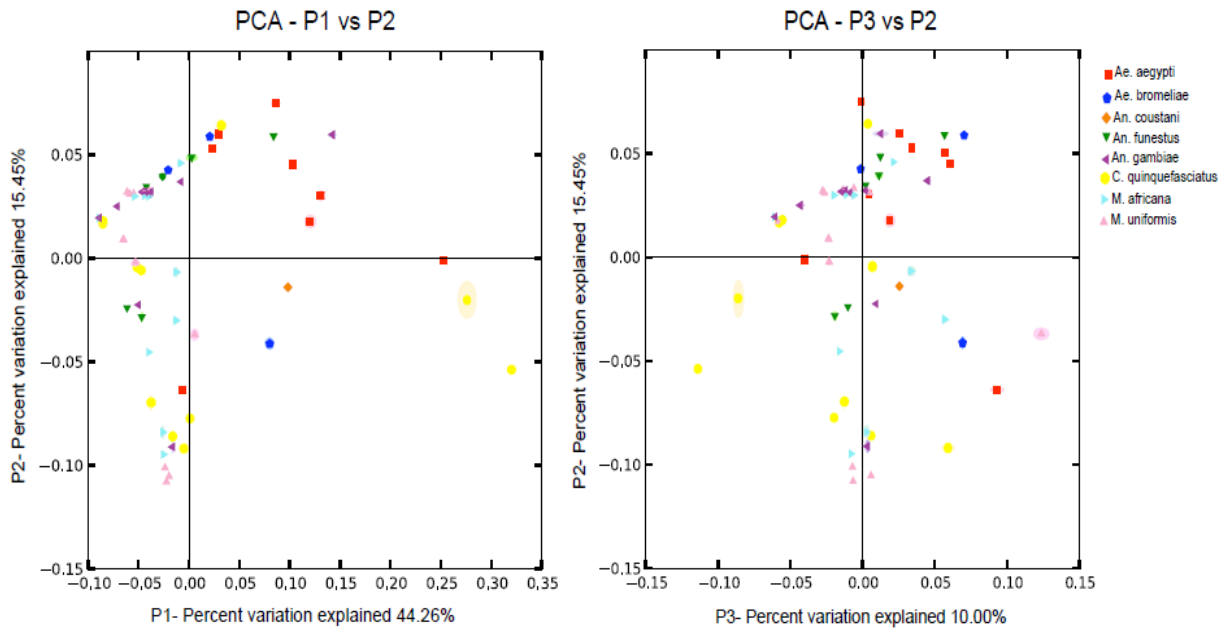
Figure 2.5: Principal component (PC) plots of gut bacteria diversity. Coloured dots represent mosquito samples. Lack of ellipsoids around dots indicates strong jackknife support values.

Classification of bacterial OTUs

Different bacterial taxa can have very different effects on the vectorial capacity of mosquitoes, so we classified our OTUs to the level of genus (Figures 2.3-B and 2.6). To do this, we compared our filtered sequence reads to 16S rRNA sequences in the Ribosomal Database Project (RDP II) Library using the Bayesian approach that is implemented by the RDP Classifier (Wang et al., 2007). This classification resulted in 144 unique bacterial genera which were mainly composed of four abundant Classes of bacteria. The four most abundant Classes of bacteria were the Gram-negative Gammaproteobacteria (62.3%), Alphaproteobacteria (18.3%) and Flavobacteria (11.6%), and the Gram-positive Bacilli (3.8%). 17.5% of the bacteria could not be classified below the level of Class (Figure 2.6). Nearly half of all the classified bacteria belonged to two genera (Figures 2.3-B and 2.6) —

Aeromonas (38.7%) and *Asaia* (13.2%). The next most abundant genus was *Chyseobacterium* (9.1%) followed by *Zymobacter* (6.0%).

Other bacterial taxa tended to have a much more patchy distribution (Figure 2.3-B). For example, unclassified Flavobacteria dominate in two individuals of *C. quinquefasciatus* (82% and 99% of sequences), but are rare or absent in the rest of our sample. *Pantoea* (Enterobacteriaceae) was dominant in a single individual of *C. quinquefasciatus* (94%), while *Pseudomonas* was at frequencies above 80% in single individuals of *Ae. aegypti*, *An. funestus* and *M. africana.* The distribution also suggests there are no host-specific bacterial genera. As was the case for the analysis of OTUs, most of the variation in the bacterial taxa was between individuals within a species rather than between species (Figure 2.3-B). Only two of the bacterial genera showed significant variation in abundance in the different mosquito species — *Aeromonas,* which varied from 49.8% in *An. funestus* to 14.9% in *M. uniformis* (Kruskal-Wallis test: χ^2= 22.53, d.f.= 7, *p*= 0.002), and *Chryseobacterium* (Kruskal-Wallis test: χ^2=33.6, d.f.= 7, *p*= 2×10^{-5}).
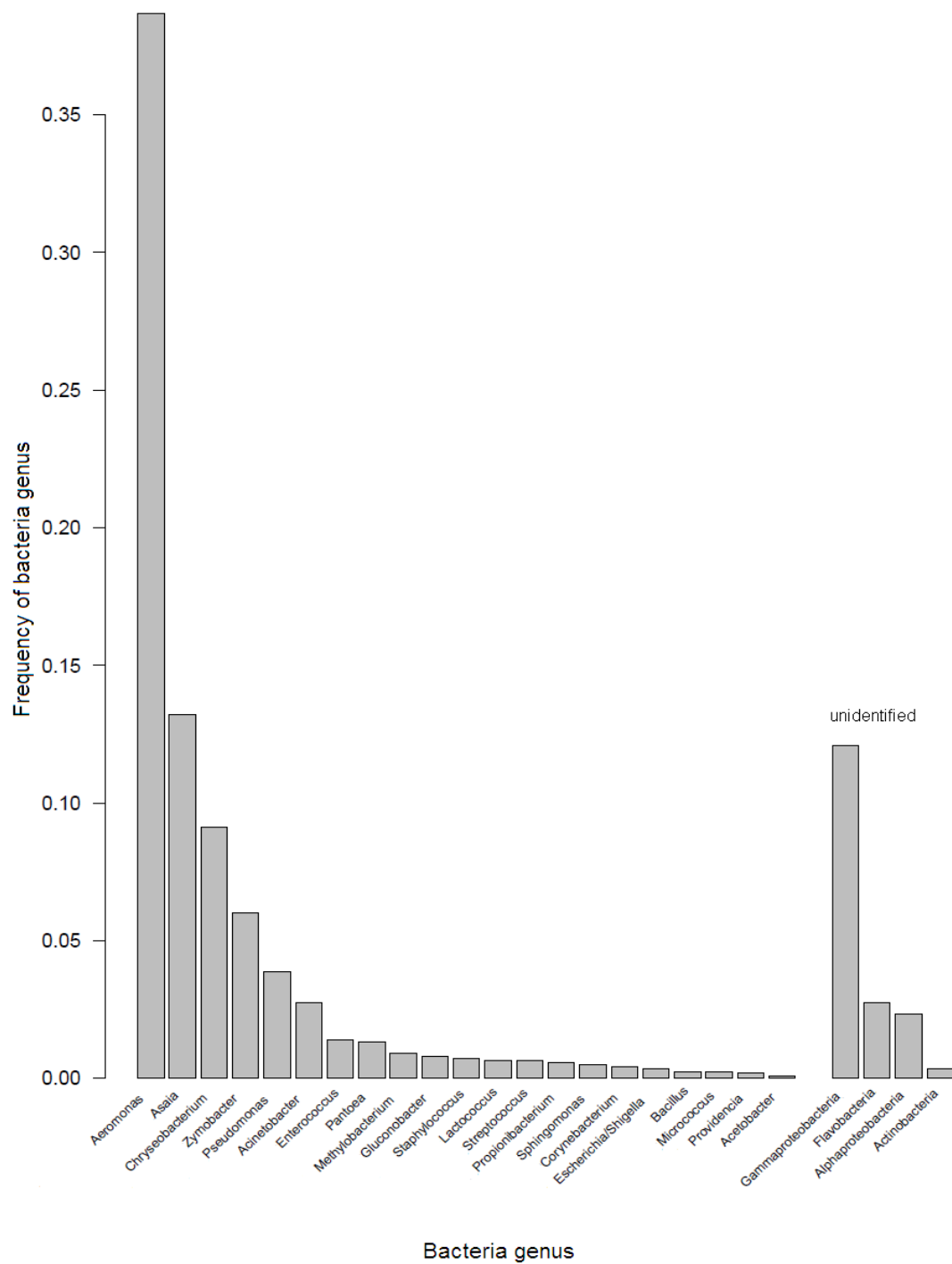
Figure 2.6: The mean frequency of bacterial genera in mosquito guts. Only bacteria that exceed 1% frequency in at least one individual are included. The mean frequencies are calculated with each individual gut being weighted equally.

2.4. Discussion

We have provided a comprehensive, unbiased, culture-independent study of the bacterial community in the guts of 8 mosquito species sampled from natural populations. We found that there is generally a very low bacterial diversity, with a single OTU typically making up two-thirds of all the bacteria. However, there are also many other rarer bacteria, with a typical gut containing 42 bacterial OTUs. Between individual mosquitoes of the same species, there is enormous variation in the bacterial taxa present, but there were few consistent differences between the different mosquito species in the composition of their gut microbiota.

The variation between individuals in the composition of their gut microbiota may affect the vector competence of mosquitoes. Several studies have found that a range of Gram-negative gut bacteria inhibit the development of *Plasmodium,* while Gram-positive bacteria do not (Cirimotich et al., 2011; Gonzalez-Ceron et al., 2003; Pumpuni et al., 1993). Furthermore, different Gram-negative bacteria have varying effects on *Plasmodium* (Cirimotich et al., 2011; Gonzalez-Ceron et al., 2003; Jadin, 1967). Some of the variation may be explained by differences in the production of certain metabolites. For example, the red pigment prodigiosin, which is produced by some Gram-negative bacteria has been shown to be effective against *Plasmodium* (Kim et al., 1999; Isaka et al., 2002; Lazaro et al., 2002). In *An. stephensi, Klebsiella* blocked the development of *P.berghei* while *Pseudomonas* did not (Jadin, 1967). In the same mosquito species, the two bacteria genera had opposite effects on *P. falciparum* (Jadin, 1967). Therefore, the differences in the gut microbiota we have observed between

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individuals within a host species could be causing variation in vector competence. They may also be affecting other aspects of host biology, such as is observed in *Drosophila melanogaster* where flies prefer to mate with individuals that have a similar bacteria community in their guts (Sharon et al., 2010).

The variation that we have observed may reflect differences in the bacteria that mosquitoes have acquired from the environment. Mosquitoes such as *Anopheles*, *Aedes* and *Culex* prefer laying their eggs in water that contains bacteria (Lindh et al., 2008; Pavlovich and Rockett, 2000; Rockett, 1987), and midgut bacteria acquired from the larval environment can then be transmitted trans-stadially to the adult gut (Jadin et al., 1966; Pumpuni et al., 1996; Briones et al., 2008). It is also possible for adult mosquitoes to acquire bacteria from their breeding water while they emerge from their pupal cases (Lindh et al., 2008). Bacteria acquired this way can then be horizontally transferred between individuals through deposition of bacteria back into laying water (Lindh et al., 2008) or via common feeding sites.

Host diet also shapes the gut microbiome. Differences in the gut microbiota of several species of *Drosophila* is strongly influenced by diet (Chandler et al., 2011), and it is possible that the lack of between-species variation we observed in the gut microbiota is because these mosquitoes tend to have rather similar diets, feeding on microbes as larvae, and blood and nectar as adults. Our samples may have included individuals that had blood fed and individuals that had not. This could cause between-individual variation, as both sugar- and blood-feeding changes bacterial abundance in mosquito midguts (Demaio et al., 1996;

Gusmão et al., 2010; Wang et al., 2011). Acetic acid bacteria, for example, are associated with many insects that have a sugar-based diet (Ashbolt and Inkerman, 1990; Mohr and Tebbe, 2006; Corby-Harris et al., 2007; Crotti et al., 2009). Blood-feeding in particular triggers the proliferation of bacteria (Gusmão et al., 2010) and, certain bacterial taxa show more increase than others. In adult *An. gambiae* that have fed on blood, there is an increase in Proteobacteria resulting in decrease in the bacterial species abundance in the gut (Wang et al., 2011). Newly emerged adults that have not fed generally have higher species richness than we observed (Wang *et al*. 2011), which suggests that we may have sampled older mosquitoes that have already fed. Although we selected individuals that were not engorged with blood, we have no knowledge of prior feeding patterns of our sample.

A less well understood influence on the bacterial community is the genetic background of the insect. Evidence for its potential role comes from *Drosophila,* where the gene *Caudal* maintains immune system homeostasis, and knock-down of the gene alters the composition of the gut microbial community, resulting in high mortality (Ryu et al., 2008). However, as the differences between individuals of the same species are far greater than between different species, this is likely to be a relatively unimportant factor.

Our deep sequencing approach has allowed us to catalogue the taxonomic diversity of the mosquito gut microbiota in great detail. In total, we found 22 genera which occur at a frequency of more than 1% in at least one of the individual guts. Consistent with previous studies, we found that the mosquito gut is dominated by Gram-negative bacteria (Cirimotich et

al., 2011; Demaio et al., 1996; Dong et al., 2009; Lindh et al., 2005; Straif et al., 1998), with Proteobacteria and Bacteroidetes constituting more than 90% of the community. As Gram-negative bacteria tend to offer greater protection against *Plasmodium* than Gram-positive bacteria (Cirimotich et al., 2011; Gonzalez-Ceron et al., 2003; Pumpuni et al., 1993, 1996), this suggests that the gut microbiota may be an important factor in reducing rates of disease transmission.

Aeromonas spp. were the commonest bacteria, representing on average over a third of the gut microbiota. *Aeromonas spp.* are common in insects, having been previously reported in house flies (Nayduch et al., 2005), tsetse flies (Geiger et al., 2011) and mosquitoes (Djadid et al., 2011; Pidiyar et al., 2004), with *Aeromonas culiciola* being the most abundant gut bacterium in *C. quinquefasciatus* (Pidiyar et al., 2002). *Aeromonas* is also commonly isolated from breeding water of mosquitoes (Smith et al., 1998), suggesting that mosquitoes are ingesting these bacteria as larvae. Trans-stadial transfer from larvae through to the adult gut is possible. Despite a reduction of bacterial numbers in the adult gut (Chavshin et al., 2012), *Aeromonas* rapidly proliferates following a blood meal (Pidiyar et al., 2002).

The second most abundant genus was *Asaia,* which is found in all the mosquito species we sampled is at an average frequency of 13%. *Asaia* is an acetic acid bacterium that has been found in the midgut, salivary glands and reproductive organs of *An. stephensi* and *An. gambiae*, two species of mosquitoes that transmit malaria (Favia et al., 2007; Damiani et al., 2010). The localization of *Asaia* in these tissues means that they may play important roles in

interacting with parasites. Furthermore, the bacterium is not only transmitted horizontally when mosquitoes feed together, but it is also transmitted sexually, maternally and paternally, so it can form stable associations across multiple generations (Damiani et al., 2008). Unusual for a vertically transmitted symbiont of insects, it can also be cultured, transformed and easily moved between host species, making it an excellent candidate for expressing anti-parasite proteins in natural populations, an approach called paratransgenesis (Favia et al., 2007; Damiani et al., 2010) (Crotti et al., 2009). Our study is the first report of natural association of *Asaia* with *M. uniformis*, *M. africana*, *Ae. bromeliae* and *An. coustani*. *Mansonia uniformis* is a competent vector of filariasis (Nelson, 1959; Ramalingam, 1968; Ughasi et al., 2012; Wharton, 1962), and *Ae. bromeliae,* transmits Yellow fever (Huang, 1986). Although *An. coustani* is previously recognized to have a zoophilic behaviour, it has recently been reported to also possess anthrophilic tendencies implicating the species in the potential transmission of human malaria (Fornadel et al., 2011). Our results suggest that *Asaia* can infect field populations of most mosquito disease vectors.

The genus *Chryseobacterium* was the third most abundant, and was particularly frequent in *Aedes* mosquitoes. Although *Chryseobacterium meningosepticum* was found in all individuals of *An. gambiae* tested by Dong et al, (2009), little is known about its effects on insects or disease transmission.

Pseudomonas has been reported to be common in mosquito guts (Jadin et al., 1966; Rani et al., 2009), but we found it had a very patchy distribution with only a few individuals infected at

frequencies above 1%. Both positive and negative effects of *Pseudomonas* on *Plasmodium* have been reported (Jadin et al., 1966; Straif et al., 1998), so the heterogeneity in infection rates may contribute to variation in disease transmission. *Pseudomonas* proliferates after a blood meal, and it has been suggested that it may be important in coping with oxidative stress after blood feeding (Wang et al., 2011).

In *Anopheles* mosquitoes*,* bacteria in the genus *Enterobacter* can dramatically reduce the intensity of *Plasmodium* infection (Cirimotich et al., 2011; Straif et al., 1998) due to the production of reactive oxygen species that affect the development of oocysts from ookinetes (Cirimotich et al., 2011). They have also been reported to be common in blood-fed *Ae. aegypti* (Gaio et al., 2011) where they have haemolytic activity that is important in digestion. Despite these bacteria having been isolated from African mosquitoes in the past, we found *Enterobacter* were rare in our sample, never occurring at a frequency over 1% in any of the guts.

The bacteria we have identified might not only be important in affecting natural rates of disease transmission, but could also be exploited to manipulate disease transmission in the wild. It may be possible to infect wild mosquito populations with bacteria that either naturally confer resistance to human parasites, or to exploit them for paratransgenesis — the concept of using insect symbionts to drive anti-parasitic factors through populations (Beard et al., 2002). Before this can be attempted we need a greater understanding of the transmission of these

bacteria in nature, and how bacteria released into the environment can compete with the natural symbionts of mosquitoes.

In summary, deep sequencing has allowed us to provide a comprehensive catalogue of the bacteria that naturally inhabit mosquito guts in this population, expanding the range of both hosts and bacteria that have been studied. We found that the mosquito microbiota has a very low bacterial diversity within an individual, but much greater variation across different individuals. Understanding the implications of this variation for disease transmission promises to be a fertile field for future research.

2.5. Acknowledgements

3. PREVALENCE AND PHYLOGENETIC ANALYSES OF *WOLBACHIA* ENDOSYMBIONTS OF WILD MOSQUITOES

This chapter has been modified and published as:

Osei-Poku, J.,Han, C., Mbogo, C.M. and Jiggins, F.M. (2012) Identification of *Wolbachia* strains in mosquito disease vectors. *PLos One*. *7* (12), e49922.

3.1. Introduction

Wolbachia are common intracellular bacteria species found in many arthropod species and nematodes (Werren et al., 2008). They are estimated to infect 40% of insect species (Zug and Hammerstein, 2012). They are generally vertically transmitted largely through infected female parents. Rare occurrences of horizontal transmission between genera and taxons have been reported (Casiraghi et al., 2005; Werren et al., 1995), but this is not an epidemiologically important mode of transmission as it only occurs over very long evolutionary timescales. *Wolbachia* usually infect the reproductive tissues of their host and manipulate the host's reproduction through cytoplasmic incompatibility (O'Neill and Karr, 1990; Clancy and Hoffmann, 1996), male-killing (Hurst et al., 1999), feminization (Rousset et al., 1992) and parthenogenesis induction (Stouthamer et al., 1993). Through these manipulations, the bacteria increase their transmission through subsequent host generations (Turelli and Hoffmann, 1991).

The relationship between *Wolbachia* and host could be physiologically beneficial to the host such as observed with filarial nematodes (reviewed in Taylor et al., 2005). Depletion

of *Wolbachia* from *Brugia malayi* by antibiotic treatment results in death of the filarial worm host (Landmann et al., 2011). In other hosts, inducing cytoplasmic incompatibility (CI) confers a selective advantage to infected females as these can successfully produce progeny with infected or uninfected males. Increased fecundity and longevity are observed in *Aedes albopictus* females infected with CI-inducing *Wolbachia* (Dobson et al., 2002). Some *Wolbachia* strains also protect their hosts against the detrimental effects of endoparasites. Positive-sense RNA viruses, in particular, have shown lowered titres in their insect hosts when hosts are co-infected with *Wolbachia* (Hedges et al., 2008; Osborne et al., 2009; Teixeira et al., 2008). In these studies, *Wolbachia*-infected *Drosophila melanogaster* and *D. simulans* have shown higher levels of tolerance or have been resistant to viral infections (Hedges et al., 2008; Osborne et al., 2009; Teixeira et al., 2008). When the *D. melanogaster Wolbachia* strain (*w*Mel) is transferred into *Ae. aegypti* or *Ae. albopictus*, it induces CI and blocks transmission of dengue and Chikungunya viruses (Blagrove et al., 2012; Moreira et al., 2009; Walker et al., 2011).

The effects of *Wolbachia* on metazoan parasites of public health importance have also been investigated. A virulent strain of *Wolbachia*, *w*MelPop, which over-replicates in somatic tissues and reduce the lifespan of infected hosts (Min and Benzer, 1997; McMeniman et al., 2009) caused an up-regulation of immune genes responsive to filarial worm infections when transinfected into *Ae. aegypti* (Kambris et al., 2009). *w*MelPop also reduced the intensity of the avian malaria parasite, *Plasmodium gallinaceum*, in *Ae. aegypti* (Moreira et al., 2009) and the rodent parasite, *P. berghei*, in *Anopheles gambiae* (Kambris et al., 2010).

In general, *Wolbachia* endosymbionts of insects possess vital characteristics that have increased interests in their use as potential mechanisms for disrupting transmission of insect-borne diseases (Beard et al., 1993b; Sinkins et al., 1997). *Wolbachia* are able to infect many somatic tissues of the host (Dean and Dobson, 2004) and have a wide host distribution (Dobson et al., 1999). They are able to spread rapidly through populations by CI induction and vertical transmission (Sinkins and O'Neill, 2000) and, can sometimes be horizontally transmitted (O'Neill et al., 1992). Most impressively, they also impair the development of disease pathogens (Moreira et al., 2009; Kambris et al., 2009, 2010). However, the choice of *Wolbachia* strain for vector manipulation needs to be carefully considered. For example, *w*MelPop which prevents the normal replication of viruses and development of metazoans, may be a good candidate, but high fitness cost to the host due to the life-shortening trait (Kambris et al., 2010) could be a threat. Decreased longevity in infected hosts implies reduced rate of bacteria spread within populations (McMeniman et al., 2009). Therefore, *Wolbachia* strains that confer resistance without life-shortening of hosts are desirable (Kambris et al., 2010).

The dynamics of *Wolbachia* strains that are introduced into an insect population may be altered by *Wolbachia* strains that already exist in the wild, as incompatibility may increase among strains (Hoffmann and Turelli, 1997). Furthermore, *Wolbachia* strains vary considerably in both the level of viral protection that they provide to their hosts (Osborne et al., 2009) and the strength of cytoplasmic incompatibility that they induce (Reynolds and Hoffmann, 2002; Sinkins et al., 2005). It is therefore important to critically assess the range of *Wolbachia* strains in natural populations of mosquitoes before beginning any control programmes with *Wolbachia*. This assessment includes investigating prevalence,

typing isolated strains of *Wolbachia* and investigating how phylogenetically related they are to each other.

Naturally-occurring *Wolbachia* in mosquitoes have been isolated in different species of mosquitoes (Kittayapong et al., 2002, 2000; Rasgon and Scott, 2004), many of which are non-vectors of human disease. The *Wolbachia* surface protein, *wsp*, is commonly used to detect *Wolbachia* infections, but increased recombination confounds the use of this gene for phylogenetic analyses (Jiggins et al., 2001). Multi-Locus Sequence Typing (MLST) of *Wolbachia* recommends the use of 5 single-copy bacterial genes (Baldo et al., 2006). These genes encode essential functional enzymes and proteins such as for aerobic metabolism and cell division so strong stabilizing selection acts on these genes with an average Ka/Ks<<1 (Baldo et al., 2006). Following the introduction of this typing system more information is being obtained on *Wolbachia* strains in different arthropod and nematode hosts (see PubMLST *Wolbachia* database at http://pubmlst/*Wolbachia*) (Jolley et al., 2004). However, a comprehensive phylogeny of *Wolbachia* has not yet been investigated using the information available.

In this study, we continue previous efforts to identify *Wolbachia* in mosquito disease vectors by examining 9 species of wild mosquitoes collected from Kenya for *Wolbachia* infections. We used amplified gene sequences to construct a phylogeny accounting for recombination events, which helped determine how mosquito *Wolbachia* strains are related to each other and other arthropod *Wolbachia* strains. We inferred how this could be applicable in the transinfection of *Wolbachia* strains among host strains.

3.2. Materials and methods

Mosquito samples

We used DNA extracted from guts of adult female mosquitoes collected from towns and villages along the Kenyan coast as described in Chapter 2 (see Section 2.2-Materials and methods). DNA from the guts of two individuals of *Aedes metallicus* collected from Matsangoni in Kilifi district was included in this experiment.

Wolbachia infection

The gene encoding the surface protein of *Wolbachia*, *wsp*, was amplified with general *wsp* primers (*wsp*81F and *wsp*691R) (Braig *et al.*, 1997). Briefly, each PCR reaction contained 2µl 10X PCR buffer (Bioline), 1µl 50mM MgCl$_2$, 2µl 2mM dNTP mix, 0.2µl each of 20µM forward and reverse primers, 1U Taq polymerase (Bioline), 1µL DNA sample and sterile water to make a final reaction volume of 20µl. The thermal cycling protocol was an initial denaturation at 95°C for 5mins; 30X cycles of denaturation at 95°C for 30s, annealing at 55°C for 20s and extension at 72°C for 20s; final extension at 72°C for 10mins and held at 4°C. The analysis was repeated on the heads and thoraces of a subset of these samples, but this did not lead to the discovery of any new infections, so the results are not reported.

As internal controls, the insect ribosomal internal transcribed spacer region-1 (ITS1) and mtDNA cytochrome oxidase I (COI) were amplified for each sample using BD1 and 4S primers (von der Schulenburg *et al.*, 2001) and universal COI primers (Folmer *et al.*, 1994) respectively, in separate reactions. The internal control reactions were set to ascertain the

quality of DNA samples and to correctly confirm the absence of *Wolbachia* in a sample. The PCR reaction for ITS1 and COI amplification was prepared as described above for *wsp.* Touchdown PCR cycling protocol was used for BD1 and 4S primers— 95ºC for 5mins, 10X cycles of 95ºC for 30s, 65ºC for 30s reducing the temperature by 1ºC after every cycle, 72ºC for 20s, 25X cycles of the thermal cycle protocol described for *wsp* amplification above— to allow amplification of different band sizes as was expected for the different mosquito species. The cycling programme for COI was the same as *wsp* except for the annealing temperature which was 48°C (Folmer et al., 1994). PCR products were visualised on 2% agarose gel stained with ethidium bromide.

wsp and ITS1 sequencing

For each mosquito species that was infected with *Wolbachia*, a maximum of 4 positive samples were selected for sequencing. PCR products for both *wsp* and ITS1 were cleaned with 4U Exonuclease I (ExoI) (NEB) and 2U Shrimp Alkaline Phosphatase (SAP) (USB Corporation). ExoI catalyses the removal of nucleotides and SAP removes 5' phosphates from DNA. Cleaned products were sequenced with the forward and reverse primers for each amplicon using ABI PRISM BigDye Terminator kit (Perkin-Elmer Corporation, U.S.A). Sequencing was done at the Source Bioscience Center, UK. Sequences were trimmed and assembled using Sequencher v4.5 (Gene Codes Corporation). Chromatograms were inspected for single and double peaks.

Multi Locus Sequence Typing (MLST)

For typing the *Wolbachia* strains detected in our infected samples, we used the multi-locus typing tool first described by Baldo et al. (2006). The protocol suggests the amplification of 5 bacteria housekeeping genes — *gatB, coxA, hcpA, ftsZ* and *fbpA.*. These genes are single copy genes and are widely distributed within the *w*Mel genome. The genes and their primer sequences are summarized on Table S2 (see Appendix). We selected 2 individuals from each of the 5 mosquito species that were infected with *Wolbachia*, except *Ae. metallicus* which had only one infected individual. The PCR set up for gene amplification using the standard MLST primers was slightly modified from that used by Baldo et al. (2006) to include final concentrations of 1X PCR buffer (Bioline), 1.5mM $MgCl_2$, 0.2mM dNTP mix (Invitrogen), 0.5µM forward and reverse primer mix and 0.5U of Taq polymerase in 20µl of reaction mix. Each reaction mix included 1µl of DNA sample. The cycling conditions were as previously described (Baldo et al., 2006) with denaturation temperature at 95°C and incubation time for annealing and extension steps reduced to 30s.

We used a nested PCR to amplify *hcpA* for *M. uniformis* samples as these failed to amplify with the *hcpA* standard primers F1/R1. Firstly *hcpA* F3/R3 primer set (Table S2) was used in a reaction with Promega GoTaq Hot Start Polymerase. We set up each reaction tube adapting the standard recommended protocol for a final reaction volume of 20µl for the Promega GoTaq Hot Start Polymerase. Then, 1µl of amplicon from the F3/R3 reaction was used in the next round of PCR using the *hcpA* F1/R1 primers as already described. The PCR resulted in multiple bands for *M. uniformis*. The correct band size was excised and purified with Qiagen Gel extraction kit. All amplicons were cleaned and prepared for sequencing as previously described.

Processing of MLST sequences

Sequences from each gene were aligned and visually inspected for defined chromatograms in Sequencher v4.5 (Gene Codes Corporation). We repeated sequencing for sequences that had undefined chromatograms to ensure correct SNP calling for all samples. We called SNPs only at positions that showed clear unambiguous peaks. As there was no difference in gene sequences between individuals of the same mosquito species, a consensus sequence for each gene per mosquito host species was obtained (except the *hcpA* and *fbpA* genes for *M. uniformis*). The *fbpA* gene was resolved for one of the two *M. uniformis* samples and *hcpA* was unresolved for both *M. uniformis* individuals. All consensus sequences were trimmed to the appropriate length for database query. We performed a BLAST search of each sequence in the *Wolbachia* MLST database (http://pubmlst.org/*Wolbachia*) (Jolley et al., 2004). Where a sequence had an exact match in the database, it was assigned the designated allele number. We submitted 6 new alleles to the database for allele number assignment which includes all the genes for the *Aedes* sp. and *hcpA* for *M. africana*. The complete isolate form containing species information and allele numbers for sequenced genes was submitted to the curator of *Wolbachia* MLST database and have been assigned ID numbers 496-501 (http://pubmlst.org/*Wolbachia*).

Running ClonalFrame

To account for the effects of recombination on the phylogeny, we analysed the dataset with ClonalFrame v1.2 (Didelot and Falush, 2007). Unlike other phylogeny analysis software, ClonalFrame estimates clonal relationships while taking into account recombination as a mode of substitution within genes. This approach can estimate the contribution made by recombination to total substitutions observed (Didelot and Falush, 2007). The complete

dataset included *Wolbachia* MLST sequences from 115 host strains obtained from PubMLST (http://pubmlst.org/*Wolbachia*), and our 5 sample species. For the downloaded data, only host strains with complete information — at least host genus and allele numbers for all 5 MLST genes — were included. To avoid repetition of MLST information, MLST profiles were critically analysed and a single unique profile was selected for each host species. For example, *Agelenopsis aperta* had been represented more than once in the database with the same profile information (http://pubmlst.org/*Wolbachia*) hence only one of these was chosen to represent that host strain.

All 5 gene sequences for the 120 sample set were aligned independently in Mauve v2.3.1 (Darling et al., 2004). To ensure convergence in our analysis, we performed 9 independent runs of our dataset in ClonalFrame v1.2 (Didelot and Falush, 2007) at 100,000 MCMC iterations after 100,000 burn-ins. The number of iterations performed between recording parameters in the posterior sample was set at 100. Default settings were used for all other parameters. For the first 8 runs we used a uniformly chosen coalescent tree as the initial tree. As a UPGMA gives a good representation of tree topology (Didelot and Falush, 2007), we performed the ninth run with parameters as previously mentioned but starting with a UPGMA tree.

The output with the UPGMA starting tree was compared with the other 8 outputs using the 'tree comparison tool' in ClonalFrame (Didelot and Falush, 2007). This tool compares nodes of an uploaded tree to other tree outputs and plots the nodes according to similarity in observed nodes. The UPGMA tree showed good convergence with the other outputs

(Figure 3.1). Good convergence was also demonstrated by the Gelman and Rubin test (Gelman and Rubin, 1992) implemented in ClonalFrame (Didelot and Falush, 2007). The UPGMA starting tree output was, therefore, used in further analyses. The posterior sample of trees was exported into MEGA 5.05 (Tamura et al., 2011) and a consensus tree with branch support values was drawn at 50% majority rule. The resulting tree was visualized and rooted in FigTree v1.3.1 (Rambaut, 2006).
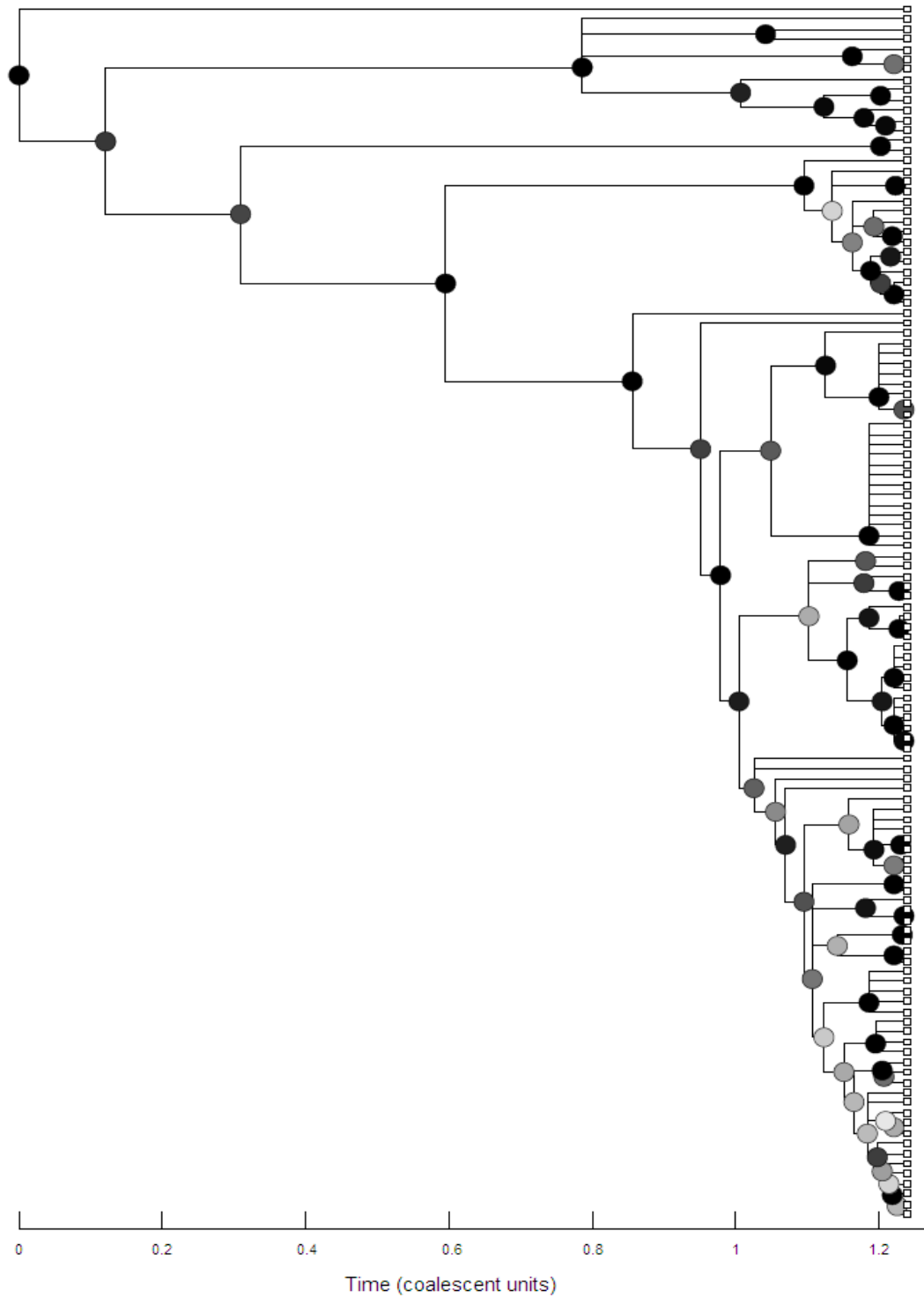
Figure 3.1: ClonalFrame 'tree comparison' output of UPGMA tree compared with the other 8 coalescent tree models. Shaded circles are proportional to the support on the nodes during the tree comparison. Black circles show nodes present in UPGMA and all the other trees, white circles show presence of nodes in UPGMA output but not in any of the other trees.

Assessing recombination events

Substitution events in the mosquito clades were investigated for probability of recombination. Plots for substitution events with high import probability were analysed further for sources of the 'imported' sequence. We used the criteria used by Didelot et al. (2009) to detect sources of imports in the mosquito clades. To do this a neighbour-joining tree was constructed in PAUP 4.10 beta (Swofford, 2003) with all 506 samples in the PubMLST database for each MLST gene under scrutiny (http://pubmlst.org/*Wolbachia*). Sequences showing high probability of recombination events were investigated for similar sequences in the database that have 2 or fewer nucleotide differences. If no sequence in the database meets this criterion then the import is said to be from an 'external source'. When similar sequences are found, that clade is said to be the source of the import. If more than one clade has host strains with similar sequences, then the import is ambiguous as the import could have come from either of these clades (Didelot et al., 2009).

3.3. Results:

Single strain Wolbachia infections

The prevalence of *Wolbachia* in the mosquito species sampled is shown on Table 3.1. Consistent with previous studies on *Wolbachia* infections in mosquitoes, none of the *Anopheles* species and *Ae. aegypti* were positive for *Wolbachia* (Kittayapong et al., 2000; Rasgon and Scott, 2004), while *Culex quinquefasciatus* and *Mansonia uniformis* were infected (Kittayapong et al., 2000). We make here a first report of *Wolbachia* infections in *Ae.*

bromeliae, *Ae. metallicus* and *M. africana*. In *Ae. bromeliae,* 75% of individuals were infected, while in the closely related *Ae. metallicus,* one of the two samples was infected. Differences in ITS1 band sizes (Figure 3.2) and sequencing of the amplicons confirmed that the infected *Ae. metallicus* individual was a distinct species to *Ae. bromeliae*. For each mosquito species, there were no nucleotide polymorphisms in the *wsp* sequences and the chromatograms showed clear single peaks, implying that a single strain was infecting these mosquitoes.

Table 3.1: Summary of mosquito species analysed for *Wolbachia* surface protein (*wsp*), ribosomal ITS1 and COI. Prevalence of *Wolbachia* in host species is shown with 95% confidence interval in parentheses.

Species	Number analysed	*wsp* positive	*Wolbachia* prevalence (%)
Anopheles gambiae s.l.	22	0	
Anopheles funestus	27	0	
Anopheles coustani	4	0	
Culex quinquefasciatus	24	10	42 (22-63)
Mansonia uniformis	19	5	26 (9-51)
Mansonia africana	22	6	27 (11-50)
Aedes aegypti	29	0	
Aedes bromeliae	16	12	75 (48-96)
Aedes metallicus	2	1	50 (1-99)
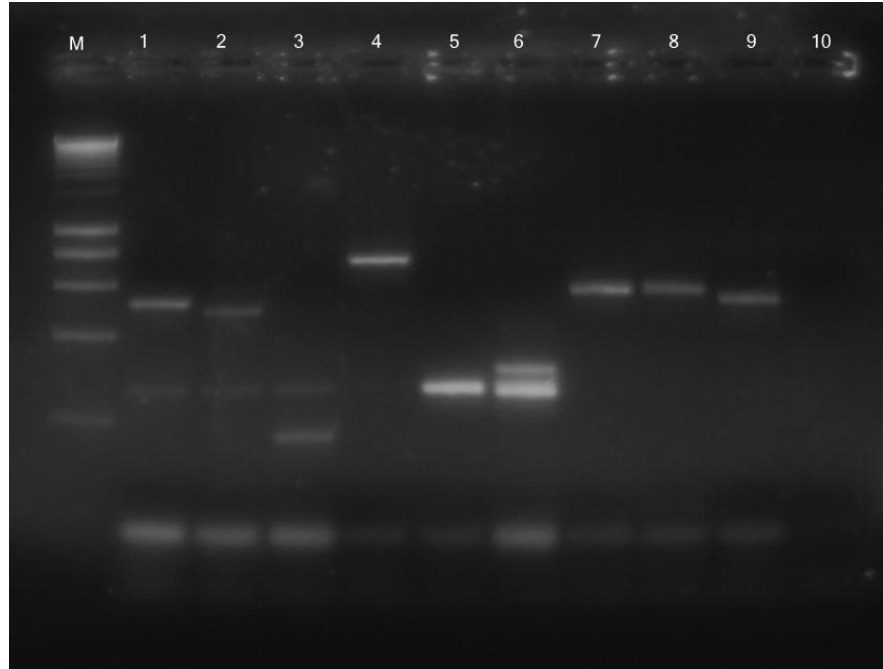
Figure 3.2: Species-specific band sizes for ITS region. M= 1X Generuler 200bp DNA ladder plus (Fermentas); 1=*Anopheles gambiae*; 2=*Anopheles funestus*; 3=*Anopheles coustani*; 4=*Culex quinquefasciatus*; 5=*Mansonia uniformis*; 6=*Mansonia africana*; 7=*Aedes aegypti*; 8=*Aedes metallicus;* 9=*Aedes bromeliae;* 10=negative control

Phylogeny

To make our tree construction more robust we used the MLST gene sequences to construct the *Wolbachia* phylogeny (Figure 3.3). The phylogeny grouped strains into supergroups A, B, F and D (Figure 3.3; supergroup D was used as the outgroup to root the tree) (Bandi et al., 1998; Casiraghi et al., 2005; Lo et al., 2002; Werren et al., 1995). The *Wolbachia* strains we identified infecting the Culicini and Masoniini tribes of mosquitoes belonged to supergroup B while those in the Aedini tribe were in supergroup A (Figure 3.3). *Wolbachia* strains in *Ae. bromeliae* and *Ae. metallicus* formed a highly supported monophyletic group, whose relationship with the strain from *Ae. albopictus* is poorly resolved. *Aedes* sp. (including *Ae. albopictus* from the database) formed monophyletic groups with the *Wolbachia*

endosymbionts of certain species of ants; *Odontomachus clarus*, *Anopolepis gracilipes*, *Lophomyrmex* sp. and those of the tortoise beetle *Acromis sparsa*. The three strains that infect *C. quinquefasciatus, M. uniformis* and *M. africana* clustered together with three strains found in the Lepitoptera (Figure 3.3). There are numerous other strains from this clade in the MLST database, many of which infect Lepidoptera, and if these are included in the tree, the relationships within the clade are poorly resolved (Figure S1).

Figure 3.3: Phylogeny of *Wolbachia* strains based on *Wolbachia* MLST genes constructed using the Bayesian ClonalFrame software. Tip labels include 115 host strains from PubMLST (http://pubmlst/*Wolbachia*) and 5 experimental mosquito host species. Red arrows indicate positions of mosquito *Wolbachia* strains. Tree was generated from a consensus of multiple trees generated after running dataset on ClonalFrame. The branch labels are support values at 50% consensus rule. Only support values above 90% are shown. Branch lengths are proportional to divergence time in coalescent units.
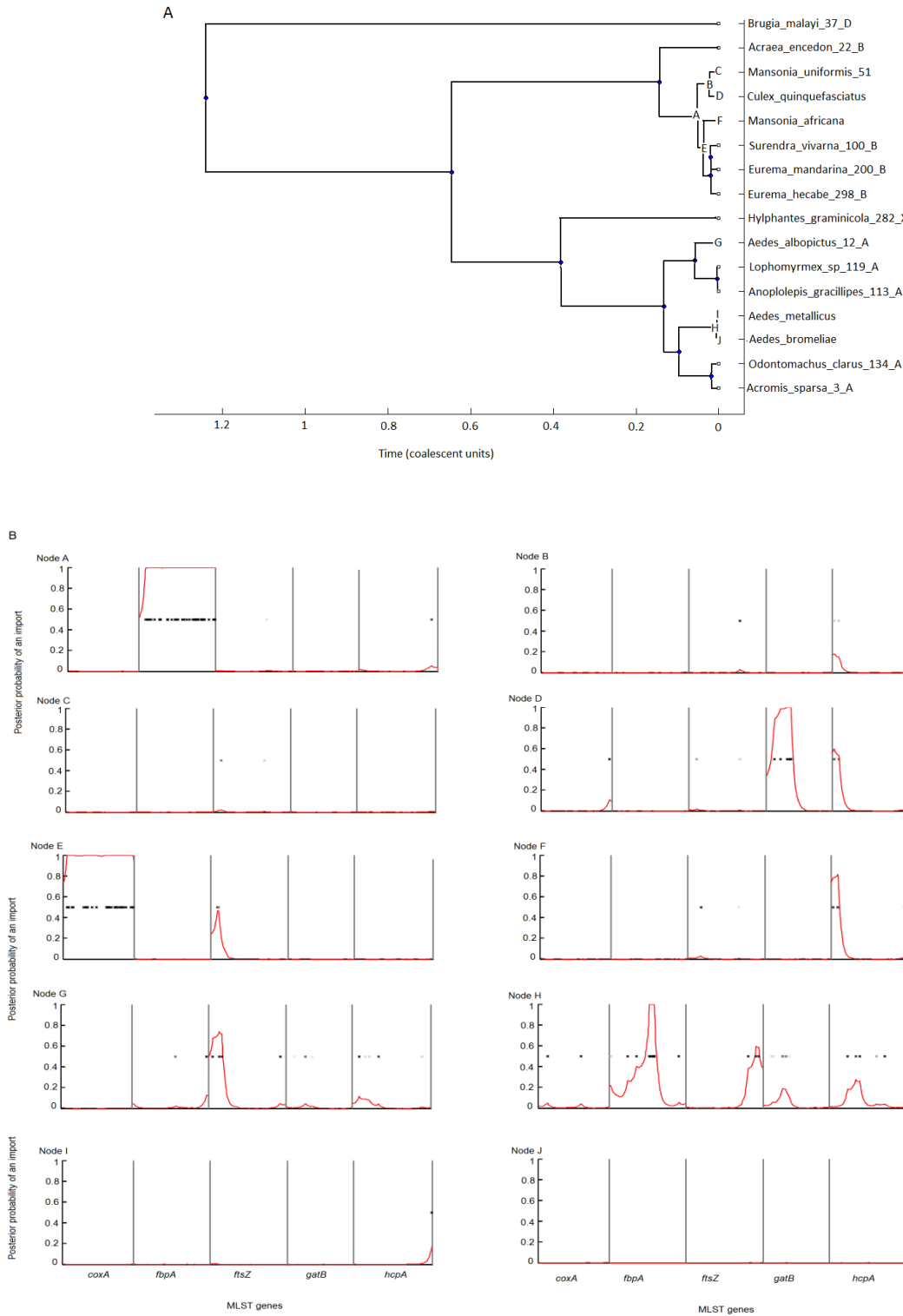
Recombination events

The topology of *Wolbachia* trees using the *wsp* and/ *ftsZ* genes have shown incongruence (Von der Schulenburg et al., 2000; Zhou et al., 1998) as a result of high recombination and positive selection on the *wsp* gene (Jiggins et al., 2001). Using MLST analyses and ClonalFrame we wanted to reconstruct the phylogeny of *Wolbachia* and make estimations of events that have occurred within *Wolbachia* strains. Across the entire tree of 120 strains, we estimate that recombination involves a mean tract length of 130bp being exchanged between strains (95% credibility interval: 100 - 168bp). We estimated that recombination (*r*) and mutation (*m*) had a similar probability of introducing substitutions into the genome of *Wolbachia* (mean *r/m*=1.3; 95% credibility interval: 0.97 - 1.73). Although both events may have equal chances of producing nucleotide substitutions, the rate at which each occurs could be different. Defined by ρ/θ (recombinational to mutational rate), point mutations were estimated to happen roughly four times more frequently than recombination (ρ/θ=0.26, 95% credibility interval: 0.18 - 0.35).

We were specifically interested in events that have led to the mosquito clades. We inspected the substitutions that had occurred on individual branches leading to various nodes in the mosquito clades (Figure 3.4-A). Few lineages had substitutions with high probability of recombination (posterior probability recombination > 0.95; Figure 3.4-B). We described events as imports when there was high confidence of recombination in more than half the length of the gene. On the branch that leads to the *Culex* and *Mansonia* clade (Figure 3.4; node A), the full length of the *fbpA* gene was imported. Similarly, the entire *coxA* gene was

imported on the lineage leading to the strains infecting *M. africana* and several Lepidoptera (Figure 3.4; node E). There was also evidence of two smaller recombination events in the mosquito clades (Figure 3.4; nodes D and H). It seems that further mutations in the *hcpA* gene distinguished endosymbionts of *M. africana* from those found in the Lepidoptera (Figure 3.4; node F). The *Aedes* clade showed less striking importation events and substitutions within the genes in this clade were of low probabilities indicating recent mutations.

Figure 3.4: Recombination events on branches leading to nodes in the mosquito *Wolbachia* clades. (A) pruned tree showing the inspected nodes and their corresponding substitution events on panel B. Positions marked 'x' are nucleotide substitutions in the genes.

Sources of recombination in mosquitoes

Since *coxA* and *fbpA* genes showed strikingly high import probabilities in almost the entire length of the gene, we investigated the possible sources of these imports. In our neighbour-joining tree analyses, we looked for taxa which had very similar sequences for each gene of interest (two or fewer differences) but appeared elsewhere on the MLST tree. Our neighbour-joining tree construction analyses for *fbpA* (Figure 3.5) showed a single clade for *Wolbachia* endosymbionts of *Culex* and *Mansonia* mosquitoes and mostly Lepidoptera. As these strains appeared at different places on the MLST tree (Appendix: Figure S1) we were unsure which clade was the source of the import. The source of the *coxA* sequence appears to be *Wolbachia* strains 355, 502, 439 and 492 in the MLST database (Figure 3.6, Appendix: Figure S1). Unfortunately, the names of the arthropod species that these strains infect have not been published.
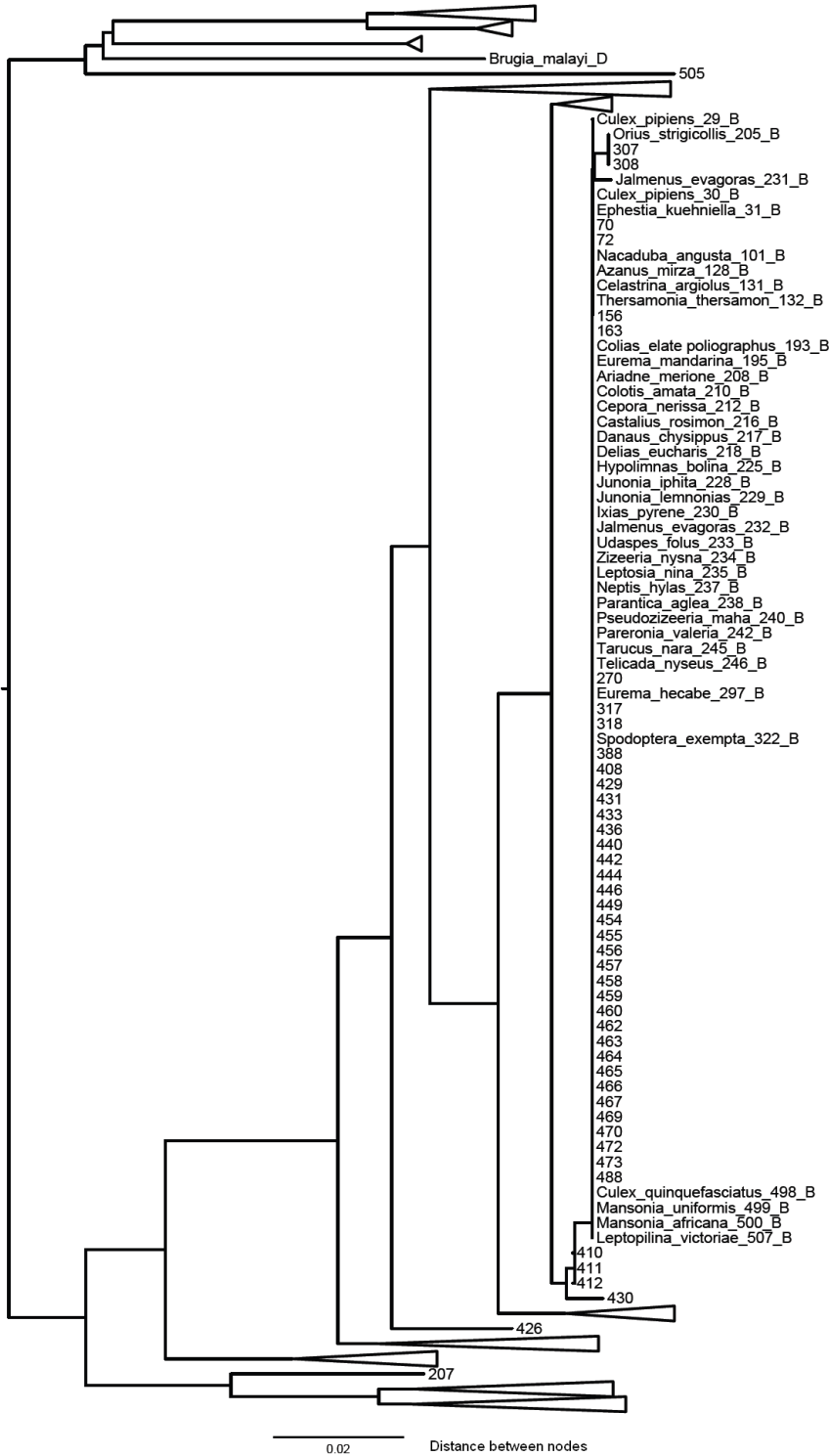
Figure 3.5: Neighbour-joining tree of *fbpA* gene showing clade for *Culex*, *Mansonia* and Lepidopteran *Wolbachia* strains. Other clades have been collapsed to show only the clade of interest. Taxa labels with only numbers are MLST *Wolbachia* database IDs with no host strain description.
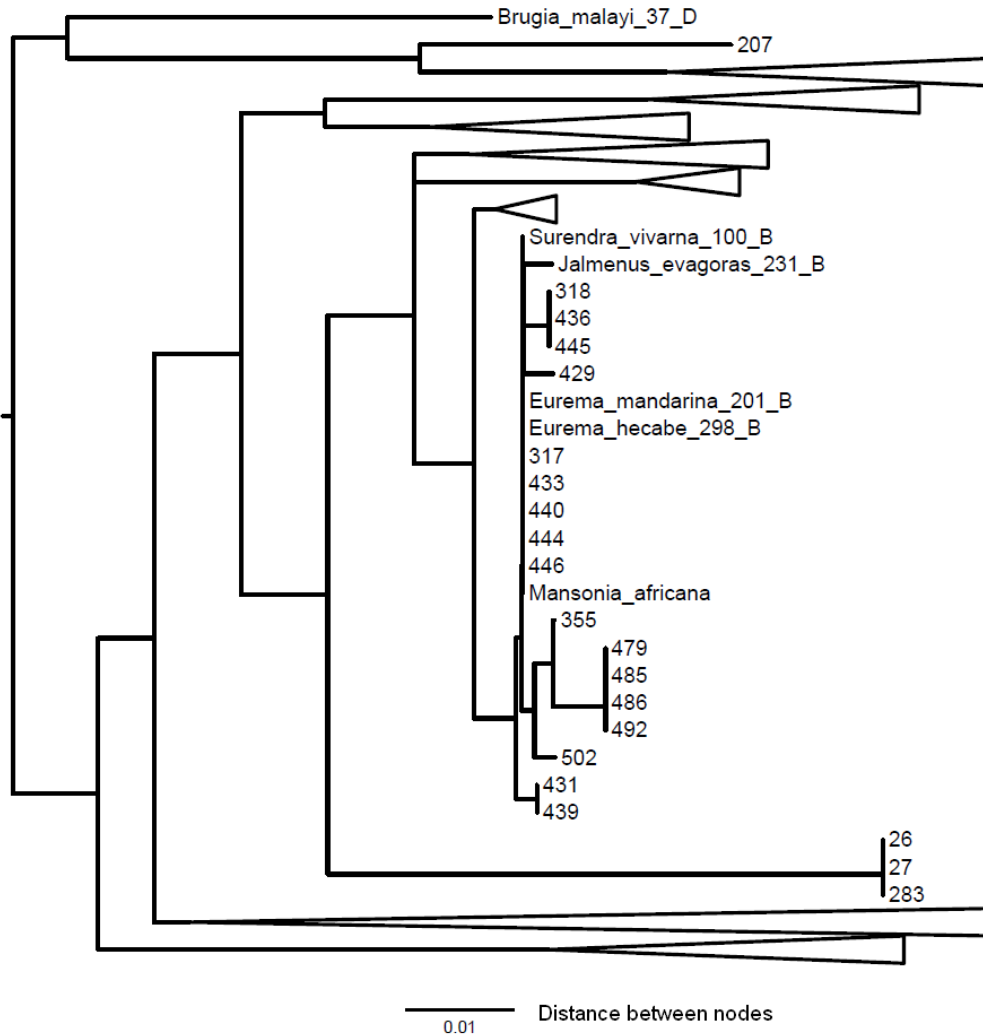
Figure 3.6: Neighbour-joining tree of *coxA* gene showing clade for *Mansonia africana* and Lepidopteran *Wolbachia* strains. Other clades have been collapsed to show only the clade of interest. Taxa labels with only numbers are MLST *Wolbachia* database IDs with no host strain description. Note that 355 and 502 are in a separate clade from *M. africana* but has fewer than 3 nucleotide differences.

3.4. Discussion

Wolbachia bacteria were first reported in *C. pipiens* (Hertig and Wolbach, 1924) and since then more strains have been reported in other mosquito species (Trpis et al., 1981; Kittayapong et al., 2000; Ricci et al., 2002; Rasgon and Scott, 2004). *Wolbachia* has the

potential to prevent mosquitoes from transmitting viruses like dengue and Chikungunya (Moreira et al., 2009; Walker et al., 2011). Furthermore, some strains of *Wolbachia* also affect metazoan parasites like *Plasmodium* (Moreira et al., 2009), hence they may also play a role in affecting the transmission of these parasites (Kambris et al., 2010, 2009). With the growing interest of using *Wolbachia* as a possible mechanism for preventing transmission of vector-borne diseases (Beard et al., 1993b; Sinkins and O'Neill, 2000; Townson, 2002), we were interested in finding *Wolbachia* infections in different species of mosquitoes collected from a common geographical area.

We assessed the prevalence of *Wolbachia* in the gut samples of wild *Anopheles*, *Aedes*, *Mansonia* and *Culex* species by amplifying the *Wolbachia* surface-protein gene, *wsp*. Due to variation in *Wolbachia* tissue tropism, some strains may go undetected. Nevertheless, we have provided a minimum estimate of the prevalence of *Wolbachia* in these mosquito species. Apart from the Anophelines and *Aedes aegypti* that have consistently been reported to have no *Wolbachia*, all the other species sampled were infected with an average prevalence of 30%. Here, we make the first report of *Wolbachia* infections in *Ae. bromeliae*, a vector of yellow fever virus (Huang, 1986) and *M. africana,* a vector of the filarial nematode *Wuchereria bancrofti* (Ughasi et al., 2012) — a major cause of lymphatic filariasis. After accounting for recombination in phylogenetic analyses, the *Wolbachia* strains in these infected mosquitoes were clearly categorized into supergroups A and B. Important import or recombination events occurred on the branch that led to the mosquito *Wolbachia* strains in supergroup B. Horizontal

transfer events between mosquito species may imply a technically easier *Wolbachia* strain for transinfecting other mosquitoes as part of control programmes.

We are increasingly understanding the importance of *Wolbachia* for their roles in resistance (Kambris et al., 2010; Moreira et al., 2009; Walker et al., 2011) and tolerance of arthropod hosts to pathogens (Osborne et al., 2009). In parasitic worms such as *Brugia* and *Onchocerca*, *Wolbachia* bacteria form an essential mutualistic relationship with the worm, maintaining the integrity of the parasite through its life cycle (Landmann et al., 2011; Townson et al., 2000). In arthropods, the *Drosophila* strains of *Wolbachia* have particularly been remarkable as they have been shown to protect hosts against the detrimental effects of positive-sense RNA viruses (Hedges et al., 2008; Osborne et al., 2009; Teixeira et al., 2008). Transinfection of *w*Mel into *Ae. aegypti* (Walker et al., 2011) and *Ae. albopictus* (Blagrove et al., 2012) has shown similar results. Few mosquito species known to vector diseases are naturally infected with *Wolbachia*. We make a first report of *Wolbachia* infections in *Ae. bromeliae*, a vector of yellow fever (Huang, 1986), its close relative—*Ae. metallicus*— and *M. africana*. Natural infection in *M. uniformis* was previously reported in samples collected from Southeast Asia (Kittayapong et al., 2000). This study has extended the incidence of *Wolbachia* in *M. uniformis* to Africa.

The sample size was not as large and diverse as used by Kittayapong et al. (2000) and Ricci et al. (2002), but we still observed comparatively similar prevalence of *Wolbachia* (42%) in *Culex* (Kittayapong et al., 2000). This suggests similar dynamic patterns of *Wolbachia* infection in *Culex* sp. across geographic areas. *Wolbachia* infection in *Ae. bromeliae* has been

first reported here with high prevalence (75%). The high number of infected individuals could have very interesting advancement in using this *Wolbachia* strain in vector manipulation of mosquitoes. The failure of all three species of *Anopheles* in our study confirms the absence of *Wolbachia* in this group of mosquitoes (Kittayapong et al., 2000; Ricci et al., 2002). Kittayapong et al. (2000) speculated that this may be due to the inability of Anophelines to physiologically support *Wolbachia*. Recent studies, however, show that *Wolbachia* transferred into *Anopheles gambiae* are able to invade some somatic tissues, but not the reproductive tissues nor the midgut (Hughes et al., 2011a). This points out that Anophelines can be infected by *Wolbachia* but not vertically transmitted.

The *Wolbachia* strains we have identified may have implications for both the natural transmission rate of human disease, and the attempts to manipulate transmission rates through the release of *Wolbachia*-infected mosquitoes. As virus protection appears to be a common trait among *Wolbachia* strains in arboviral hosts (Hedges et al., 2008; Moreira et al., 2009; Teixeira et al., 2008), it is possible that these strains we have detected in the yellow fever vector *Ae. bromeliae* may reduce arboviral transmission rates in the wild. This has the potential to be significantly important since 75% of individuals were infected. These strains also have the potential to be transinfected into key vector species such as *Ae. aegypti*. This is likely to be far easier than transfers of strains from distantly related species like *Drosophila,* as transinfection is known to have higher success rates between more closely related species of insects (Russell et al., 2009). Finally, these resident *Wolbachia* strains might interfere with attempts to introduce novel strains into the population as part of control programmes.

One of the major problems involved in using phylogeny to type *Wolbachia* strains is with the utilization of the *wsp* gene which has been shown to be under strong positive selection (Jiggins et al., 2002b). Sometimes the use of the slowly-evolving cell division gene, *ftsZ*, has been used in conjunction with *wsp* (Von der Schulenburg et al., 2000) or on its own (Werren et al., 1995) to resolve the phylogeny. Both genes were used to categorize the *Wolbachia* strains of *C. quinquefasciatus*, *M. uniformis*, *Ae. albopictus* and *Aedes* (Stegomyia) sp. into supergroups A and B with some mosquitoes showing super-infections (Kittayapong et al., 2000). Homoplasy and increased substitutions in *wsp* and *ftsZ* sequences could lead to low confidence in inferred phylogenetic analyses (Von der Schulenburg et al., 2000). This could result in undefined categorization of *Wolbachia* strains isolated from their hosts. We used Multi-Locus Sequence Typing (Baldo et al., 2006) and ClonalFrame (Didelot and Falush, 2007) to help avoid such issues in our phylogenetic analyses. The phylogeny of *Wolbachia* presented here by using these two methods grouped the mosquito *Wolbachia* strains into supergroups A and B; the Aedini in A and Culicini and Mansoniini in B. Our *Culex* MLST profile matched exactly those of *C. pipiens* on the PubMLST database (http://pubmlst/*Wolbachia*).

Although supergroups in *Wolbachia* give little information on the functions of the bacteria in its host (Bordenstein et al., 2009), certain inferences may be made based on how closely related unknown strains are to defined strains. For instance, it is suggested that *Wolbachia* strains that are closely related to *w*Mel can also protect their natural host, *D. melanogaster*, against DCV (Hedges et al., 2008; Teixeira et al., 2008). In view of this, it is likely that *Mansonia* strains of *Wolbachia* are inducing cytoplasmic incompatibility as they form a

monophyletic group with *C. quinquefasciatus Wolbachia* strains. It is difficult to try to infer anything about the characteristics of the bacteria in the *Aedes* mosquitoes from their positions within the phylogeny. *Aedes albopictus*, for example, has high prevalence of super-infections in nature with evidence of cytoplasmic incompatibility (Kittayapong et al., 2002). It is not exactly known what the functions of the supergroup A strains are in *Ae. albopictus* mosquitoes as the study that showed the influence of *Wolbachia* on viral titres was done by eliminating both strains A and B from experimental mosquitoes (Mousson et al., 2010). Despite the gap in the knowledge of strain A *Wolbachia* in *Aedes* mosquitoes, the high number of infected individuals in *Ae. bromeliae* may suggest host reproductive manipulations which usually results in rapid spread and increased numbers of infected individuals. This, however, needs to be investigated and confirmed.

Our analyses in ClonalFrame also provided estimations on relative time since coalescence of the *Wolbachia* strains. Generally, supergroup B is relatively younger than supergroup A (Figure 3.3) suggesting that the evolution of cytoplasmic incompatibility in *C. pipiens* (Yen and Barr, 1971), male-killing in *Acraea encedon* (Hurst et al., 1999), occurred more recently than viral protection in *D. melanogaster* and *D. simulans* (Hedges et al., 2008; Osborne et al., 2009; Teixeira et al., 2008).

This work has also shown that mosquito species cluster together with respect to their *Wolbachia* endosymbionts. Two hypotheses could explain this: co-speciation and horizontal transmission. A common ancestor may have harboured a type of *Wolbachia* endosymbiont

which co-evolved with mosquito species. This implies that two common ancestors of mosquitoes existed; one for the Culicini and Masoniini tribes and the other for the Aedini tribe. Horizontal transfer between related hosts may seem more probable. Horizontal transfer most commonly occurs between the closest related host species (Baldo et al., 2008; Jiggins et al., 2002a) and unlike speciation, horizontal transmission may not require evolutionarily long time scales for both the endosymbiont and the host to adapt to each other and establish a mutual relationship.

This study has shown that there is high prevalence of *Wolbachia* infections in mosquitoes in the wild, some of which are important disease vectors. Using phylogeny we have shown the relationship between the isolated strains among mosquitoes and with other host strains. This has highlighted that it may be much easier to move *Wolbachia* strains between closely related hosts. For example, *Wolbachia* strains from *Ae. bromeliae* may be transinfeced into *Ae. aegypti*. We propose further work on functions of *Wolbachia* strains of *Ae. bromeliae*, *Ae. albopictus* and *Mansonia* sp. as these could potentially be used in reducing pathogen transmission by mosquitoes, especially if these strains are causing variation in vector competence among their natural host populations.

3.5. Acknowledgements

4. FREQUENCY OF SUSCEPTIBILITY TO *BRUGIA MALAYI* AMONG *AEDES AEGYPTI* POPULATIONS ALONG THE KENYAN COAST

4.1. Introduction

Mosquito species vary widely in their ability to transmit diseases, resulting in competent and non-competent vectors of diseases such as malaria, filariasis and dengue. Although there are about 470 Anopheline species, just over 60 of these vector malaria (Service, 1993). *Anopheles quadriannulatus*, for example, is not an important vector of malaria, (Habtewold et al., 2008) despite its existence in malaria endemic regions together with important malaria vectors such as *An. gambiae s.l*. Across geographic regions, differences in vector competence may also be observed within species. For example, *C. quinquefasciatus* transmits bancroftian filariasis in East Africa (Mwandawiro et al., 1997; White, 1971), while the same species of mosquito is a non-vector in West Africa (Appawu et al., 2001). Such variations in transmission of diseases by mosquitoes are important factors in epidemiology and may have implications on vector control programmes. For instance, reduction of filariasis transmission by mass drug administration (MDA) as part of the Global Lymphatic Filariasis Elimination Programme (GLFEP) will not be very effective in East Africa as *C. quinquefasciatus* exhibits 'limitation', thus can transmit the disease even when parasitemia is reduced in the human population (Subramanian et al., 1998).

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Aedes aegypti aegypti is the predominant form of *Ae. aegypti* found outside Africa (Powell et al., 1980; Brown et al., 2011) and the incidence of yellow fever and dengue outbreaks in many countries is correlated with increased urbanization and spread of *Ae. aegypti*. There is evidence that strains of *Ae. aegypti* from different geographic regions vary in their competence to transmit these viral diseases (Aitken et al., 1977; Beaty and Aitken, 1979). The ancestral *Ae. aegypti formosus* has a lower competence to yellow fever (Lorenz et al., 1984; Tabachnick et al., 1985; Wallis et al., 1985) and dengue (Miller and Mitchell, 1991). Although *Ae. aegypti* is not a natural vector of *Brugia* sp. of filarial nematodes, variations in susceptibility is observed in laboratory stocks (Hawking and Worms, 1961; Ramachandran et al., 1960); which previously allowed a susceptible line of *Ae. aegypti* to be selected (Macdonald, 1962b). Later, a study of geographic strains of *Ae. aegypti* revealed that strains were generally refractory to *Brugia pahangi,* except those sampled from East Africa (Rodriguez and Craig, 1973). The authors concluded that, the high susceptibility status among East African populations was largely contributed by the ancestral, sylvan *Ae. aegypti formosus* (Rodriguez and Craig, 1973).

The observed status in East Africa may not be a simple case of two distinct subpopulations with one being refractory and the other susceptible. This is because in East Africa, which is the only region in the world where the two forms of *Ae. aegypti* occur in sympatry, an intermediate ecological population of *Ae. aegypti* also exists (Trpis and Hausermann, 1975). Even though both the sylvan and domestic forms of the *Ae. aegypti* have separate habitats, they occasionally spill over into a common ecological zone (Trpis and Hausermann, 1978). Crosses between the sylvan and domestic forms in this new zone is likely to result in the

intermediate population usually referred to as peri-domestic (Trpis and Hausermann, 1978). Peri-domestic populations are often found outside houses, breeding in coconut groves and other disturbed habitats such as open cans and tyres (Trpis and Hausermann, 1975).

Two hypotheses have been proposed to explain the observations in East African strains of *Ae. aegypti*: balanced polymorphism and diversifying selection. Observations made by Townson (1971) from performing laboratory crosses between refractory and susceptible strains led to the postulation of balancing polymorphism as a likely explanation for the susceptibility status in East African strains (Rodriguez and Craig, 1973). Heterozygotes resulting from laboratory crosses seemed to have a higher survival advantage over homozygote refractory progeny (Townson, 1971), so that selective advantage of heterozygotes may result in the maintenance of susceptible homozygotes in the population. There is also the diversifying hypothesis which contradicts the balancing polymorphism hypothesis. The diversifying selection hypothesis suggests that extreme phenotypes are selected for when subpopulations of a species are geographically or sexually isolated. In this case, different alleles at the susceptibility locus may be selected for due to differences in parasite exposure. The diversifying hypothesis for selection also suggests that resistance may come with high costs, which may cause a trade-off with other physiologically important genes. Fitness cost may also result in high mortality, reduction in the number of eggs or less viable offspring. Rodriguez and Craig (1973) suggested that the latter hypothesis seems to better explain the high susceptibility in the East African region.

Diversifying hypothesis will hold only if the sylvan and domestic subpopulations of *Ae. aegypti* have sufficiently low gene flow for selection to maintain genetic differences. It is not clear if this is the case in East Africa. Although studies of some genetic polymorphic sites have suggested some degree of restricted gene flow between *Ae. aegypti aegypti* and *Ae. aegypti formosus*, the measures of genetic distance is small and does not strongly support this phenomenon (Tabachnick et al., 1979; Wallis et al., 1983). Multiple inversions on chromosome-1 in *Ae. aegypti formosus* is evident (Bernhardt et al., 2009) and may result in lack of recombination in certain regions of the chromosome— a characteristic that is likely to favour diversifying selection.

In this study, the frequency of susceptibility to *B. malayi* is investigated in peri-domestic populations of *Ae. aegypti* sampled from communities along the Kenyan coast. Two sylvan populations were also sampled to enable comparison of susceptibility between the peri-domestic and sylvan populations. Results are compared with similar studies done on Kenyan populations infected with *B. pahangi*.

4.2. Materials and methods

Sample collection

Mosquito eggs were collected from peri-domestic habitats in towns and villages in three districts along the Kenyan coast (Figure 4.1). The town centre of Malindi and Mombasa are approximately equal distances (≈50km) from the Kilifi town centre. Mombasa is the most

urbanized among the three districts and has a high tourism acknowledgement. Within these districts, two forests were also sampled for sylvan *Ae. aegypti*. The Jaribuni forest is located in the Kilifi district about 40km west of the Kilifi town. Few villages are located in close proximity to this forest. Arabuko-Sokoke forest is a nature reserve that lies between Kilifi and Malindi districts (Figure 4.1).

Sampling was done in the months of June and July. This period was ideal as the month of June comes just after the heavy rainfall season, providing puddles of water for breeding of mosquitoes. I prepared simple oviposition traps (ovitraps) for collecting eggs. An ovitrap consisted of a black plastic cup, hay infusion, a strip of creped cardboard paper and paper clip. Hay infusion was prepared from dried grass and water at a ratio of 4g to 1L, and left standing for about 4 days. The hay was then removed by pouring the infusion through a fine mesh. Black plastic cups were two-thirds filled with the infusion and a strip of cardboard paper was clipped to the cup with one end dipping into the infusion. Each ovitrap was clearly labelled on the strip of cardboard paper with a three letter code each for district and locale and a single letter for replicate ID. The ovitraps were positioned in cool, shady places, for example, under shrubs outside houses and in tree crevices and tree holes in the forests. Sampling was done in replicates in any one compound or sampling site (Table 4.1). The ovitraps were set in the morning and retrieved 3 days later. Eggs were counted, left to dry in the insectary (27±5°C, 80±5% humidity) for a day and sealed in plastic bags for shipping.
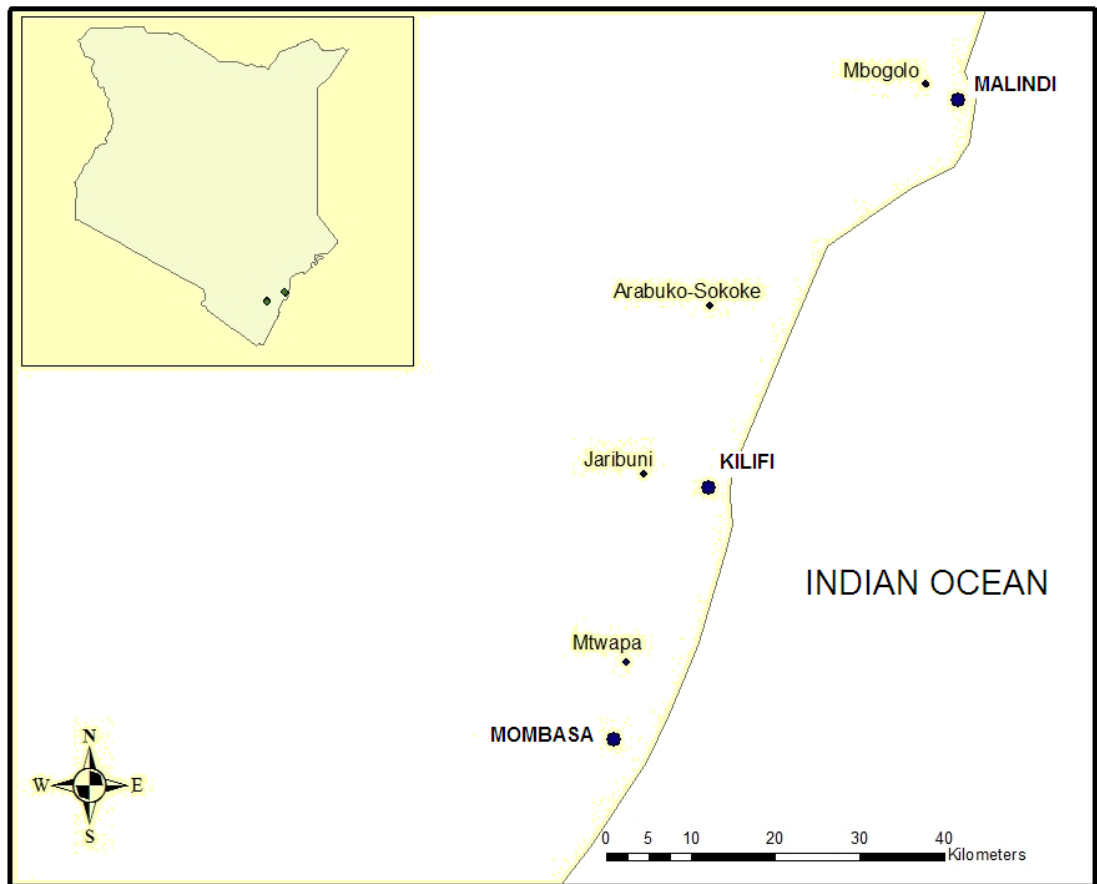
Figure 4.1: Map of the coast of Kenya showing the 3 major districts from which sampling of Aedes eggs was done. Districts are shown by big rounds dots and uppercase names. Locales and towns within the Kilifi district could not be shown on this map due to the size and scale of the map.

Table 4.1: Summary of results from ovitraps set to collect *Aedes aegypti* eggs from sampling sites in 3 districts along the Kenyan coast. Suburbs marked with symbols show places where other species of mosquitoes collected as larvae from the ovitraps. These were reared for identification (listed below table).

District	Suburb	Status	Number of traps set	Number of traps retrieved with eggs	Range of eggs collected	Total number of eggs collected
Kilifi	KEMRI	peri-urban	19	17	1-96	676
Kilifi	St. Thomas*◙†	peri-urban	10	9	7-148	504
Kilifi	Mnarani	peri-urban	10	6	10-99	282
Kilifi	Mkwanjuni	peri-urban	10	6	4-34	109
Kilifi	Mabirikani †	peri-urban	4	4	43-123	349
Kilifi	Charo-wamae †	peri-urban	8	6	10-68	286
Kilifi	Mtaani	peri-urban	7	4	22-87	208
Kilifi	Jaribuni forest	forest	20	19	1-121	764
Kilifi	Jaribuni village	rural	5	4	19-148	351
Malindi	Muthangani	peri-urban	8	8	10-179	722
Malindi	GK prison	rural	4	4	9-65	123

Table 4.1 *continued*

District	Suburb	Status	Number of traps set	Number of traps retrieved with eggs	Range of eggs collected	Total number of eggs collected
Malindi	Kibokoni	peri-urban	6	5	19-547	1307
Malindi	Kwandamo	peri-urban	4	1	125	125
Malindi	Arabuko-Sokoke♦	forest	28	16	3-200	728
Mombasa	Mtwapa	peri-urban	27	16	5-111	749

* *Eretmapodite chrysogaster*; ▣*Toxorhynchites* sp; ♦ identified *Culex* sp; † *Ae. bromeliae*

Laboratory rearing

In the Evolutionary Genetics Lab (EGL), Cambridge, each paper strip of eggs was hatched separately in a plastic cup of tap water. Cups were placed in a vacuum for 30 minutes to stimulate hatching. Larvae were fed with yeast on the day of hatching and with desiccated liver powder on subsequent days till pupation. Adult females that emerged were maintained on 10% fructose solution. Females were blood fed after a week using the Hemotek blood feeding system (Discovery Workshops, Accrington, UK), but with a stretched piece of pig intestine instead of parafilm. Parafilm seemed less attractive to the wild mosquitoes and they refused to feed. Slow adaptation to laboratory conditions resulted in the loss of some of the populations, especially the sylvan populations.

It was also observed that some populations that had hatched poorly had skewed numbers of adult males and females. To improve numbers and encourage breeding, some replicates or collections made in the same locale were pooled to maintain representatives from each district, at the least. Females did not lay when offered cups with tap water; they preferred to lay in hay infusions, consistent with observations made by Trpis and Hausermann (1975). Mosquitoes eventually became adapted to parafilm feeding and were offered hay infusion each time to lay eggs. Females were blood-fed every 4-5 days and eggs were collected 3-4 days after blood feeding. Adult mosquitoes were maintained on fructose solution (10% fructose + 1% Para-aminobenzoic acid) in between blood meals. Surviving populations were reared for 5-6 generations at insectary conditions of $27\pm1°C$ and $80\pm5\%$ humidity.

Hatching for parasite infection

F_5 or F_6 eggs from Kenyan populations were hatched for parasite infection. Unfortunately, no eggs from sylvan populations were obtained for this experiment. Observations prior to this experiment revealed that larval density is especially important in the survival of wild mosquito larvae. When larval density was not controlled developmental time was prolonged. Adults that eventually emerged were small in size and ingested little blood, affecting the number of eggs that was later laid. The larval density for this experiment was, therefore, strictly controlled. A day after hatching the eggs, larvae were counted and transferred from beakers into larger larval trays (70 x 210 x 145mm) at a density of 10 larvae per 100ml of tap water. Larvae were fed everyday with liver powder until they were pupae. The black-eyed Liverpool (LVP) strain, maintained at the Filariasis Research Reagent Resource (FR3) Center, Atlanta, Georgia for keeping the *B. malayi* worm cycle, was used as control. This was hatched and treated similarly. I refer to this strain as GEORGIA from here.

Brugia infection

Female adults were 6-9 days old when they were fed on infected blood. *Brugia malayi* microfilariae were obtained in RPMI medium from Mark Taylor's lab at the Liverpool School of Tropical Medicine (LSTM). Microfilariae had been harvested from infected gerbils 2 days prior to infection. To avoid bacterial contamination, the medium had been mixed with Gentamicin at a final concentration of 0.1mg/ml. The parasites transferred from the medium into blood and fed to the mosquitoes at a dose of 400-450 microfilariae / 20µl of blood.

GEORGIA and Kenyan populations were fed simultaneously using the Hemotek blood feeding system (Discovery Workshops, Accrington, UK), switching the blood feeders between cages every 10 minutes. Blood feeding was much better in GEORGIA than in the Kenyan populations. Few individuals of the Kenyan populations had fully engorged abdomens. Half-filled abdomens could imply few ingested parasites and may result in false record of refractoriness. Such individuals were excluded. Female mosquitoes with fully-engorged abdomens were transferred into cages and provided with water and fructose. Mosquitoes were fructose-fed every other day and checked for mortalities.

At 11 days post infection (PI) the head, thorax and abdomen of infected mosquitoes were dissected and scored for parasites. Briefly, each cage was moved to a 4°C walk-in room to knock-out the mosquitoes and allow handling. Each mosquito was separated at the thorax and abdomen to prevent flying during handling at room temperature. Each sample was dissected on a microscopic slide in 50µl of 1X Grace's insect cell culture media (Invitrogen, UK). Carcasses were covered with a clover slip and analysed at 4X objective power of a Leica DFC420 light microscope. Individuals were scored for presence or absence of live matured worms (L2, L3). Live worms were counted.

4.3. Results

Evaluation of the ovitraps

In total, I set 176 ovitraps and retrieved 131 of them with eggs. Some ovitraps had been toppled over while others had no eggs at all. In Arabuko-Sokoke, one of the traps had had the cardboard paper eaten and destroyed by a snail which was still attached to the paper when the trap was retrieved. Few traps had less than 10 eggs and these were excluded from the laboratory rearing process. A total of 7,607 *Aedes* eggs were counted from the ovitraps with a median number of ≈42 eggs per trap. Malindi traps resulted in significantly higher number of eggs with a mean of 101 eggs ($F_{(3, 13)}= 9.56$, *p*= 0.0014). Kilifi and Mombasa had an average of 53 and 47 eggs per trap, respectively. More eggs were collected from the peri-urban sites (mean= 77) than from the rural (mean= 59) and forest (mean= 43) sites ($F_{(3, 13)}= 7.51$, *p*=0.036).

Besides *Ae. aegypti* eggs, the ovitraps also attracted oviposition from other mosquito species. Egg rafts were found in 10 out of 27 traps set in Arabuko-Sokoke (data not shown), 7 of which had no *Ae. aegypti* eggs. The laying of rafts by another species of mosquitoes may have deterred *Ae. aegypti* from laying in the same breeding water. The eggs rafts from some of these pots were reared in the laboratory and identified as *Culex* sp. Kilifi showed high species richness in mosquitoes by assessment of the ovitraps. Some traps set in the sampling locales in Kilifi had larvae of *Eretmapodites chrysogaster* and *Toxorhynchites* sp. which were identified after rearing in the laboratory in Kenya Medical Research Institute (KEMRI), Kilifi. During

rearing of eggs in the laboratory in Cambridge, *Ae. bromeliae* emerged from some *Aedes* eggs collected also from Kilifi.
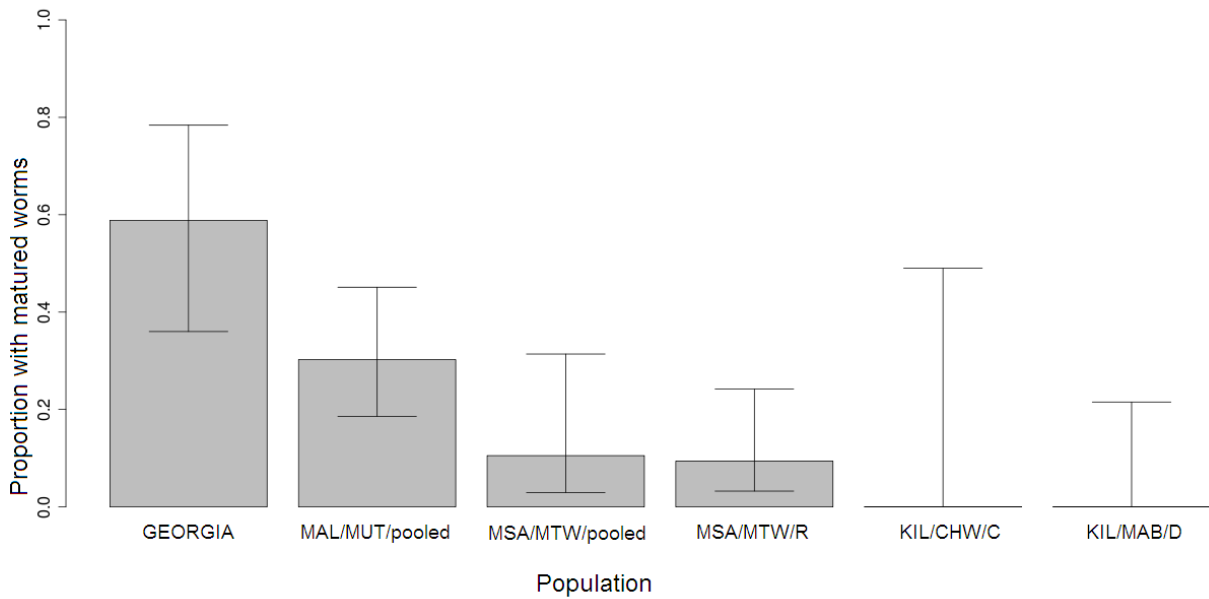
Susceptibility recorded in peri-domestic populations

Following dissection of individuals at 11 days post-infection, most individuals observed with worms harboured L3s. Few individuals had both L2 and L3 worms. For analyses, L2 and L3s were counted together as matured worms. Table 4.2 and Figure 4.2 summarise results of parasite load and number of infective individuals within each population. GEORGIA recorded 58% infective individuals while there was 0-30% range of infective individuals in the Kenyan populations. The proportion of infective individuals in GEORGIA was significantly higher than the combined results from Kenyan populations (Fisher's exact: $p= 0.0002$). However, differences between Kenyan populations was also significant (Fisher's exact: $p= 0.034$). The Malindi population recorded the highest number of positive individuals (30%) among the Kenyan populations, which seemed to have contributed to the significant difference in susceptible individuals among the wild populations (Figure 4.2).

Table 4.2: Parasite load and worm developmental stage in mosquitoes 11 days after infections. Kenyan population names relate to the district/town/replicate ID. Refer to Table 4.1 for name details.

Population	Number dissected	Individuals with matured worms	L2	L3
GEORGIA	17	10	3	84
MAL/MUT/pooled	43	13	4	24
MSA/MTW/R	32	3	0	4
MSA/MTW/pooled	19	2	0	2
KIL/MAB/D	14	0	0	0
KIL/CHW/C	4	0	0	0

Figure 4.2: Proportion of susceptible individuals in each population. Error bars are lower and upper limits at 95% confidence interval.

Number of developing worms is reduced in peri-domestic populations

The number of matured worms detected varied widely, especially in GEORGIA where some individuals harboured as high as 23 worms while others had none. The highest number of parasites detected in the Kenyan populations was 7 from the Malindi population. This population had a mean number of worms of 2.3, while the other Kenyan populations had a mean of 1 (Figure 4.3)

To test the observed variation in the number of matured worms detected, individuals that recorded zero worms were excluded and a linear model was fitted to the number of worms counted, given the population. The result showed that the number of developed parasites varied significantly between the Kenyan populations and GEORGIA ($F_{(4, 24)} = 9.776$, $p=7.73 \times 10^{-5}$). Between the Kenyan populations parasite load was also significant ($F_{(3, 24)} = 4.5845$, $p= 0.011$).
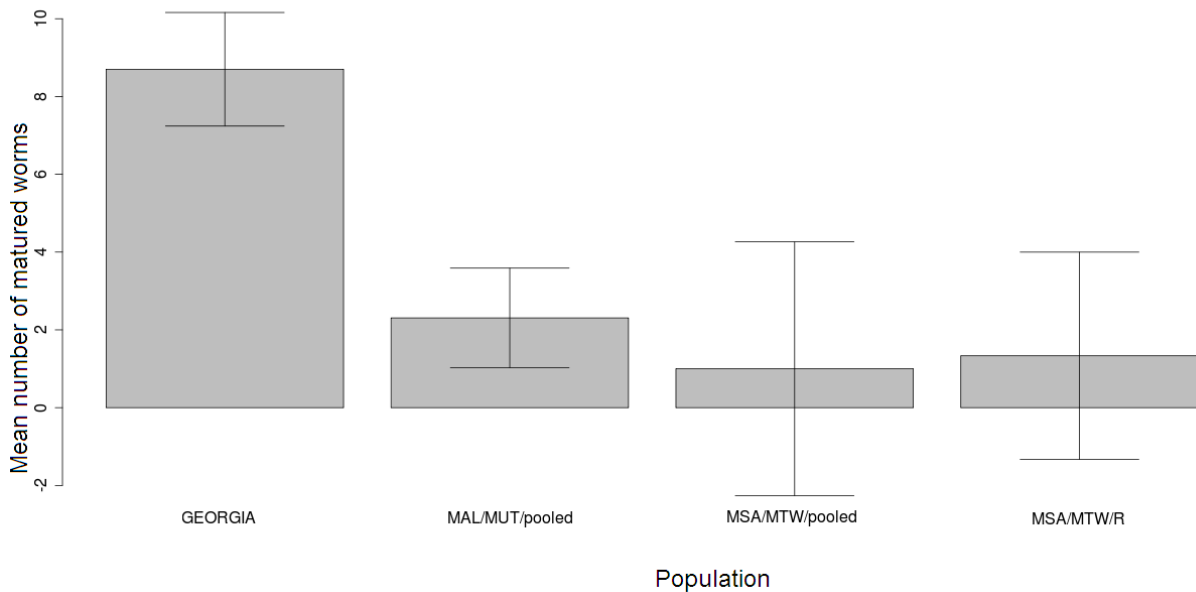
Figure 4.3: Summary of worm load in GEORGIA and Kenyan populations. Barplots show the mean number of worm found in each population and error bars are standard errors.

4.4. Discussion

Aedes aegypti is known to naturally transmit two genera of worms; *Dirofilaria* and *Foleyella* (Hawking and Worms, 1961). Although not a natural vector of *Brugia* sp. filarial worms, *Ae. aegypti* is used extensively in the laboratory as vectors of *B. malayi* and *B. pahangi* to study various aspects of mosquito-filarial parasite interactions. This study shows that the peri-domestic populations of *Ae. aegypti* collected from towns along the coast region of Kenya can support the development of *B. malayi,* with up to 30% of individuals harbouring matured infective worms 11 days after infection. Even though these individuals with matured worms could be categorized as susceptible because they were found to have, at least, a developed L2 or L3 worm, they harboured significantly lower numbers of infective worms than the

laboratory LVP (GEORGIA) strain. The results are consistent with previous susceptibility tests of East African strains of *Ae. aegypti* with *B. pahangi* (Paige and Craig, 1975; Rodriguez and Craig, 1973).

The peri-domestic habitat is an ecological zone of contact for the domestic and sylvan forms of *Ae. aegypti* (Trpis and Hausermann, 1978). Breeding places in this zone are formed when rainwater collects in open containers such as cans, tyres and tanks so that, peri-domestic populations of *Ae. aegypti* are common during the rainy season (Trpis and Hausermann, 1978). Egg collection for this experiment was done within a period where rapid population expansion was likely. The month of June-July is a period following the rainy season in Kenya and by setting ovitraps outside homes, convenient habitats for both forms of the mosquito were created. Hay infusions promoted growth of bacteria which some mosquitoes use as a cue for suitability of breeding water (Lindh et al., 2008). It was observed during the laboratory rearing that tap water was unattractive to blood-fed females of the wild population and a good indication that the peri-domestic population samples did comprise a high sylvan composition. In the Rabai village of Kenya, the months of June and July have previously recorded the highest percentage of *Ae. aegypti* sampled within a year to be of the sylvan form (Trpis and Hausermann, 1986). The strong preference for hay infusion observed may also imply that the peri-domestic samples consisted of hybrids that were exophilic rather than endophilic, as observed by Trpis and Hausermann (1978).

Susceptibility in East African populations of *Ae. aegypti* to *B. pahangi* has been attributed to sylvan sub-populations. Paige and Craig (1975) and Rodriguez and Craig (1973) in separate experiments, observed that high proportions of mosquitoes collected from tree holes were able to support the development of *B. pahangi* in the laboratory. Individuals collected from peri-domestic habitats show intermediate susceptibility when compared with domestic and sylvan populations (Paige and Craig, 1975). Sylvan populations were not available for comparison in this study, but populations collected from peri-domestic habitats in three districts showed significant differences in the number of infected individuals. Kenyan populations showed none or few susceptible individuals, except Malindi which had 30% susceptible individuals, comparable to observations made by Paige and Craig (1975) .

Chromosomal rearrangements is a source of differentiation and evidence of such events have been documented in mosquito species such as *An. gambiae* (Coluzzi et al., 2002, 1979) and *Ae. aegypti* (Bernhardt et al., 2009). In *Ae. aegypti*, chromosomal inversions could explain observed restricted gene flow between *Ae. aegypti aegypti* and *Ae. aegypti formosus* (Tabachnick and Powell, 1979; Wallis et al., 1983), as is the case in *Anopheles* species (Coluzzi et al., 1979). Despite this, *Ae. aegypti aegypti* and *Ae. aegypti formosus* mate to form viable offspring (Trpis and Hausermann, 1978). As there is no evidence of assortative mating existing between the two distinct forms (Moore, 1979), random mating will continue if the two forms exist in the common habitat. If there are no chromosomal restrictions in the locus conferring susceptibility to *Brugia* parasites, then peri-domestic F_1 progeny will be heterozygous for the gene. Continuous migration of the two forms into the peri-domestic zone

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will lead to increase in random mating and mixing of genes. Heterozygotes resulting from crosses between susceptible and refractory individuals show a survival advantage over homozygotes (Townson, 1971). Refractory homozygotes have high mortality 24 to 48 hours after feeding on infected blood, but mortality levels out with heterozygotes after this period (Townson, 1971). High frequency of heterozygosity leads to maintenance of susceptible individuals within the peri-domestic population.

Hardy-Weinberg equilibrium model for selection implies that, as recessive alleles become rare in a population, the ratio of heterozygotes to homozygote recessives becomes increasingly higher. This experiment showed that an average of 16% of the Kenyan population is susceptible to *B. malayi*, and assuming that this phenotype is conferred by a single recessive gene, it can be inferred that this percentage represents the frequency of recessive homozygotes. By extrapolation, this will suggest that there are three times as many heterozygote individuals as there are recessive homozgotes. This suggests that individuals carrying the recessive gene conferring susceptibility to *Brugia* are in high frequency. It will be interesting to find out how these frequencies may change with subsequent generations as this will help determine the dynamics within populations and whether the recessive alleles have reached equilibrium.

Parasite intake varies among individuals of a species and between mosquito species. For example, *Culex quinquefasciatus* and *Ae. aegypti* were observed to ingest more worms than *An. gambiae* when presented with similar microfilaria loads (McGreevy et al., 1982).

Individuals of a strain may ingest similar number of parasites, but the degree to which they support the development of the parasite may differ, so that individuals that are more susceptible have higher loads of developed parasites. Despite Kenyan populations having individuals that harbour matured worms, parasite load was significantly lower in these populations compared to GEORGIA controls. GEORGIA has been used to maintain *Brugia* at FR3 and is highly susceptible. It was expected that at least 90% of the individuals will be susceptible. Paige and Craig (1975) observed that susceptibility in their black-eyed LVP controls only went below 90% when the parasitemia in gerbils was below 20 microfilariae / 20µl of blood. The parasite dose I used was quite high to ensure high numbers of individuals ingested worms when they blood-fed. As the control strain, it was unexpected for some GEORGIA individuals to have no matured worms and others to have as high as 23 worms. This could be as a result of clumping of worms in the blood offered to the mosquitoes rather than a case of reduced frequency in number of susceptible individuals.

Nevertheless, variation in number of matured worms was significantly different between GEORGIA and Kenyan populations. Increased use of GEORGIA for maintaining the parasite cycle makes the strain a more competent vector than the wild population. Among the Kenyan populations, worm load was also significantly different although not as high when they are all compared to GEORGIA. Again, the average matured parasites in susceptible individuals in the Kenyan populations was comparable to previous results with *B. pahangi* (Paige and Craig, 1975).

This study has confirmed previous reports of susceptibility in East African strains of *Ae. aegypti* to *Brugia* sp. (Paige and Craig, 1975; Rodriguez and Craig, 1973). While previous studies used *B. pahangi*, results from this study with *B. malalyi* has shown consistent results. With this susceptibility established in the population collected from Kenya, it will be interesting to compare polymorphisms associated with susceptibility within these populations. Results could also be compared to polymorphisms observed in laboratory strains to determine if similar genes responsible for the trait are observed in both laboratory and wild populations.

4.5. Acknowledgements

5. THE *AEDES AEGYPTI* LINKAGE MAP: TOWARDS MAPPING THE GENE ASSOCIATED WITH *BRUGIA MALAYI*

This chapter is a collaborative work with Punita Juneja (Frank Jiggins group, Cambridge) and, Arnab Pain and Shwen Ho (King Abdullah University of Science and Technology)

5.1. Introduction

Aedes aegypti, as a model system for studying mosquito-parasite interactions, shows variation in susceptibility to *Brugia* parasites in both natural and laboratory populations, as shown in Chapter 4 of this thesis and the literature (Macdonald, 1962b; Paige and Craig, 1975; Rodriguez and Craig, 1973). This has allowed interesting findings on the complexities of mosquito-filarial worm interactions including trait inheritance (Macdonald, 1962a; Macdonald and Ramachandran, 1965; Wattam and Christensen, 1992), infection dynamics (Christensen and Sutherland, 1984; Ewert, 1965a) and expression profiling of mosquito defence proteins during infection (Erickson et al., 2009). Particularly of epidemiological interest is the discovery that the genetic control of susceptibility to *Brugia* is similar to the more pan-tropic filarial parasite *Wuchereria bancrofti* (Macdonald and Ramachandran, 1965) which causes about 90% of human lymphatic filariasis cases. This genetic susceptibility of *Ae. aegypti* to *Brugia* infections has been shown to follow a Mendelian mode of inheritance (Macdonald, 1962a).

The major effect locus for *Brugia* resistance in *Ae. aegypti* is sex-linked (Macdonald, 1962a) and maps onto chromosome-1 of *Ae. aegypti* (Severson et al., 1994). This locus has been defined to a 10 cM region which is estimated to cover ≈17Mb chromosomal region (Brown et al., 2001; Severson et al., 1994), a relatively small region considering the size of the *Aedes* genome (Nene et al., 2007). Mapping and isolation of the gene could potentially provide a useful tool for comparative analyses of vector populations and encourage a better understanding of vector competence in different mosquito populations. It can also provide an easy method for screening vectors in natural populations, just as the identification of the knock-down resistance gene (*kdr*) in *Anopheles gambiae* has enabled the detection of insecticide resistance within mosquito populations (Donnelly et al., 2009).

To be able to isolate the genetic components of vector competence, knowledge of the genome organization of the mosquito host is essential. Among the sequenced genomes of the 'big three' mosquito vectors, *An. gambiae* is the most completed and organized genome draft. The *Aedes* and *Culex* genomes are still highly fragmented with large numbers of supercontigs not assigned to chromosomes (reviewed in Severson and Behura, 2012). At 1.38 Gigabases, the *Ae. aegypti* genome is the largest among these sequenced mosquito genomes. The genome is currently organized into 4,758 supercontigs with only a few of these assigned to chromosomes (representing 31% of the genome) (Nene et al., 2007). Furthermore, the order and orientation of supercontigs that have been assigned to chromosomes through genetic and physical mapping are still unknown (Brown et al., 2001; Nene et al., 2007). The reason for the difficulty in assembling of the Culicine genomes is attributable to their large sized

chromosomes and the presence of a high percentage of repetitive transposable elements (Arensburger et al., 2010; Nene et al., 2007). Such a large portion of the supercontigs not yet assigned to chromosomes confounds attempts to physically map genes of interest.

The advent of improved DNA-based technology for genetic and linkage mapping (Loxdale and Lushai, 1998; Severson, 1994) have enhanced the identification of loci that affect the vector competence of mosquito vectors. For example, an integrated genetic map based on microsatellite analyses identified 3 loci as responsible for melanotic encapsulation of *Plasmodium cynomolgi* in *An. gambiae* (Zheng et al., 1996; Zheng, 1997). In *Ae. aegypti*, susceptibility to *Brugia malayi* was found to be associated with 2 quantitative trait loci (QTL) using Restriction Fragment Length Polymorphism (RFLP) (Severson et al., 1994). The major effect, sex-linked locus, *fsb* [LF178], is located on chromosome-1 and the minor effect locus, *fsb* [LF98], on chromosome 2 (Severson et al., 1994).

We contribute to efforts to improve the genetic map of *Ae. aegypti* by using **R**estricted-site **A**ssociated **D**NA (RAD) tag sequencing (Baird et al., 2008). The technique identified some supercontigs that have previously been misassigned to chromosomes, misassemblies of contig into scaffolds and enabled the new assignments of supercontigs to chromosomes. We were also able to order the supercontigs on the chromosomes. This development has provided a better tool for the mapping of the genetic susceptibility of *Ae. aegypti* to *B. malayi*.
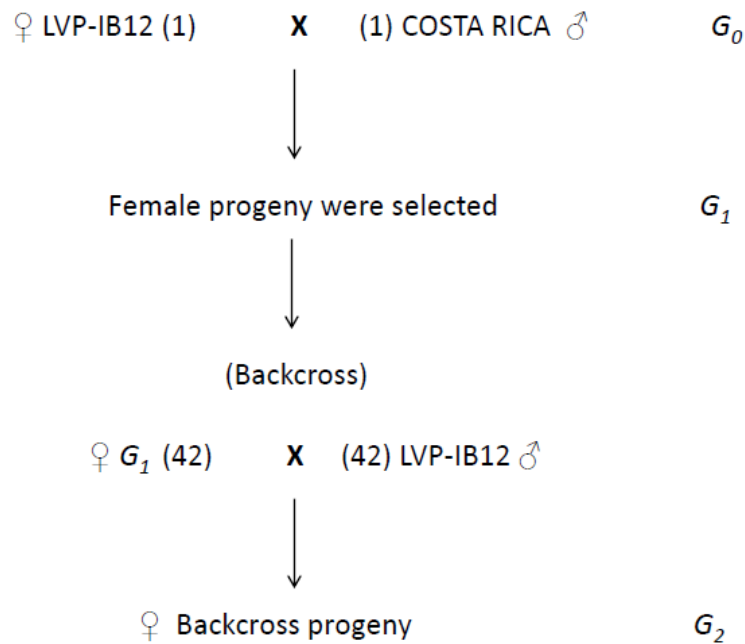
5.2. Materials and methods

Mosquito strains

We obtained LVP-IB12 and COSTA RICA laboratory strains of *Ae. aegypti* from the Malaria Research and Reference Reagent Resource Center (MR4) (ATTC Manassas Virginia). The LVP-IB12 is an inbred line from a stock (LVP) which had previously been selected for susceptibility to *B. malayi* (Macdonald, 1962b), and is the reference genome sequencing strain (Nene et al., 2007). LVP-IB12 is, however, not very susceptible when we tested it in the laboratory. We confirmed that this was not as a result of contamination in our laboratory by testing the same strain obtained from David Severson's lab. COSTA RICA is a wild-type stock reported to be insecticide susceptible (Perich et al., 2003). To be sure we had a strain with high susceptibility, we also obtained LVP from the NIAID/NIH Filariasis Research Reagent Resource Center (FR3) (Altlanta, Georgia). At FR3, LVP is used to maintain the *B. malayi* cycle (Michalski et al., 2011). To distinguish between the two Liverpool strains, we refer to the strain from MR4 as LVP-IB12 and that from FR3 as GEORGIA throughout this chapter. We initially tested all 3 strains for their susceptibility status to *B. malayi*; COSTA RICA showed 0% susceptible individuals (n=46), LVP-IB12 (16%, n=30) and GEORGIA (50%, n= 20). To obtain homogeneity in the genome of this refractory stock, we inbred COSTA RICA by single pair sib-mating for two generations.

Crossing design for linkage map

We used LVP-IB12 and COSTA RICA for setting up crosses for this experiment. A single female LVP-IB12 was mated to a single male COSTA RICA (G_0). Individual female progeny

(G_1) were collected, put in a single cage and backcrossed to LVP-IB12 males (Figure 5.1). The

LVP-IB12 and COSTA RICA parents (G_0) and female backcross progeny (G_2) were stored at

−80C until DNA extraction.

♀ LVP-IB12 (1)   **X**   (1) COSTA RICA ♂ G_0

Female progeny were selected G_1

(Backcross)

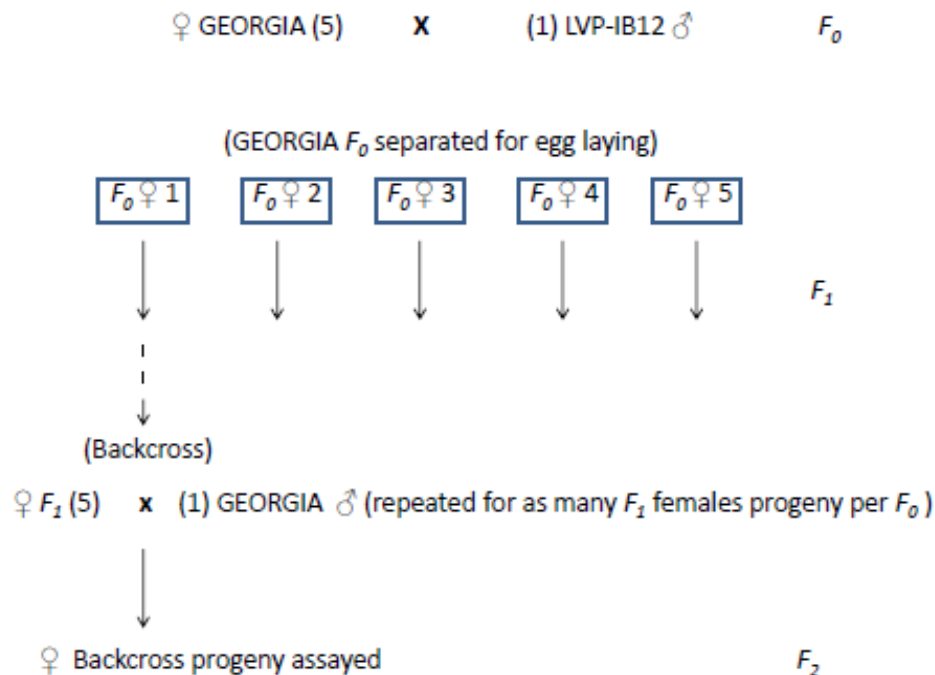♀ G_1 (42)   **X**   (42) LVP-IB12 ♂

♀ Backcross progeny G_2

Figure 5.1: Summary of the crossing design for linkage map assembly of *Ae. aegypti*.

Crossing plan for bulk-segregant analyses

Since GEORGIA was being used in FR3 for maintaining *B. malayi*, the expectation was that

this strain was highly susceptible. For the purpose of having a common genetic background

for the bulk-segregant analyses, we used the two Liverpool strains (LVP-IB12 and

GEORGIA) in this cross. We mated 5 GEORGIA females to an LVP-IB12 male (F_0) (Figure

5.2). Once the females had a blood meal they were separated and allowed to lay eggs

individually. The F_0 male was placed into a 1.5ml Eppendorf tube and stored at −80C. After

multiple egg collection from the F_0 females, these females were also stored for DNA and their

eggs were hatched. F_1 females from the same F_0 female were selected and set up in pools of 5.

Each pool was backcrossed to a single GEORGIA male (Figure 5.2). The GEORGIA male

was removed from the cage after the first blood-meal was taken by the F_1 females, and kept in

a 1.5ml Eppendorf tube at -80°C until DNA extraction. F_1 females that survived the full period

of feeding and egg collection were also stored similarly.

♀ GEORGIA (5)   X   (1) LVP-IB12 ♂ F_0

(GEORGIA F_0 separated for egg laying)

| F_0♀1 | F_0♀2 | F_0♀3 | F_0♀4 | F_0♀5 |

F_1

(Backcross)

♀ F_1 (5)   x   (1) GEORGIA ♂ (repeated for as many F_1 females progeny per F_0)

♀ Backcross progeny assayed F_2

Figure 5.2: Crossing experiment for bulk-segregant analyses for mapping genes associated with susceptibility of *Ae. aegypti* to *B. malayi*.

Backcrossed female progeny from two sets of F_0 parents were infected with *B. malayi* for

further analyses. To ensure that the parasite was infective we included GEORGIA as a control

for reasons stated above. *Brugia malayi* was obtained from Mark Taylor's lab at the Liverpool

School of Tropical Medicine (LSTM). Mosquitoes were fed on blood containing parasites at a concentration of 125 microfilariae/20µl of blood. Females were 5-7 days old on the day of infection. We separated fed individuals from unfed ones, selecting only individuals with fully engorged abdomens. This was to ensure we accurately recorded individuals as refractory. As a high number of microfilariae never make it across the midgut (Michalski et al., 2010), a partially-filled abdomens could imply very low numbers of parasites are ingested, causing a false representation of refractoriness. Selected mosquitoes were returned to a cage and maintained on 10% fructose solution.

At 11 days post-infection, all surviving individuals were scored for parasites. We used a different approach to score infections as quality DNA was required for downstream analyses. Instead of dissecting each individual on a microscopic slide as described in Chapter 4, the thorax and abdomen were separated while the mosquitoes were immobilised in a 4°C room. The head and thorax of each individual were then returned to a 1.5ml Eppendorf tube containing 100µl of 1X PBS. The tubes and contents were incubated on a heat block at 37°C for an hour. The supernatant was moved to microscopic slides, covered with a cover slip and viewed under a light microscope. The mosquito carcass was immediately stored at -80C until DNA could be extracted. Individuals with at least one matured worm (L2/L3) were scored as susceptible. The results of the infection are summarised on Table 5.1.

Table 5.1: Summary of the infection statuses of backcross progeny used in bulk-segregant analyses.

F_0 GEORGIA ID	F_2 backcross progeny ID	Refractory	Susceptible
4	4/8	3	6
4	4/9	13	9
4	4/12	7	3
4	4/5	14	6
4	4/6	47	20
4	4/3	14	18
4	4/11	12	11
4	4/10	4	3
21	21/6	16	9
21	21/2	35	13
21	21/7	20	4
21	21/5	4	5
21	21/4	46	19
21	21/3	39	13
TOTAL		**274**	**139**

RAD library preparation

We adapted the RAD-tag protocol developed by Baird et al. (2008), making minor changes as this was the first time the protocol was being used on *Aedes aegypti*. Briefly, RAD uses a

restriction enzyme to digest the genome of the organism under study and incorporates barcoded P1 adaptors to the compatible ends of the short fragments. The adaptors have unique barcodes, allowing each individual or pools of individuals to be easily identified. Insert sizes between 300-500bp are selected by gel electrophoresis and excision. Libraries are then sequenced on an Illumina platform by either single or paired-end sequencing.

For the linkage map assembly, we estimated that sequencing a pool of 9 G_2 individuals per lane will be sufficient to produce 30-fold coverage of RADtags. By using PstI —a 6-base pair restriction enzyme— we obtain 345,658 RADtags. PstI is predicted to have approximately 172,829 cut sites in the *Aedes* genome (RADtag counter available at www.wiki.ed.ac.uk/display/RADSequencing).

DNA was extracted from G_2 females and G_0 male using QiaAmp MicroDNA kit (Qiagen), following manufacturer's recommendation. As the G_0 female is the reference strain, only the G_0 male was prepared for sequencing. Genotyping will be based on the reference genome (LVP-IB12) and our G_0 COSTA RICA male. DNA was eluted in 50μl AE buffer and 1μl of elute was quantified with Qubit 2.0 fluorimeter (Invitrogen). The amount of DNA ranged between 0.07-1.8μg. Since the G_0 male was to be prepared individually, sufficient DNA was required as starting material. We obtained 0.38 μg of DNA for the G_0 male and to increase this amount, we performed whole genome amplification (WGA) using V2 Genomiphi kit (GE Healthcare, UK). To increase the DNA yield, WGA was done in 3 replicates. Final combined elution volume was 60μl with a concentration of 61.4ng/μl.

We used PstI-HF (NEB) to digest the DNA samples. High fidelity (HF) enzyme is preferred because, it has reduced star activity and allows fast digestion, hence reducing library preparation time. Each digestion reaction tube contained 5µl of Buffer 4 (NEB), 0.2µl PstI-HF (NEB), Xµl DNA (0.17-1µg) and deionized water to make 50µl total reaction volume. 7.9µl of P1 adaptor was ligated onto the DNA fragments with T4 ligase (NEB). Multiplexing of G_2 was done at this point to include samples with approximately equal DNA amounts and different barcodes. DNA was sheared for 6mins 30secs at high intensity with a Bioruptor Sonication System (Diagenode). An insert size between 300-500bp was excised from an agarose gel after loading sheared samples and running for 45mins at 100V. The DNA was extracted from the excised gel with QIAquick MinElute Gel Purification kit. Next, a blunt-end repair reaction (Quick Blunting Buffer, NEB) was performed to create blunt ends from sticky ends that resulted from shearing. To enable ligation of P2 adaptor, it is required to add a poly-A tail to the 3′ end of the blunt-end phosphorylated DNA using Klenow fragment (NEB). The final step of the library preparation enabled a P2 adaptor (this is not barcoded) to be ligated onto the DNA. 4µl of DNA library was amplified in a 100µl reaction volume as described (Baird et al., 2008). Final library concentration ranged from 1.26-29.2ng/µl.

RAD library preparation for the bulk segregant analyses was slightly different. For this experiment, we chose a less frequent 8-base pair cutting enzyme– SbfI. This enzyme cuts the *Aedes* genome at 6,239 sites i.e. 12,478 RADtags (RADtag counter available at www.wiki.ed.ac.uk/display/RADSequencing). We combined DNA of each F_2 based on the phenotype (susceptible or refractory). Since susceptible and refractory individuals were

unequal for each F_2 group, we allowed 8-13 individuals per pool depending on the number of susceptible individuals. We also matched up the number of samples in a susceptible and refractory pool for each F_2 group. As there were more refractory females, we prepared extra pools of refractory individuals for each F_2 group. F_0 parents, backcross GEORGIA males and a few F_1 females were treated individually. Each pool of DNA had a starting amount of ≈1µg. All other DNA ranged between 0.3-1µg.

Restriction site digestion was performed as previously described. Each pool was then given a single unique P1 adaptor (adaptor was used only once). After ligation, we pooled equal amounts of barcoded DNA (susceptible and refractory) from each F_0 group into a single tube. The extra pools of refractory females were treated similarly. We also pooled barcoded F_0 females (2), F_0 males (2), F_1 females (4), and backcross males (12) into separate tubes. This resulted in 7 different libraries. Library preparation followed what has already been described above. Final library concentration ranged between 2.90-26.6ng/µl. Finally, all the amplified libraries were pooled into a tube in equimolar concentrations for sequencing after checking each library for insert quality on a Bioanalyzer Agilent 2100 (Appendix: Figure S2).

Libraries for the linkage map assembly were sequenced on HiSeq2000 at KAUST. We have performed 5 lanes of sequencing for backcross (G_2) progeny and 1 lane for the COSTA RICA male. Sequencing for the bulk-segregant analyses is done at EASIH, Addenbrooke's Cambridge. From this point, down-stream analyses will only be for the linkage map study, as data from EASIH has not yet been received.

Processing of sequences

We obtained an average of 11 million reads per individual with 10 fold coverage. Sequences were sorted by barcodes, eliminating sequences with base errors in the barcodes. Nucleotides with base Phred scores < 20 were trimmed off the ends of the sequences resulting in reads with variable lengths. Sequences were aligned to the reference genome (Lawson et al., 2009) using the Burrows-Wheeler Aligner (BWA) software (Li and Durbin, 2009). An average 86% of all the reads mapped to the reference genome. Indel regions were identified and sequences were re-aligned in GATK (McKenna et al., 2010). Sequences with indels were then removed due to low confidence. PCR enrichment of libraries could potentially introduce sequence duplication and reduce marker diversity. On average, 46% of reads were PCR duplicates. Duplicate removal was performed with Picard (Li et al., 2009). SNP calling and filtering were performed in GATK (DePristo et al., 2011) and VCFtools (Danecek et al., 2011), respectively. SNPs near indels were removed due to low confidence. We also removed SNPs with a quality score < 20 and coverage < 10x. SNP filtering enabled variant calling and genotype assignment.

Obtaining markers

After applying all our quality filtering parameters, we kept 31 G_2 individuals with the most mapped reads with an arbitrary threshold of 15 million reads. For these individuals we removed markers that had missing genotype information for more than 5 individuals. Accounting for heterozygosity in our parental genomes and the use of multiple LVP-IB12 males in the backcross, we only retained markers where (1) the COSTA RICA G_0 male was

homozygous for the non-LVP allele and, (2) fewer than 10% of the progeny were homozygous for the COSTA RICA allele since homozygosity for the COSTA RICA allele in our backcrossed progeny implies a high frequency of the COSTA RICA allele in the LVP-IB12 population.

Drawing the linkage map

We employed MSTMap algorithm (Wu et al., 2008) to our data to reconstruct linkage group assignments for the markers we had obtained. To make our results as stringent as possible while still capturing as many markers, the *p*-value for estimating linkage groups was set to 0.0001. This resulted in 3 big linkage groups with 7 other minor groups (Appendix: Table S3). Results of the maps were visualised and compared to established maps (Nene et al., 2007) using MapInspect (available at www.plantbreeding.wur.nl/UK/software_mapinspect.html).

5.3. Results

Chromosomal assignments

We constructed a genetic map of *Aedes aegypti* with 168 markers from 31 backcrossed progeny. A summary of the results produced by the algorithm implemented in MSTMap is shown on Table 5.2. 90 % of markers fell into 3 linkage groups (Appendix: Table S3). As we had no way of identifying which chromosomes these new markers had been assigned to, we used the assignments of previously mapped supercontigs as a way to detect a chromosome. We report here mapping of 79 previously unmapped supercontigs to the 3 chromosomes of *Ae.*

aegypti (Figure 5.3). These newly mapped supercontigs contained 105 markers and represent 122.4Mb of the genome. In detail, 11 of the newly assigned supercontigs (18.2Mb) mapped to chromosome-1, 56 (54.2Mb) to chromosome-2 and 38 (50Mb) to chromosome-3.

Some of the markers we obtained had previously been assigned to chromosomes. We were able to map 45 out of 50 of these markers. Of these 45, 31 (22 supercontigs) were correctly re-assigned to the chromosomes they had already been mapped to (Figure 5.3). Our analyses suggested that 8 previously mapped supercontigs have been misassigned to their chromosomes (Fisher's exact test: $p= 0.00042$) (Table 5.3, Figure 5.3). Although we did not have large numbers of markers per supercontig, we identified a few cases where contigs had been misassembled into supercontigs. If scaffolding was correct, then markers/contigs within the same supercontig should segregate together. Markers within supercontigs 1.1 and 1.96 on chromosome-1 and 1.113 on chromosome-2 were mapped onto different loci on their respective chromosomes (Figure 5.3).

Table 5.2: Summary of markers mapped to chromosomes by MSTMap (Wu et al., 2008).

Feature	Number
Total number of markers	168
Previously assigned markers	50
Total number of markers assigned to chromosomes by MSTMap	150
Newly assigned markers	105

Table 5.3: Summary of supercontig assignments to chromosomes, comparing results from MSTMap analyses to previously assigned supercontigs. Each pairwise comparison indicates number of supercontigs that have been correctly re-assigned or misassigned by our analyses.

MSTMap assignments

| Previously assigned		Chromosome-1	Chromosome-2	Chromosome-3
	Chromosome-1	4	2	2
	Chromosome-2	0	14	1
	Chromosome-3	1	2	4
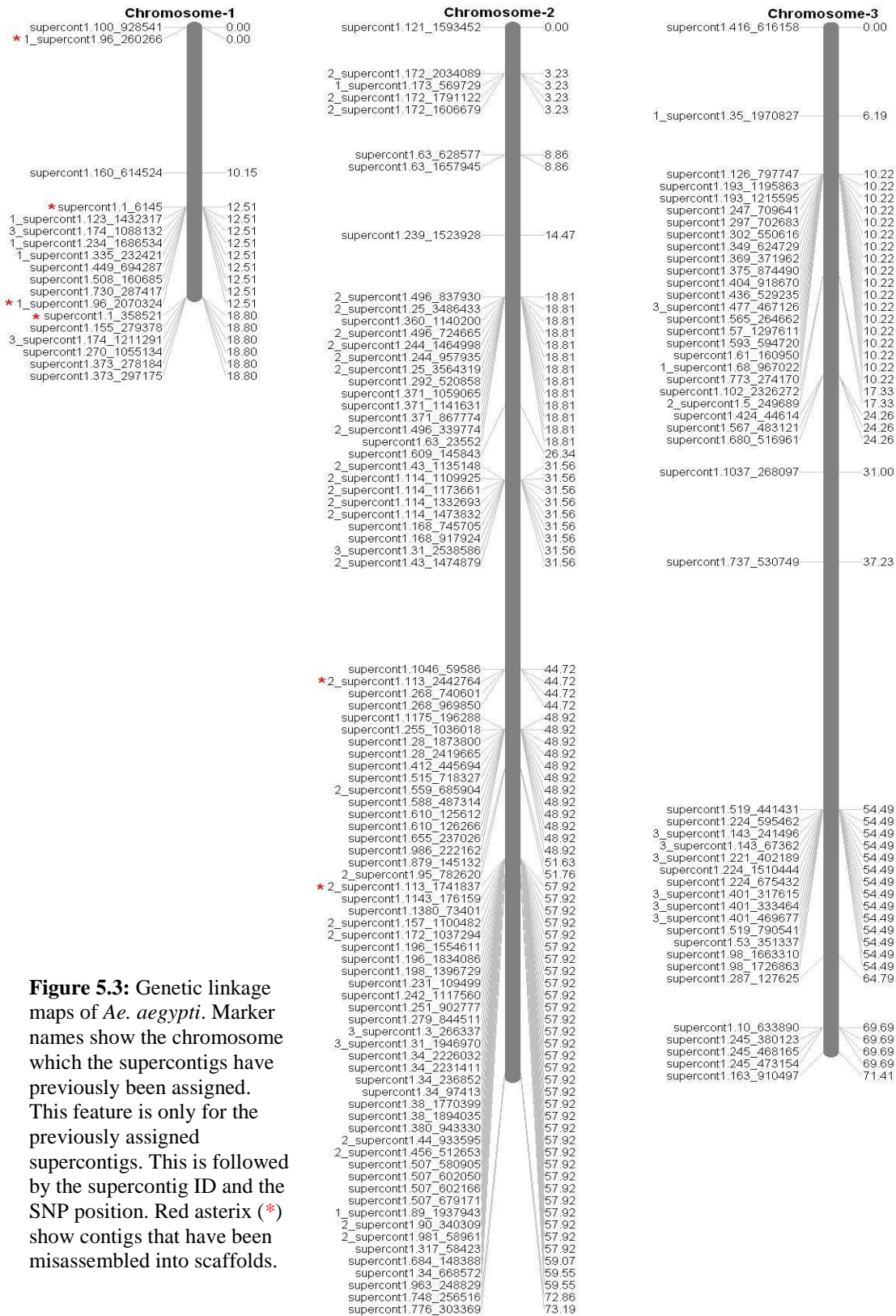
Figure 5.3: Genetic linkage maps of *Ae. aegypti*. Marker names show the chromosome which the supercontigs have previously been assigned. This feature is only for the previously assigned supercontigs. This is followed by the supercontig ID and the SNP position. Red asterix (*) show contigs that have been misassembled into scaffolds.

Comparing genetic maps

As the intention of the study is to later map genes related to pathogen resistance, we were interested in comparing our linkage groups to two comprehensive linkage maps of *Ae. aegypti*; one of which includes an integration between the genetic and physical maps (Brown et al., 2001; Nene et al., 2007). Our maps were more comparable to those presented in the *Aedes* genome paper (Nene et al., 2007) as the maps reported here are also an update of the maps from Brown et al. (2001). Chromosomes-1 and 3 showed the best comparisons (Figure 5.4). Generally, most of the markers had comparable positioning on the chromosomes. However, markers that were shown to be segregating together in our results were split onto different positions on the chromosomes shown by Nene et al. (2007). This may be due to our relatively low number of markers per chromosome and low frequency of observed recombination due to small number of individuals with genotype information.

It also became evident that a huge chunk of chromosome-1 was missing from our data, resulting in a large proportion of our markers mapping only to small parts of the chromosome (Figure 5.4-A). The missing markers are probably on the small linkage groups and we just do not yet have the marker density to link the small linkage groups to the big ones. However, we were still able to spot another case of misassembly on chromosome-1 of Nene et al. (2007). On chromosome-1, supercontig 1.123 maps onto two different loci which are 23.2cM apart (Nene et al., 2007) (Figure 5.3-A, Chromosome-1*). More lanes of sequencing will increase the number of markers and genotyped individuals. This will improve the genetic maps and map comparisons.
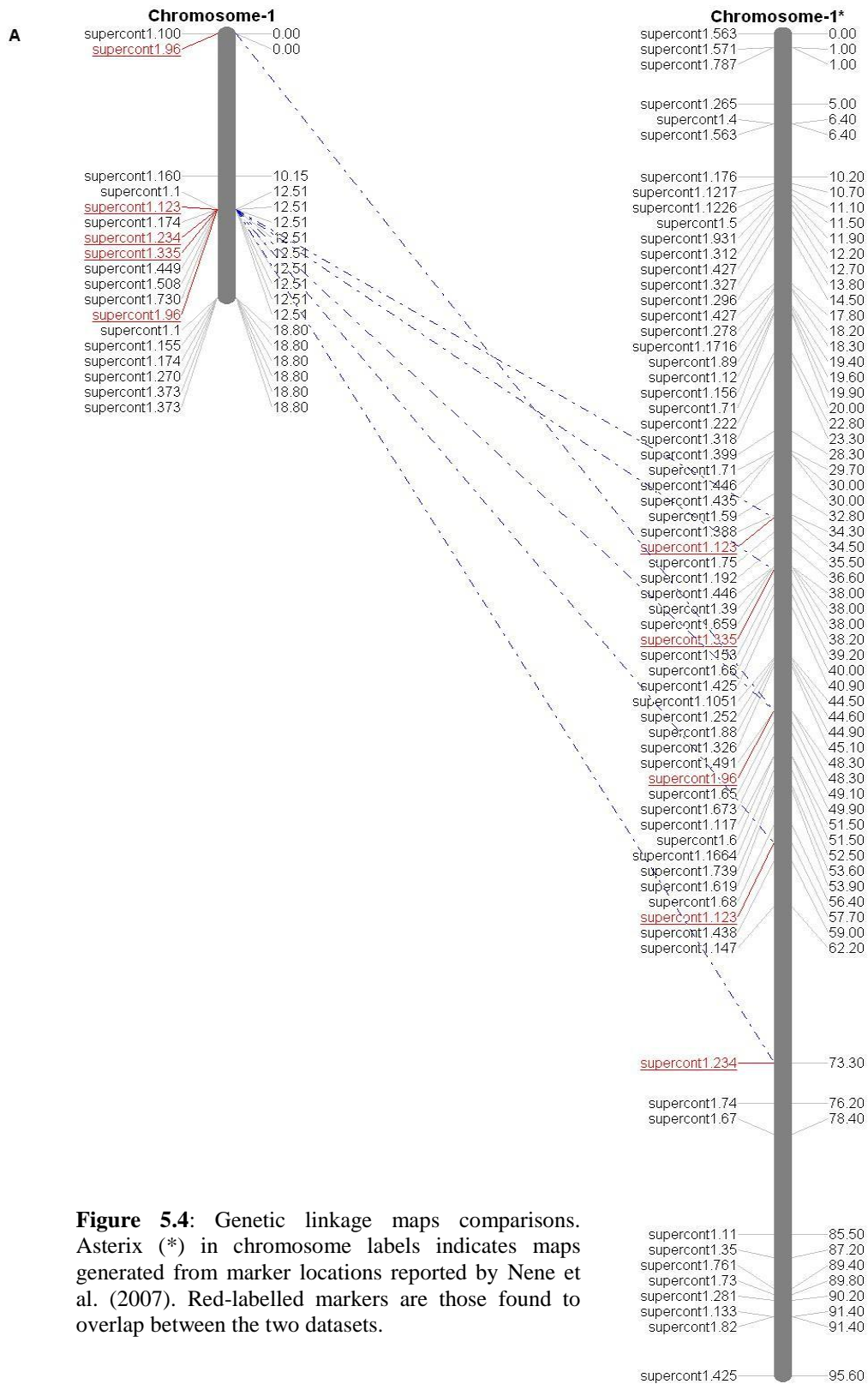
Figure 5.4: Genetic linkage maps comparisons. Asterix (*) in chromosome labels indicates maps generated from marker locations reported by Nene et al. (2007). Red-labelled markers are those found to overlap between the two datasets.
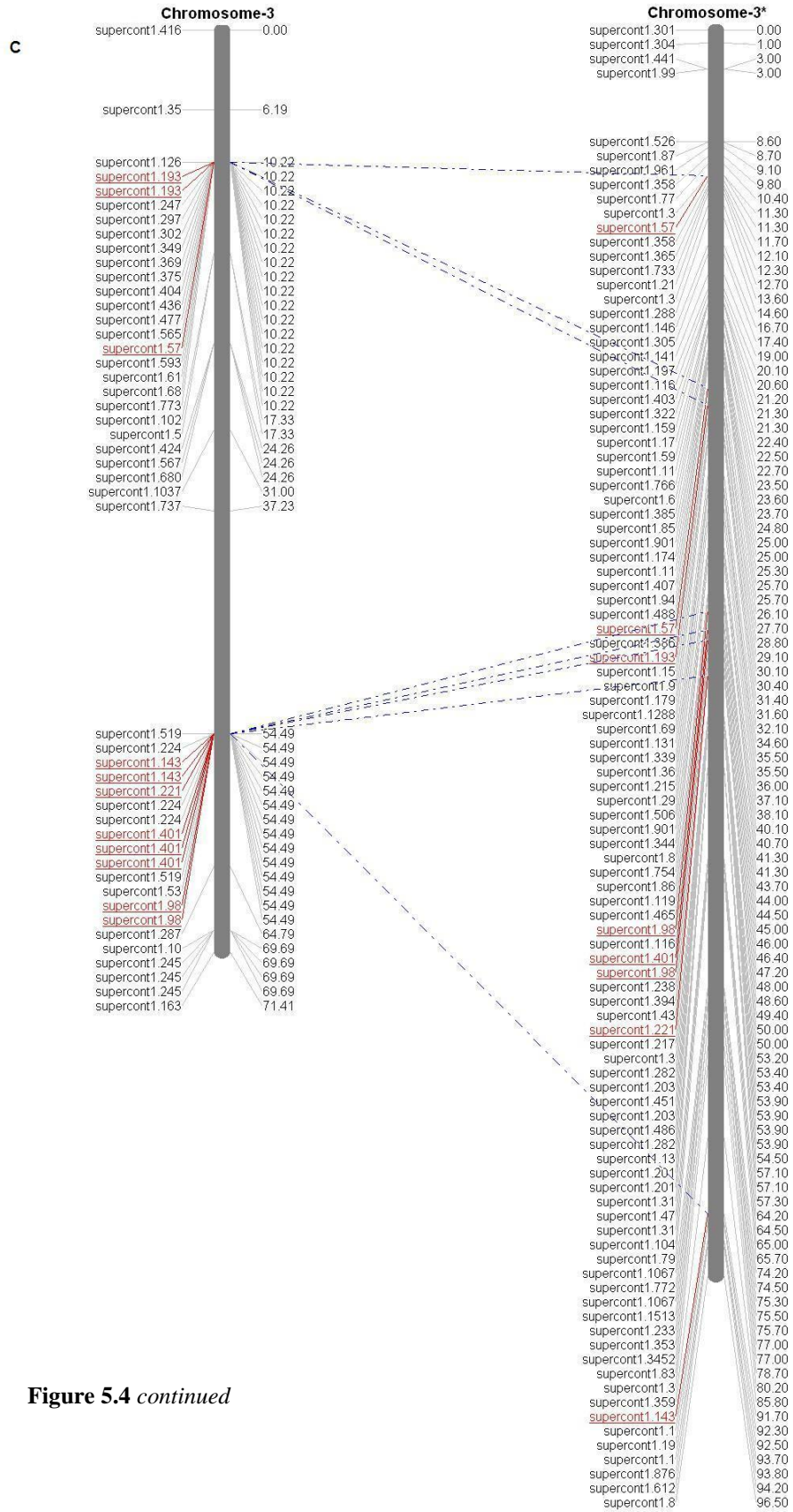
B

Figure 5.4 *continued*

Figure 5.4 *continued*

Additional markers

We later received 4 more lanes of sequencing from G_2 individuals, bringing the total number of sequenced backcross progeny to 99. This increased the average number of reads to 17 million and we have almost doubled the number of backcross individuals (n=54) that have reads above our set threshold of 15 million. With these individuals we have obtained a 7-fold increase in the number of markers (n=1213).

To estimate how this additional sequencing has improved our previous results, we obtained genotype information from the top 60 individuals with the most SNP calls using the software described. We obtained 284 markers from these. 179 of these markers are in supercontigs that have not previously been assigned to chromosomes. These unassigned supercontigs account for 177Mb of the genome, and if these are assigned to chromosomes by MSTMap we will increase the percentage genome mapped by ≈12%.

Note, however, that these are preliminary overview of what the extra lanes of sequencing add to the data already analysed. By modifying the parameters to suit the new data, we may be able to increase the number of useful markers and individuals to improve our results and the genetic maps.

5.4. Discussion

The sequencing of the *Ae. aegypti* genome (Nene et al., 2007) provided a more advanced platform for continuing efforts on developing a comprehensive genetic and physical map (Brown et al., 2001) for this mosquito species. Having the largest genome of the three most important mosquito disease vectors sequenced and with a highly repetitive genome content, a well organized genome is far from complete. We have contributed to efforts to improve the assembly of the *Ae. aegypti* genome by using new DNA-based genome sequencing technology. We have mapped 79 previously unmapped supercontigs consisting of 105 markers. This represents ≈122Mega basepairs of the genome, increasing the percentage of supercontigs assigned to chromosomes to ≈40% of the genome. With few individuals and low number of markers, we were unable to confidently order these supercontigs on their respective chromosomes. However, we were able to identify significant ($p<0.00001$) misassignment of supercontigs to chromosomes by comparing our results to previous data (Nene et al., 2007). We envisage that these results can greatly be improved with more sequenced individuals and markers.

In this study, we used the LVP-IB12 reference genome strain which is an inbred strain, as one of our parental in the crossing design. After 12 generations of inbreeding, we expected a fairly homogenous genome for this strain which would enable genotype calling in our COSTA RICA parent and backcross progeny. However, we experienced high levels of heterozygosity in the reference strain which confounded our ability to assign genotypes to our individuals.

This resulted in lots of genotypes being designated as missing data and greatly reducing the depth of markers even though high numbers of SNPs were observed (data not shown).

Despite the number of markers used in this study, the data set was able to show the 3 linkage groups of *Ae. aegypti*, which are comparable to what the current states of the chromosomes are known to be (Nene et al., 2007). RFLP and cDNA-based genetic markers seem to capture high densities of the euchromatin region of the chromosomes (Brown et al., 2001; Severson et al., 2002). By performing a search for the gene annotations associated with the markers we obtained, we found that 34% of the markers we mapped were in annotated genes (VectorBase: Lawson et al., 2009).

We had used strains of two different genetic backgrounds with the intention of capturing some markers that could potentially aid in the identification of candidate genes near the filarial worm susceptibility marker. Unfortunately, due to little overlap between our markers and markers from two other reported maps (Brown et al., 2001; Nene et al., 2007), this could not be investigated. Nevertheless, supercontig 1.174 from our data is close to the LF178 marker region (supercont 1.59) (Brown et al., 2001; Nene et al., 2007; Severson et al., 1994) and contains a gene encoding serine protease (VectorBase ID: AAEL005787). Serine proteases in the midgut could affect dengue viruses in *Ae. aegypti* (Brackney et al., 2008). Also among our supercontigs, we identified one that mapped close to the QTL associated with midgut penetration by *B. malayi*, LF98 (supercont1.151) (Nene et al., 2007; Beerntsen et al., 1995). Supercont1.507 is 1.2cM from supercont1.151 on chromosome-2 (Figure 5.4-B; chromosome

2*) and mapped to similar positions when we compared our genetic map to the established map from the *Aedes* genome paper (Nene et al., 2007). Although our data seems to suggest 23 more markers are segregating with this locus (Figure 5.3-B; chromosome-2), these markers are split up on chromosome 2* (Figure 5.3-B). A closer investigation of candidate genes within supercont 1.507 show two serine protease genes (VectorBase: AAEL010769, AAEL010773) and a leucine-rich repeat protein (VectorBase: AAEL010772), which could be involved in *Ae. aegypti* immune defense against pathogens (reviewed in Strand, 2008).

We have shown the use of RAD-sequencing to improve the genetic map of *Ae. aegypti*, which is currently still highly fragmented. Our data set shows preliminary efforts to resolve the chromosomal mapping and order of scaffolds. Further sequencing will increase the diversity of markers already obtained and also increase our confidence in these maps. We have also generated more RADtag libraries for mapping the susceptibility gene (s) which should further resolve the maps and gene location.

5.5. Acknowledgements

6. INVESTIGATION OF RECOGNITION GENES IMPORTANT IN *AEDES AEGYPTI* IMMUNITY AGAINST SEPHADEX BEADS

6.1. Introduction

Melanotic encapsulation is a primary means by which an adult mosquito protects itself from metazoan parasites (Beerntsen et al., 1989). It is a form of melanisation that has been thought to be targeted to parasites that are too large to be phagocytosed (Wang et al., 2005), but it has also been observed in response to certain bacteria (Hillyer et al., 2003a). The mechanism is effective in eliminating *Plasmodium* (Collins et al., 1986; Hillyer et al., 2003b) and filarial worm infections in some mosquito hosts (Kobayashi et al., 1986; Yamamoto et al., 1985; Nayar et al., 1989). Melanotic encapsulation can be described as an interplay between the cellular and humoral immune responses of the insect immune system (Christensen and Forton, 1986). It involves the recruitment of haemocytes (cellular) to the surface of the pathogen that has invaded the insect body and initiation of the phenoloxidase cascade (humoral) leading to production and deposition of melanin on the parasite's body (Cho et al., 1998; Hillyer and Christensen, 2002). However, in the adult mosquito where there is a reduced number of haemocytes compared to earlier developmental stages, melanisation occurs with less involvement of haemocytes (reviewed in Beerntsen et al., 2000). Haemocytes in the adult mosquitoes do not form large cellular capsules engulfing the parasite's body (Hillyer et al., 2003b), but are probably involved in pathogen recognition that lead to activation of melanisation (reviewed in Beerntsen et al., 2000).

Recognition of pathogens as non-self is a crucial step in the insect immune response to pathogens that invade the body. This step mediates the invasion of the pathogen and the activation of appropriate proteins and enzymatic cascades to kill the pathogen and prevent pathogen infection and development (reviewed in Beerntsen et al., 2000). Subsequently, the transmission of infective stages of mosquito-borne parasites is reduced. Pattern Recognition Receptors (PRRs) are complement-like molecules that are responsible for distinguishing non-self microbial or pathogenic cells from the host's own cells (Janeway, 1989). PRRs detect Pathogen-Associated Molecular Patterns (PAMPs) which are components, such as bacterial lipopolysaccharide (LPS) and peptidoglycan and fungal β-glucan (Janeway, 1989), that form part of the surface membrane of pathogens. Some families of recognition genes, for example, thioester-containing proteins (TEPs) have been shown to be important determinants of vectorial capacity in *Anopheles gambiae* (Blandin et al., 2004), hence contributing to observed variations in disease transmission among mosquito populations. This makes recognition genes potential candidates for manipulating susceptible populations of mosquitoes for effective immunity against disease pathogens.

The identification of recognition genes in mosquito vectors has gained a lot of research attention because these genes have been shown to initiate strong immune responses that reduce the intensity of *Plasmodium* oocysts in *Anopheles* hosts (Christophides et al., 2004; Dong et al., 2006; Habtewold et al., 2008; Osta et al., 2004). Thioester-containing proteins (TEPs) have been identified as important anti-*Plasmodium* recognition molecules (Levashina et al., 2001). Knockdown of TEP1 results in significant increase in the number of developing

Plasmodium oocysts in susceptible mosquitoes and melanisation ceases in refractory mosquitoes (Blandin et al., 2004). Leucine-rich repeat (LRR) proteins are another family of immune proteins that are effective against *Plasmodium*, particularly Leucine-Rich Immune protein-1 (LRIM1) and the *Anopheles-Plasmodium* responsive Lecuine protein (APL1) (Osta et al., 2004; Riehle et al., 2006). Gram-Negative Binding Proteins (GNBP), though known to be a family of proteins quite specific against bacteria, have been associated with resistance to *P. berghei* in *An. gambiae* (Dong et al., 2006).

More work on recognition protein identification has been done with *Anopheles* species than any other mosquito species. Studies with other species of mosquitoes have shown the importance of other recognition genes besides TEPs and LRRs in killing of metazoan parasites. For example, in the host species *Armigeres subalbatus*, β-1,3-glucan recognition proteins binds to *Dirofilaria immitis* and initiates the elimination of the parasite by melanotic encapsulation (Wang et al., 2005). It will be a collateral benefit for the gene manipulation control strategy if TEPs and LRR proteins are also found to be important elements of immunity in other species of mosquitoes especially *Aedes* and *Culex* which are important vectors of disease.

Aedes aegypti and *An. gambiae* share similarities in their immune gene families (Garver et al., 2008). They are orthologous at most of the TEP genes with TEP1 being the only gene which is *An. gambiae* specific (Waterhouse et al., 2007). *Aedes aegypti* has been a good laboratory model for studying mosquito interactions with filarial worms (Beerntsen et al., 1989;

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Macdonald, 1962b; Macdonald and Ramachandran, 1965; Nayar et al., 1992; Severson et al., 1994) and thus, can also be used in determining the genes that are important in recognising filarial infections. The use of recognition genes as possible means of rendering susceptible population refractory to disease pathogens will be more worthwhile if similar genes are effective in eliminating both eukaryotic parasites– *Plasmodium* and filarial worms– that are transmitted by mosquitoes. To be able to compare how similar recognition genes can affect activation of immune response on *Plasmodium* and filarial worms, the recognition genes responsible for *Plasmodium* killing in *Ae. aegypti* need to be identified, as has already been established in *An. gambiae* (Blandin et al., 2008; Osta et al., 2004; Riehle et al., 2006).

Sephadex beads have been very useful in studying immune responses in mosquito species. In *An. gambiae*, similarities in immune responses to Sephadex beads and *Plasmodium* challenge was observed (Gorman et al., 1996). Inoculation of Sephadex beads shows differences in immune response in *Plasmodium*-refractory and –susceptible strains (Gorman and Paskewitz, 1997; Paskewitz et al., 1998; Paskewitz and Riehle, 1994). Various effects on the melanisation response in *Ae. aegypti*, including cost of immunity (Schwartz and Koella, 2004) and physiological effects (Boëte et al., 2002; Voordouw et al., 2008), have been studied in *Ae. aegypti* using Sephadex beads.

In this study, I investigate the regulation of candidate genes in 2 families of recognition genes. I used Sephadex beads as 'pathogens' to evoke an immune response, first performing a time-course experiment to detect the optimal time point for melanisation of the beads, and using

real-time PCR to determine regulation of the candidate genes. I attempted to use RNAi to investigate the importance of significantly up-regulated genes in the melanisation of the beads.

6.2. Materials and methods

Mosquito rearing

The *Ae. aegypti* strain used in this experiment was the LVP-IB12 strain obtained from The Maizels Lab, University of Edinburgh, where it had been maintained for several generations. Eggs were hatched in a beaker of tap water. The beaker and its contents were placed under a vacuum for about 30mins to trigger hatching. The larvae were transferred to a larval tray containing about a litre of tap water and larvae were provided with 1g of yeast (Sigma-Aldrich, UK). The next day, larvae were picked and transferred into a new tray of water and fed with 1g of desiccated liver powder (Sigma-Aldrich, UK) to reduce overcrowding and allow late eggs to hatch. The contents of the first larval tray were discarded 2 days after the first larvae were collected, as unhatched eggs were considered non-viable. About 0.5g of liver powder was added to the trays 2 days after larvae collection. Water levels were checked and topped-up *ad libitum*. Pupae were picked and transferred into cages from the 6th day after hatching.

Age control of mosquitoes

Throughout the experiment, the age of the adult female mosquitoes used was controlled, as age of mosquitoes affects their melanisation response (Chun et al., 1995). Once adult

mosquitoes had emerged from pupae, they were aspirated and kept in a cage labelled with the day of emergence. Adult mosquitoes were a day old on the morning they were collected, and all female mosquitoes that were inoculated with the bead were approximately 3-4 days old. Adult mosquitoes were fed with 10% fructose (Sigma-Aldrich, UK) mixed with 1% PABA (Sigma-Aldrich, UK). Each cage was provided with a plastic cup containing water and a piece of filter paper fan slipped into the water. This provides access to drinking water and helps maintain humidity in the cage (Christophers, 1960). Mosquitoes were maintained in a controlled room with conditions of 27±1°C and 80±5% humidity, and a 12 hour photoperiod cycle.

Blood feeding female adult mosquitoes

Two to three day old adult female mosquitoes were starved of sugar for 24 hours. The plastic cup containing water and filter paper was removed from the cage about an hour before blood feeding, and the mosquitoes were exposed to light (Christophers, 1960) by turning on a lamp. At the time of performing this experiment, equipment for maintaining constant blood temperature such as the Hemotek blood feeding system (Discovery Workshops, Accrington, UK) used in previous experiments, was not available. I prepared 'feeders' by stretching a piece of parafilm onto the open end of a plastic cylindrical container and making a hole in the opposite closed end through which to pass blood. Once the 'feeders' were filled with blood, they were checked regularly to warm up the blood.

Inoculation of Sephadex bead

The day after blood feeding, blood-fed females were transferred into a paper cup covered with a fine white mesh. These were placed on ice briefly to immobilise them. CM Sephadex C-25 beads (40-125μm) (Sigma-Aldrich, UK) which had initially been left in Sephadex rehydration solution (1.3mM NaCl, 0.5mM KCl, 0.2mM CaCl2, 0.001% methyl green, pH=6.8) were selected for inoculation. The smallest beads were selected by visual inspection with the aid of a microscope. A single bead in about 0.5μl of rehydration solution was inoculated into the thorax of each mosquito. Inoculation was done with the aid of CellTram Oil microinjector (Eppendorf) and a heat-pulled glass capillary (1mm diameter). Each post-treatment paper cup contained mosquitoes that had been inoculated within a 30 minutes period and this was noted on each cup. A piece of cotton soaked in sugar solution and a piece of wet paper towel were placed on top of the cup to provide food and humidity, respectively. It was observed that inoculated mosquitoes dried out and died when the wet paper towel was not provided.

Melanisation time-course experiment

At periods corresponding to 5, 6, 8, 10, 12 and 16 hours post-inoculation, mosquitoes that survived the injection were randomly selected, transferred into another paper cup, and killed by freezing. The thoraces of the mosquitoes were teased in 1X PBS and 0.01% methyl green solution to expose the Sephadex beads and make unmelanised beads easy to detect. Beads were observed for deposition of melanin and were categorized into unmelanised, partially or fully melanised (Figure 6.1)
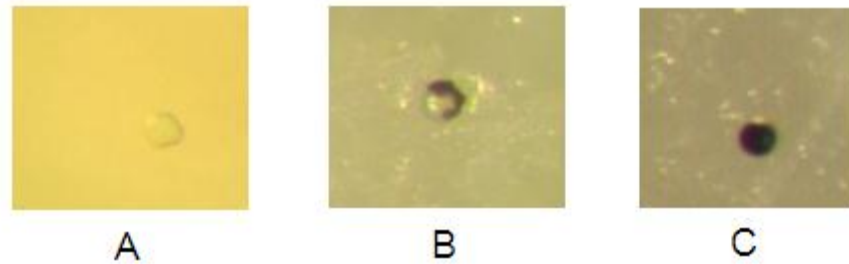
Figure 6.1: Categories of recovered Sephadex beads from mosquito thoraces. Unmelanised beads (A) were clear and hardly visible. Patchy or partially melanised (B) beads had patches of melanisation or were lightly melanised. Fully melanised (C) beads were dark and uniformly melanised.

Inoculation of mosquitoes for gene expression

Preparation of mosquitoes for injection was the same as described for the time-course experiment. In addition, each inoculated mosquito had a control mosquito which was treated similarly (placed on ice together with the inoculated mosquito and returned to a paper cup simultaneously) but without inoculation. Each post-treatment paper cup contained inoculated or control mosquitoes that had been handled within a 30-minute period. The post-treatment cups were treated as previously described. Inoculated samples and their controls were transferred into a freezer for a few seconds after 1, 2, 6, 12 and 24 hours. Samples were immediately homogenized in Trizol (Invitrogen) in 1.5ml microcentrifuge tubes and stored in a freezer at -80°C till RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted from homogenized samples adapting the protocol provided by Bogart and Andrews (2006). Each experimental time comprised of 15 injected mosquitoes and 15 controls, in pools of 5. RNA pellets were re-suspended in 35µl of RNA storage solution (Ambion) and stored at -80°C. 1µl of RNA was reverse transcribed with M-MLV Reverse

Transcriptase (Invtirogen) following manufacturer's protocol. All complementary DNA (cDNA) were tested for DNA contamination with end-point PCR by amplifying the Actin gene (primer sequence on Table 6.1). The primers for this gene had been designed to amplify a region that spans parts of two exons and an intron to help distinguish DNA from RNA.

Candidate gene selection

Gene information and sequences for the following *Ae. aegypti* immune genes were obtained from ImmunoDB (Waterhouse et al., 2007) and VectorBase (Lawson et al., 2009): 7 Thioester-containing Proteins (TEPs) and 2 Leucine Rich Repeat (LRR) proteins (Table 6.1). At the time of performing this experiment, LRR with VectorBase ID AAEL012086 was described as an orthologue of *An. gambiae* LRIM1. All genes were checked for their VectorBase description and orthology with *An. gambiae* (Table 6.1).

Quantitative PCR

Primer sequences for the amplification of candidate genes (Table 6.2) were designed using Primer3 software (Rozen and Skaletsky, 2000). Each primer pair was designed to produce an amplicon size between 100-150bp within an exon. I prepared a 'Homemade' SYBR green reaction mixture (Pellissier et al., 2006) to use in the real-time reactions. I compared the efficiency of the 'Homemade' mix to the Supermix reagent (Biorad) (Figure 6.2) and confirmed the 'Homemade' mix worked efficiently. The qPCR reactions were optimized to obtain adequate working primer efficiencies (95-105%) by using serial dilutions from pooled

cDNA of immune–challenged (inoculated) samples. Primer pairs with efficiencies outside the expected range were re-designed.

Each pool of 5 individuals was analysed in triplicate. Each reaction well contained 2µl of 10X 'Homemade' buffer (10mM Tris HCl (pH=8.5), 20mM KCl, 3mM MgCl$_2$, 0.15% Triton X-100), 2µl of 2mM dNTP mix, 1µl each of 10µM forward and reverse primer, 0.5µl formamide (BDH Lab supplies), 0.4µl of 500nM Fluorescein Reference passive Dye (USB, Product code: 75767), 0,4µl of 10mg/ml bovine serum albumin (BSA) (Biolabs), 0.1µl of 0.01 diluted (in DMSO) SYBR green dye (Invitrogen), 1U Taq, 1µl of cDNA template and distilled water to make a final reaction volume of 20µl. Reactions were performed using the Bio-Rad iQ5 real-time machine. Cycling conditions included an initial denaturation at 95°C for 3min, 45 cycles of 95°C for 30s, 55-60°C for 30s and 72°C for 30s. A melting curve analysis was performed at the end of the reaction cycle for 81 cycles at 55°C for 15s. Samples were held at 16°C after both analyses. Gene expression was analysed using the Pfaffl method (Pfaffl, 2001), employing the static efficiency equation:

$$\text{Ratio of gene expression} = \frac{2^{\wedge(\text{test-control})}_{\text{target gene}}}{2^{\wedge(\text{test-control})}_{\text{Actin}}}$$

All statistical analyses were performed using custom scripts in R statistical analysis software (R Development Core Team, 2008).
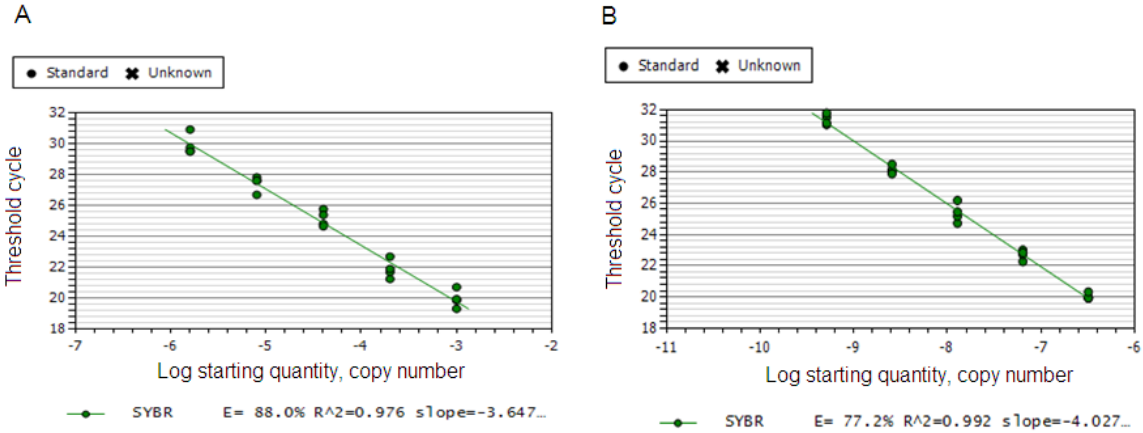
Figure 6.2: Comparison of working efficiency between Bio-Rad Supermix (A) and 'Homemade' SYBR green mix (B).

Table 6.1: Thioester-containing genes obtained from ImmunoDB (Waterhouse et al., 2007) and their VectorBase (Lawson et al., 2009) *An. gambiae* orthologues. All the IDB TEP genes used in the study are macroglobulin/complement genes.

Ae. aegypti IDB gene name	IDB ID	*Ae. aegypti* VectorBase ID	VectorBase description	*An. gambiae* orthologues	*An. gambiae* gene name
-	Aaeg:TEP3	AAEL014755	TEP2	AGAP008364, AGAP008368, AGAP008654	TEP15, TEP14, TEP12
TEP13	Aaeg:TEP1	AAEL012267	Macroglobulin	AGAP008407	TEP 13
TEP15	Aaeg:TEP2	AAEL014755	TEP2	AGAP008364, AGAP008368,	TEP15, TEP14
TEP20	Aaeg:TEP4	AAEL001794	Macroglobulin	AGAP010812, AGAP010814, AGAP010815, AGAP010816, AGAP010818, AGAP010819, AGAP010830, AGAP010831, AGAP010832, AGAP008368	TEP4, TEP6, TEP1, TEP3, TEP11, TEP10, TEP9, TEP8, TEP19, TEP14
TEP21	Aaeg:TEP5	AAEL001802	Macroglobulin	Same as for *Ae. aegypti* TEP20	Same as for *Ae. aegypti* TEP20
TEP22	Aaeg:TEP6	AAEL000087	Macroglobulin	Same as for *Ae. aegypti* TEP20	Same as for *Ae. aegypti* TEP20
TEP23	Aaeg:TEP7	AAEL001163	Macroglobulin	AGAP008368	TEP14

Table 6.2: Primer sequences for reference gene (Actin) and candidate genes used in quantitative PCR analyses. Two LRR genes were included which were orthologous to LRIM1 and 2, respectively, of *An. gambiae* at the time of performing this experiment.

Gene name	VectorBase ID	Forward primer	Reverse primer	Amplicon size
Actin	AAEL015309	ACGTGGCCAAGGATATGAAG	CTTGCTTGGAAACCCACATC	123
TEP 2	AAEL014755	ATAACTCTCGCATCGCTCGT	CCTTAGGTGATCGCTGCTTC	123
TEP 13	AAEL012267	CTTCAACCTTCCGCGACTAC	AACCGTTGACTGTTCAACCA	138
TEP 15	AAEL014755	AAACCATCCAAGTTGGGTCA	CGAACGAAGCCTTATTGACG	130
TEP 20	AAEL001794	GTGGCCTTATGCCAAGTTGT	GTCGGAAGCTTTTACGGTGA	103
TEP 21	AAEL001802	GGATTCATACGTTGCGTTCA	CGTGGAACAAGTCAAACTCG	143
TEP 22	AAEL000087	CGGACATCAGAAGTTCAGCA	CCGAAGAACTCGAAATCCAA	150
TEP 23	AAEL001163	AGCGACAGATGCCATACAAC	TCCATACGTTGCAGTTCTCG	148
LRR1	AAEL012086	TTCATGCGATTGTTCAAGGA	AATTGGGAAGAAACCGTGTCA	110
LRR2	AAEL009520	GACGCATTTTCCAAAACCAC	AGCGGCTAATTGATTGTTCTCT	102

RNAi

I tested two TEP genes for their importance in bead melanisation using RNA interference (RNAi). First discovered in *Caenorhabditis elegans* (Fire et al., 1998), double-stranded RNA (dsRNA) has shown to be an effective mechanism for gene and transcriptional silencing in many organisms (reviewed in Novina and Sharp, 2004). Briefly, dsRNA is cut up into short interference RNA (siRNA) which are 20-25 nucleotides long by an enzyme known as Dicer (Hamilton and Baulcombe, 1999). The antisense strand of the siRNA binds to the complementary strand of the targeted mRNA, with the aid of a protein complex and the target gene is destroyed.

Primers for dsRNA synthesis were designed with a T7 promoter sequence extension (5′ TAA TAC GAC TCA CTA TAGG 3′) at the 5′ ends, and to amplify transcripts between 500-600 bp (Table 6.3).

Table 6.3: Basic primer sequences for dsRNA synthesis. Each was modified to include the T7 promoter sequence and to amplify between 500-600bp of the coding gene sequence.

Gene	Forward primer	Reverse primer	Amplicon size
dTEP2	GGACCCTCAGGTGAACAAAA	CCTTAGGTGATCGCTGCTTC	581
dTEP20	GTGGCCTTATGCCAAGTTGT	GGAACTCCTGGTCGAAATGA	531

The MEGAscript RNAi Kit (Ambion) was used to synthesise dsRNA. According to the manufacturer's protocol, there are two ways of designing primers for dsRNA synthesis; primers with the T7 promoter sequence on the 5′ ends of forward and reverse primers or, the promoter sequence could be placed on the 5′ end of either the forward or reverse of a primer pair. The former gives a pair of primer for each dsRNA to be synthesised and the amplification of the gene is performed in one reaction while the latter provides two pairs of primer and requires two amplification reactions. An example of a set of designed primers for dTEP2 (dsRNA for TEP2) using the second approach is shown below:

```
Forward primer 1:    TAA TAC GAC TCA CTA TAGGG GGACCCTCAGGTGAACAAAA
Reverse primer 1:                            CCTTAGGTGATCGCTGCTTC

Forward primer 2:    GGACCCTCAGGTGAACAAAA
Reverse primer 2:    TAA TAC GAC TCA CTA TAGGG CCTTAGGTGATCGCTGCTTC
```

Synthesised dsRNA was eluted in elution buffer (10 mM Tris-HCl pH 7, 1 mM EDTA) and quantified with the Nanodrop ND 1000 (Thermo Fisher Scientific). Using a set of primers in a single reaction produced low concentrations of dsRNA (dTEP2=136ng/µl, dTEP20=76ng/µl). The yield of the final product increased 2-fold for dTEP2 and 8-fold for dTEP20 when the second approach was used, and these were used in the experiment. I diluted dTEP20 by a dilution factor of 2.5 to obtain a similar concentration as dTEP2. Samples were aliquoted and stored at -80°C until use.

Injections were performed in a 4°C room where mosquitoes were immobilised without placing them on ice. Day-old adult female mosquitoes were injected on the left side of the thorax with 69nl dsRNA using Nanoinject (Drummond, USA) and returned to a cage to recover. Controls were injected with elution buffer. Cages were covered with plastic bags to keep the cage moist, preventing the mosquitoes from drying out. All surviving mosquitoes were provided with a blood meal on the second day following injection (3 days after emergence). All blood-fed mosquitoes (dsRNA and controls) were injected with a bead on the right side of the thorax the following day. After 6 hours mosquitoes that could fly or stand were selected for gene expression. They were immobilized, homogenized in Trizol and RNA extracted as previously described. The remaining samples were kept for 16 hours after which they were killed for bead melanisation assay.

6.3. Results

Time course of melanisation

A total of 161 surviving mosquitoes were selected for scoring bead melanisation. Out of this 97 beads (60.2%) were recovered and scored. This percentage of recovered beads could have been due to experimental errors which occurred during injections. It was realised at some point during injection that the beads remained attached to the internal walls of the capillary needle while the rehydration solution was dispensed. After this was noticed, mosquitoes were discarded if the bead was left behind in the needle after injection. The total number and state of beads recovered at each experimental time are summarised on Table 6.4. Fully and partially melanised beads were grouped as melanised for analyses.

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In total, 48 melanised beads were observed accounting for 49.5% of the total number of beads retrieved. The proportion of melanised beads increased significantly (Fisher's exact: 95% confidence, p=0.018) with increasing incubation time (Figure 6.3). There was a steep increase in the number of melanised beads from 5 to 6 hours (12.5%- 33.3%). At 10hours, 58% of the beads were melanised showing that this post-inoculation time point may be ideal for assaying bead melanisation. Melanisation of beads reached a threshold after 12hours incubation time where about 71% of recovered beads were melanised.

Table 6.4: Summary of Sephadex beads recovered at each time post-inoculation. Partially melanised beads were grouped together with full melanised beads for analyses.

| Time PI (hours)	Samples dissected	Beads recovered	Fully melanised	Partially melanised	Unmelanised
5	23	16	2	0	14
6	24	18	2	4	12
8	35	15	3	4	8
10	29	12	2	5	5
12	26	17	5	7	5
16	24	19	8	6	5
TOTAL	**161**	**97**	**22**	**26**	**49**
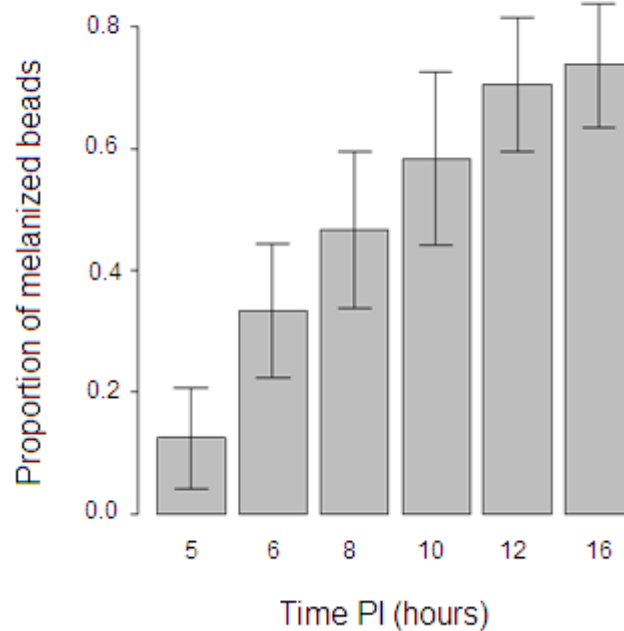
Figure 6.3: Plot showing the increase in the number of melanised beads with incubation time. Each bar represents the proportion of melanised beads recovered from the thoraces of inoculated mosquitoes. Error bars are upper and lower limits at 95% confidence interval. A steep increase in the number of melanised beads is observed from 5 to 6 hours. An asymptote is reached between 12-16 hours with 0.74 of beads melanised.

Gene expression

Although the candidate genes showed their strongest up-regulation at different time points, a general trend in their profiles is observed. Generally, most of the genes seem to be constitutively expressed in the mosquitoes tested i.e. ratio of expression equals 1 when gene expression is compared between injected and non-injected individuals an hour after immune-challenge. TEPs 15, 20, 21 and LRR2, on the other hand, showed up-regulation in inoculated samples even at 1 hour (Figure 6.4). Gene expression peaked between 2-12 hours, with TEP20 showing the highest up-regulation with a 6-fold increase in gene expression at 6 hours (Figure 6.4). TEPs 13 and 23 were not up-regulated at any of the time points tested. Rather, TEP 23

was downregulated i.e. ratio of expression < 1. When all the Ct values for the controls and immune-challenged individuals are considered per gene, TEPs 15, 20, 21 and 22 showed significant expression profiles (Figure 6.4).
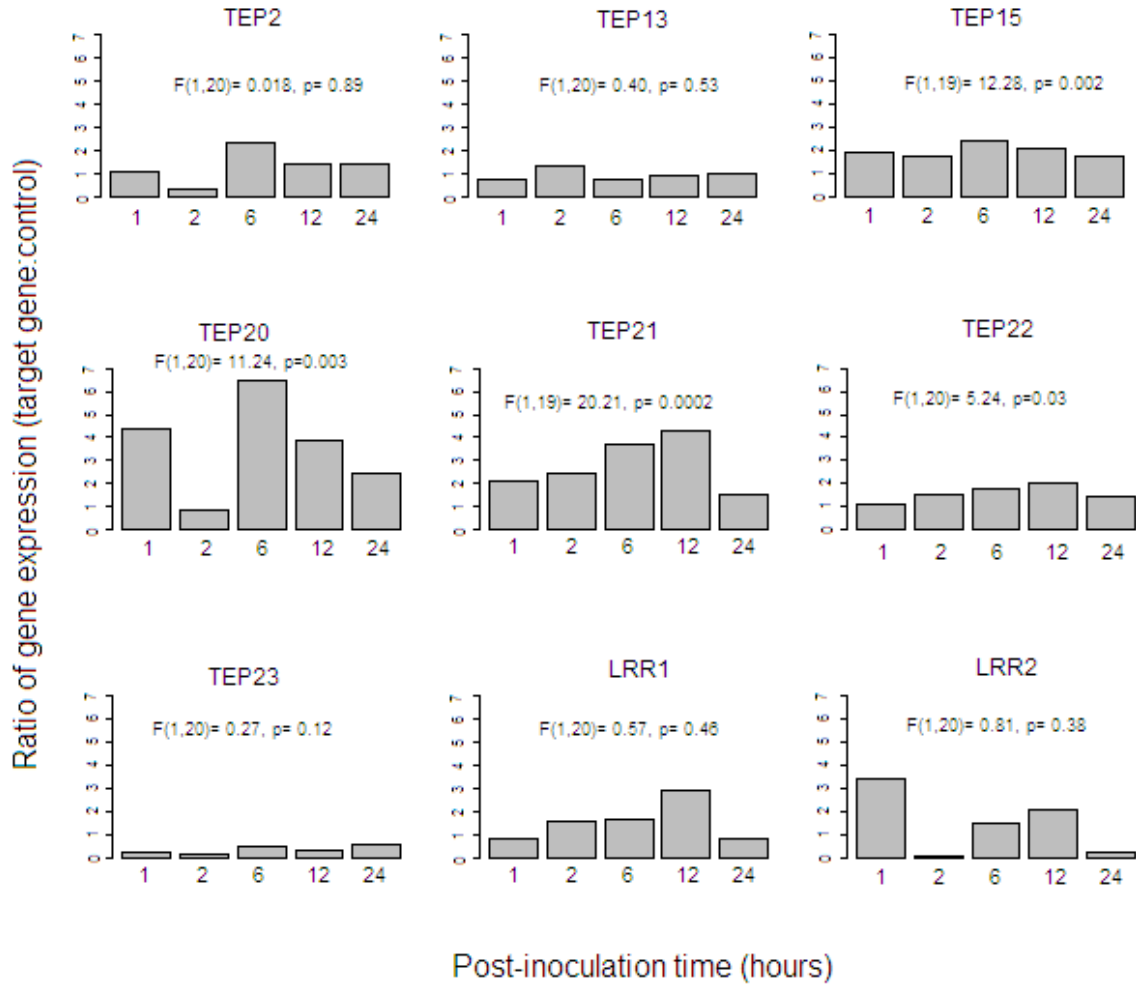
Figure 6.4: Expression levels of recognition genes at different time points after Sephadex bead inoculation. The amount of transcript in immune-challenged samples is compared to control to determine up- or down-regulation. Ratio=1 indicates no difference between injected and control; ratio >1 indicates up-regulation; ratio<1 indicates down-regulation. *F*-statistic shows a test of significance of the overall gene regulation through the experimental time points.

RNAi

All the dsRNA-injected samples had unmelanised Sephadex beads. However, the controls were found not to have melanised beads either hence it cannot be definite to say that the RNAi had worked. The Ct values from TEP2 and TEP20 gene expression from the dsRNA-injected and control samples confirmed that the injection of dsRNA constructs did not result in a significant knockdown of the gene. The Ct values were rather similar between elution buffer injected and dsRNA injected samples (Figure 6.5). Knockdown of TEP20 seems to have worked marginally better than that of TEP2. This is indicated by p-values in Figure 6.5. Amplification of TEP20 in dTEP20 samples showed significantly higher Ct values when compared to TEP20 expression in dTEP2 samples (Wilcoxon=0, p=0.002). The difference in expression was not as significant when the comparison is made between the dTEP20 and elution buffer controls (Wilcoxon=4, p=0.03).
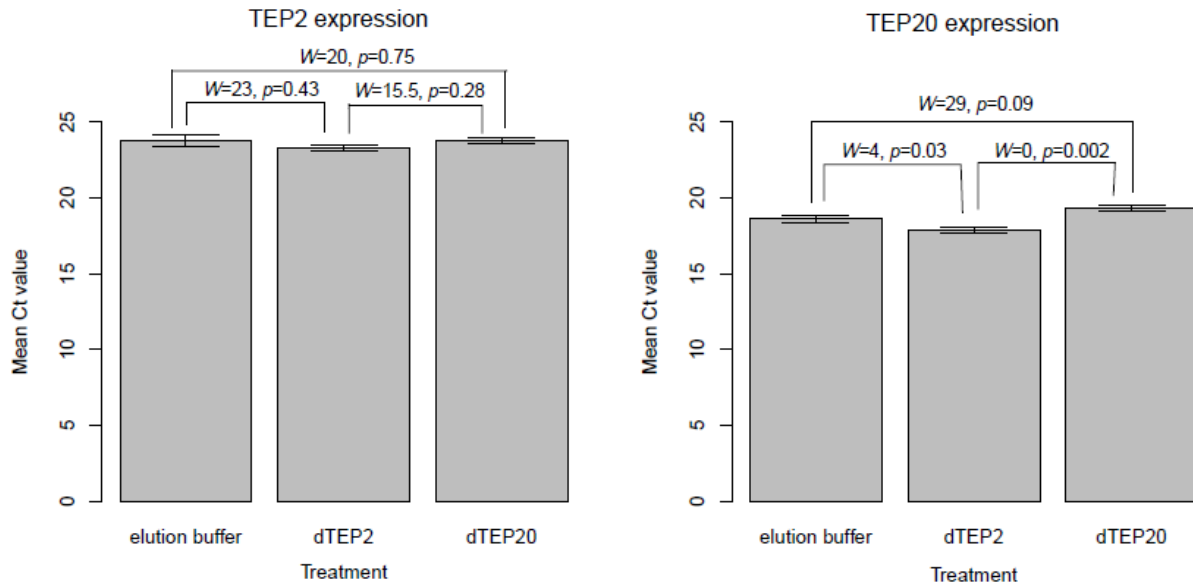
Figure 6.5: Mean Ct values of each group of treated samples after real-time amplification of TEP2 and TEP20. Error bars are standard errors of the mean Ct under each treatment. Statistics shown is the Wilcoxon test for significance between each pair of mean Ct value.

6.4. Discussion

Recognition genes are important for eliciting immune responses against pathogens. Thioester-containing proteins and leucine-rich immune genes are particularly effective on *Plasmodium* parasites in *An. gambiae* (reviewd in Volohonsky et al., 2010). Using Sephadex beads as foreign body to elicit an immune response, I show in this study that at least 50% of laboratory strains of *Ae. aegypti* are able to melanise Sephadex beads 10 hours after bead inoculation. Some TEPs are significantly up-regulated in response to these Sephadex beads following the immune-challenge. TEP15 and TEPs 20, 21 and 22 —orthologs of TEP1 in *An. gambiae* — generally showed increase in gene expression in inoculated samples, with $\approx$ 7-fold increase in

TEP20. This emphasises the importance of TEPs, and most probably *An. gambiae* TEP1 orthologs, in the immune response of mosquitoes.

Sephadex beads are used to elicit an immune response in laboratory experiments to study different aspects of immunity in mosquitoes. For example, beads were used to investigate cost of immunity in *Ae. aegypti* (Schwartz and Koella, 2004) and also useful in distinguishing refractory and susceptible individuals of *An. gambiae* (Gorman and Paskewitz, 1997). The inoculation of Sephadex beads into *Ae. aegypti* could be likened to infection with *Plasmodium* as both have been observed to elicit similar immune activation in *An. gambiae* (Gorman and Paskewitz, 1997). The study has shown variation in melanisation response to Sephadex beads in *Ae. aegypti* with about 50% of the retrieved beads being melanised, an indication of the immune response in the vector when *Plasmodium* sp. is ingested through a blood meal.

Thioester-containing proteins are important in the melanisation of *Plasmodium* parasites in *Anopheles* sp. and a determinant of vectorial capacity (Blandin et al., 2004; Zhang et al., 2011). In *An. gambiae*, leucine-rich repeat (LRR) proteins are known to form a complex with cleaved TEP1 to direct TEP1 to the parasite surface (Povelones et al., 2011). The detection of highly expressed TEPs which are othologous to *An. gambiae* TEP1 emphasises the evolutionary importance of TEPs in mosquito immunity (Waterhouse et al., 2007). While the expression of some TEPs increased significantly, LRR proteins showed no significant increase. This is consistent with the hypothesis of LRR-TEP binding complex such that, LRR are depleted as more TEPs are expressed (G. Christophides, 2012, pers. comm.). The role of

these genes would have been more definite from this study if RNAi had been successful and knockdown of TEP20 had resulted in the inability of the mosquito to melanise the beads, for example.

This study has not only emphasised the evolution of immunity in related species, but has specifically shown the importance of TEPs in mosquito immune responses against parasites. I propose further work on evaluating the importance of these TEPs in melanisation in *Ae. aegypti*, especially to filarial parasites such as *Brugia malayi* as they are extensively used together as a model system. It will also be important to investigate if these TEPs are determinants of vectorial capacity in *Ae. aegypti* populations.

7. GENERAL DISCUSSION

7.1. Summary of field

Mosquito-borne diseases are major public health concerns. Malaria, for example, killed an estimated 655,000 people in 2010, most of these being children (WHO, 2011b). An estimated 40 million people worldwide show clinical manifestations of human lymphatic filariasis (WHO, 2011a). Over the decades, vector control strategies that have been implemented to eliminate risks of these diseases have proven ineffective in certain regions for various reasons. One reason that has encouraged discovery of alternative methods for vector control is evolution of resistance by mosquitoes to insecticides (Mitchell et al., 2012). We have only recently begun to harness the natural mechanisms utilised by the mosquito hosts to eliminate parasites as potential strategies for blocking disease transmission. Now, novel ways of reducing disease transmission are sought with a primary aim of killing parasites within the mosquito hosts, rather than killing of the hosts.

Remarkable advancements have been made in this quest. The innate immune responses against pathogens have been comprehensively studied in mosquito vectors (Castillo et al., 2011; Cirimotich et al., 2010; Kumar and Paily, 2008). In most of the studies involving the immune system, bacteria had been widely used, but it was only quite recently that the role played by bacteria in immune response against other pathogens was discovered (Azambuja et al., 2005; Dong et al., 2009; Kambris et al., 2010; Moreira et al., 2009; Mousson et al., 2010).

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Differences in immune gene expression probably due to differences in bacteria composition in the host are important factors that influence variation on diseases transmission. These components have great potential as useful mechanisms to exploit in alternative strategies for reducing disease transmission (Walker et al., 2011). Above this, the genetic components of host-parasite interactions cannot be oblivious to us. Genome sequencing of three important mosquito vectors (Arensburger et al., 2010; Holt et al., 2002; Nene et al., 2007) has allowed comparative analyses of genetic components that cause variation in vector competence between mosquito species. It has also provided the platform to isolate gene loci that explain observed phenotypes such as susceptibility to disease pathogens (Brown et al., 2001; Nene et al., 2007).

7.2. Research overview

In this thesis, I have continued efforts in these areas by studying natural and laboratory populations. It is important that as we study laboratory strains in controlled environmental conditions, these studies are also extended to natural populations to give a true picture of mechanisms in the wild. The first part of the project presented in this thesis investigated wild populations (Chapter 2-4) while the second part (Chapter 5 and 6) involved laboratory strains of *Aedes aegypti*.

Gut bacteria

Bacteria form essential parts of the life history of the mosquito, first being a cue for selecting appropriate breeding water (Lindh et al., 2008), providing food for larvae (Lindh et al., 2008) and playing important roles in the physiological functions of the adult (Dong et al., 2009). The comprehensive study of gut microbiota in mosquitoes is the first reported use of 454 pyrosequencing to investigate bacteria diversity in different mosquito species from one geographical area (Chapter 2). It was possible to account for species richness and relative proportions of bacteria taxa which was previously impossible to obtain with the methods that were in use (Pidiyar et al., 2004; Straif et al., 1998). Although various factors such as age and feeding history were unaccounted for, it is clear that species are generally similar in their gut microbial composition. This result indicates that if specific gut bacteria are to be fed to mosquito populations as a new control strategy it will be easily applicable in different mosquito species. *Asaia* sp. is a most likely candidate for this control approach due to its versatility and ability to colonize many hosts species (Chapter 2; Chouaia et al., 2010; Crotti et al., 2009). Its ability to colonize many host tissues, including the reproductive tissues, enables the bacteria to also be horizontally and vertically transmitted (Crotti et al., 2009) and maintained in host populations.

Wolbachia

Another group of bacteria with seemingly greater potential as a candidate for use in control programmes are *Wolbachia* endosymbionts. *Wolbachia* is widespread among arthropod species (Zug and Hammerstein, 2012), however it is not present in two of the most important

mosquito disease vectors— *Anopheles* sp. and *Aedes aegypti* (Kittayapong et al., 2000; Rasgon and Scott, 2004; Chapter 3). The inability of *Anopheles* to harbour *Wolbachia* in the wild or establish stable transinfections in germlines is still not completely understood. *Anopheles* may have selected against the ability to sustain *Wolbachia* due to unfavourable effects the bacteria has on its immune transcriptome profile during parasite infection (Hughes et al., 2011b). On the other hand, as *Wolbachia* seem unable to infect ovaries of infected *Anopheles* (Hughes et al., 2011a) being in this host species is an end point for bacteria transmission. As a result both host and bacteria have diverged from each other. I have demonstrated that horizontal transmission has occurred in the mosquito *Wolbachia* lineage in supergroup B (Chapter 3). This makes it possible to transinfect *Anopheles* with a strain from supergroup B rather than from supergroup A (Hughes et al., 2011a) as these seem to show horizontal transfer events likely to make transinfections technically easier.

Successful transinfection into *Aedes aegypti* has yielded promising results (Walker et al., 2011) however, there is need for careful consideration of the dynamics and sustainability of the introduced *Wolbachia* in natural populations (Hancock et al., 2011). One concern is that *Wolbachia* already existing in natural populations may alter dynamics of the introduced strain and vice versa (Hoffmann and Turelli, 1997). This is more likely to happen with strains that belong to different clades of the *Wolbachia* phylogeny rather than those that cluster together. In effect, the newly discovered *Wolbachia* strains from the *Aedes bromeliae* and *Mansonia uniformis* and *Mansonia africana*— all vectors of disease (Huang, 1986; Ughasi et al.,

2012)— could be a better choice in vector control programmes as they already exist in natural populations of mosquitoes.

The Aedes genome and genetic susceptibility to Brugia

Human lymphatic filariasis belongs to a group of infectious diseases classified as Neglected Tropical Diseases by the World Health Organization. Although it is transmitted by mosquitoes it seems to receive less attention than malaria and dengue fever. *Aedes aegypti* is not a potential threat to spread of filariasis because *Ae. aegypti* is active during the day while the parasite moves to peripheral blood in the mammalian host at night. However, *Ae. aegypti* has still given remarkable information on transmission dynamics of the filarial parasite. As it is also a vector of major arboviruses, this encouraged the sequencing of the *Aedes* genome (Nene et al., 2007).

In their conclusion to the report of the draft genome of *Aedes* Nene et al. (2007) highlighted the prioritization of providing a high resolution of the genetic maps of *Ae. aegypti* for comparing variation between field and laboratory strains. The LVP-IB12 inbred line used in the draft sequence was derived from the Liverpool line formally selected for *Brugia* susceptibility (Macdonald, 1962b). If susceptibility is fixed in the line used in the inbreeding selection then, LVP-IB12 should be susceptible. However, this observation was not made in two different isolates of the line obtained from two different sources (Chapter 5). Although inbred, the genome of LVP-IB12 shows a lot of heterozygosity compromising the genotype calling for backcross individuals set up for linkage mapping. Nevertheless, RADtag

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sequencing has proven to be an effective DNA-based method for improving the genetic maps and can be equally effective in providing a comprehensive list of candidate genes that influence vector competence.

I am inclined to agree with previous suggestions that the incidence of high frequencies of susceptible individuals is mainly associated with the ancestral *Ae. aegypti formosus* (Rodriguez and Craig, 1973). This is because in other parts of the world where the ancestral form does not exis,t susceptibility is between 0-2% (Rodriguez and Craig, 1973). West African *Ae. aegypti* cluster with this ancestral form from East Africa (Brown et al., 2011), but shows low frequency of susceptibility to *Brugia* (Rodriguez and Craig, 1973). This is an indication of domestication in West African strains with associated evolution of refractoriness. Nevertheless, East African strains offer an excellent source for mapping variations associated with susceptibility in natural populations.

Immunity

Another aspect of vector competence that is actively studied is mosquito immunity. The network of proteins and signalling pathways has evolved in Diptera in response to various pathogens (Waterhouse et al., 2007). In the malaria vector, *An. gambiae*, TEP1 is important in *Plasmodium* killing (Blandin et al., 2008, 2004). While this important function of immune proteins is established in *An. gambiae* the same cannot be said for the yellow fever mosquito, *Ae. aegypti*. If immune proteins are to be exploited for rendering susceptible populations of mosquitoes refractory, having orthologous immune proteins that affect various pathogens in mosquito disease vectors can be very beneficial. As such the investigation of gene expression

in *Aedes* after immune-challenge is a step to identifying how proteins encoded by these candidate genes have similar functions.

TEP1 and LRIM1 have only quite recently been shown to function together in a complex to get rid of *Plasmodium* in *An. gambiae* (Povelones et al., 2011). In *Ae. aegypti* a similar mechanism may exist as inoculated *Ae. aegypti* show as up-regulation of TEPs and down-regulation in leucine-rich repeat (LRR) immune proteins (Chapter 6). The functions of TEPs and LRRs seem to be conserved in these two mosquito species. TEP1 and LRIM1 were previously known to be *An. gambiae* specific (Waterhouse et al., 2007) however, this may not entirely be the case.

7.3. Future directions

We are increasingly gaining understanding of how these factors interplay in killing parasites and block disease transmission. Other factors not investigated in this project will improve what is already known, leading to a better way of utilising these to eradicate diseases. In the next few paragraphs, I make brief suggestions of what future studies could contribute to this area of study.

Effects of environment

Several environmental factors affect the distribution of mosquito species and in effect, disease distribution (de Souza et al., 2010). For example, presence of *An. gambiae s.s.* is known to be heavily dependent on rainfall (Yawson et al., 2004). As availability of breeding places increase during rainy seasons this may also be directly correlated with an increase of bacteria in the breeding water. It will be very informative to determine, then, how changing dynamics of bacteria in the environment can influence disease transmission. This will further emphasis the role of bacteria in the epidemiology of mosquito-borne diseases and also, give an indication of how sustainable the use of some bacteria species in control programmes are likely to be.

Functions of Aedes and Mansonia Wolbachia strains

Drosophila Wolbachia strain, *w*Mel, has successfully been transferred into *Ae. aegypti* in the laboratory (McMeniman et al., 2009). Vectors such as *Anopheles* have repeatedly failed to

establish *Wolbachia* in their germline (Hughes et al., 2011b). The problem may be that there have not been a lot of naturally-occurring *Wolbachia* strains tested, especially from mosquitoes. This has limited the preferred choice for transinfection of mosquito vectors to *w*Mel. The study in Chapter 3 has added 2 strains of *Wolbachia* from mosquito disease vectors (*Aedes bromeliae* and *Mansonia africana*). One important study will be to first confirm the functions of these *Wolbachia* strains in their natural vectors, especially testing their effects on disease parasites and if they manipulate their host reproduction. Once these have been established, transinfection could be tried on *Anopheles* and *Ae. aegypti*.

Comparing natural and laboratory populations

Aedes aegypti is a good model for studying mosquito relationships with disease parasites. As it is easier to maintain in the laboratory, this tends to drive our focus away from natural populations. It is important that we use new methods and techniques to address questions in laboratory strains, but also extend these to wild populations and natural mosquito-parasite systems. With improved genome assembly, genes can be mapped and comparative analyses will be more definitive. I suggest that while RADtag sequencing has proven effective in improving the *Aedes* genome, this effort is continued. Similarly, mapping of polymorphisms associated with variations in disease transmission should be evaluated in natural systems, for example, *Anopheles-Wuchereria bancrofti* and *Culex-Wuchereria bancrofti*. This will contribute tremendously to understanding how genetic susceptibility to various pathogens in mosquito hosts are related to each other, especially in vectors that transmit more than one disease.

7.4. Conclusion

My research has looked at both natural and laboratory populations of mosquitoes, attempting to piece together some of the factors that contribute to variability in susceptibility to disease-causing pathogens. It is evident that vector competence is a complex attribute influenced by both ecological and genetic factors. All these need to be critically considered and evaluated in our search for appropriate, alternative methods to controlling mosquito-vectored diseases.

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APPENDIX

Chapter 2: Gut bacteria

Table S1: Taxonomic information on the OTUs shown on heatmap (Figure 2.3A).

OTU ID	Family	Genus
1	Aeromonadaceae	*Aeromonas*
2	Acetobacteraceae	*Asaia*
3	Acetobacteraceae	*Asaia*
5	Halomonadaceae	*Zymobacter*
6	Halomonadaceae	
7	Halomonadaceae	
13	Pseudomonadaceae	*Pseudomonas*
14	Methylobacteriaceae	*Methylobacterium*
16	Enterococcaceae	*Enterococcus*
66	Staphylococcaceae	*Staphylococcus*
75	Streptococcaceae	*Streptococcus*
76	Enterococcaceae	*Enterococcus*
100	Aeromonadaceae	*Aeromonas*
109	Flavobacteriaceae	
110	Enterobacteriaceae	*Providencia*
111	Acetobacteraceae	*Asaia*
112	Pseudomonadaceae	*Pseudomonas*
117	Flavobacteriaceae	*Chryseobacterium*
136	Flavobacteriaceae	*Chryseobacterium*
160	Xanthomonadaceae	
163	Acetobacteraceae	*Asaia*
164	Halomonadaceae	
165	Moraxellaceae	*Acinetobacter*

Table S1 *continued*

OTU ID	Family	Genus
168	Propionibacteriaceae	*Propionibacterium*
196	Micrococcaceae	*Micrococcus*
209	Enterobacteriaceae	*Escherichia/Shigella*
215	Enterobacteriaceae	
219	Enterobacteriaceae	*Pantoea*
279	Novispirillum	
295	Aeromonadaceae	*Aeromonas*
300	Aeromonadaceae	*Aeromonas*
308	Moraxellaceae	*Acinetobacter*
310	Aeromonadaceae	*Aeromonas*
314	Aeromonadaceae	*Aeromonas*
320	Aeromonadaceae	*Aeromonas*
321	Aeromonadaceae	*Aeromonas*
327	Aeromonadaceae	*Aeromonas*
381	Moraxellaceae	*Acinetobacter*
382	Moraxellaceae	*Acinetobacter*
383	Moraxellaceae	
387	Halomonadaceae	*Zymobacter*
392	Acetobacteraceae	*Asaia*
404	Sphingomonadaceae	*Sphingomonas*
411	Acetobacteraceae	*Asaia*
424	Acetobacteraceae	*Asaia*
426	Acetobacteraceae	*Gluconobacter*
434	Acetobacteraceae	*Gluconobacter*
435	Acetobacteraceae	*Gluconobacter*

Table S1 *continued*

OTU ID	Family	Genus
455	Halomonadaceae	*Zymobacter*
463	Halomonadaceae	
467	Halomonadaceae	*Zymobacter*
500	Pseudomonadaceae	*Pseudomonas*
602	Halomonadaceae	*Zymobacter*

Chapter 3: *Wolbachia* in mosquitoes

Table S2: Primer sequences for Multi-locus Sequence Typing (MLST) genes. *These were used in a nested PCR with standard primers as suggested (Jolley et al., 2004).

Gene	Primer sequences (5'-3')	Product size, bp
gatB	gatB_F1: GAK TTA AAY CGY GCA GGB GTT	471
	gatB_R1: TGG YAA YTC RGG YAA AGA TGA	
coxA	coxA_F1: TTG GRG CRA TYA ACT TTA TAG	487
	coxA_R1: CT AAA GAC TTT KAC RCC AGT	
hcpA	hcpA_F1: GAA ATA RCA GTT GCT GCA AA	515
	hcpA_R1: GAA AGT YRA GCA AGY TCT G	
	*hcpA_F3: ATT AGA GAA ATA RCA GTT GCT GC	524
	*hcpA_R3: CAT GAA AGA CGA GCA ARY TCT GG	
ftsZ	ftsZ_F1: ATY ATG GAR CAT ATA AAR GAT AG	524
	ftsZ_R1: TCR AGY AAT GGA TTR GAT AT	
fbpA	fbpA_F1: GCT GCT CCR CTT GGY WTG AT	509
	fbpA_R1: CCR CCA GAR AAA AYY ACT ATT C	
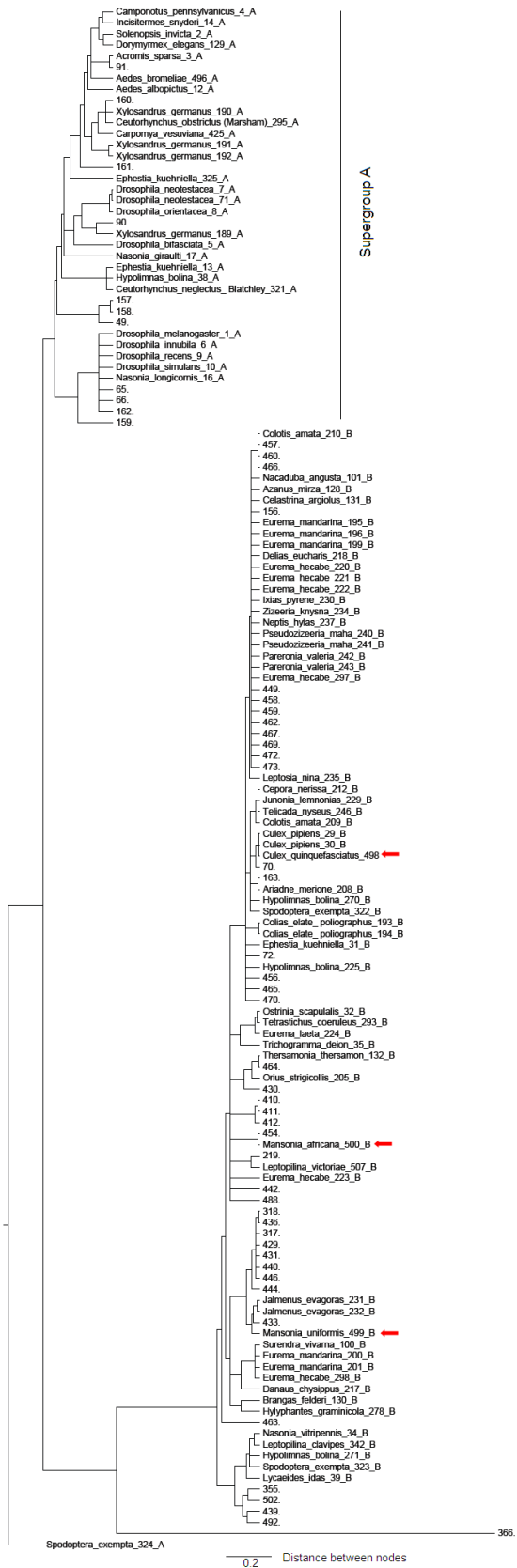
Figure S1: MLST analyses on supergroup B *Wolbachia* strains (http://pubmlst/*Wolbachia*). Red arrows show *Culex* and *Mansonia* *Wolbachia* strains

Chapter 5: *Aedes* linkage map

Figure S2: Validation of libraries used in bulk-segregant analyses.
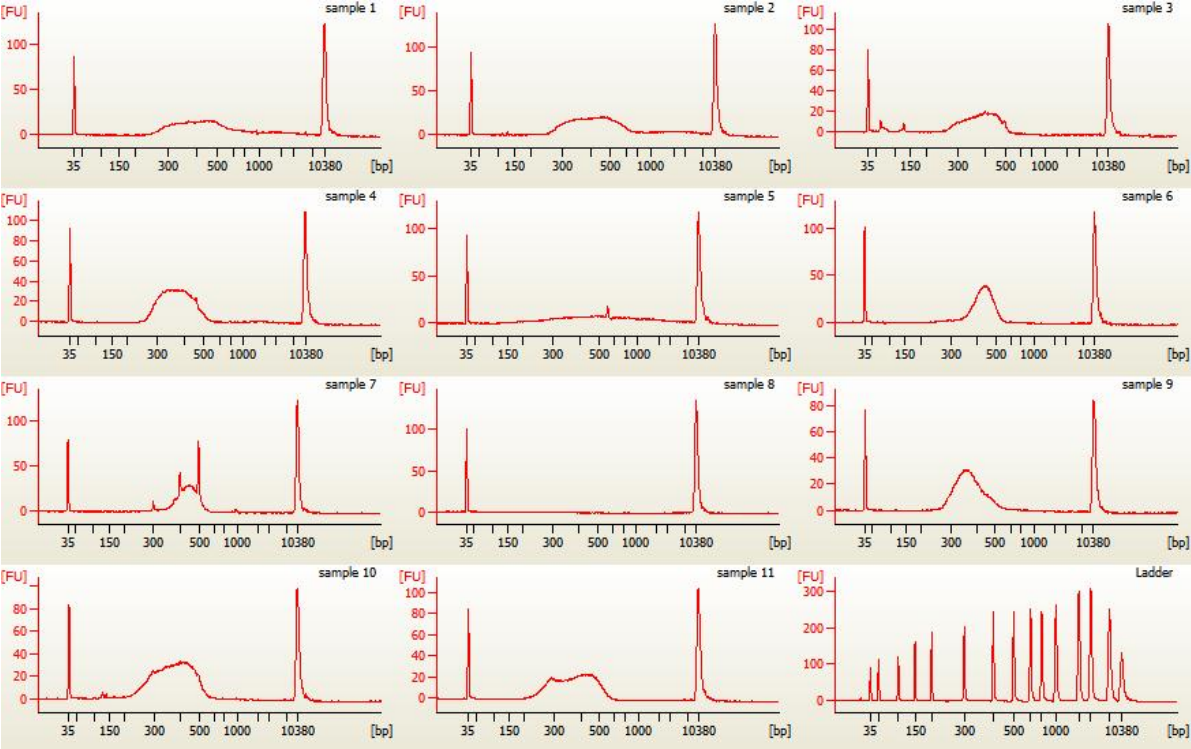
Table S3: Linkage group results from MSTMap analyses. V1-V31 are the 31 backcross progeny from which markers were obtained. Genotypes are in clouored cells to show segregation patterns. 'A' represents homozygotes for the LVP-IB12 reference genome, 'B' is the homozygous for the COSTA RICA male and, 'U' are missing data.

Linkage group 1

Locus_name	position (cM)	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29	V30	V31
supercont1.100	0	U	B	U	A	B	A	B	A	A	B	B	U	A	B	B	B	B	A	B	B	B	B	B	B	U	B	B	B	B	B	B
supercont1.96	0	B	B	U	A	B	A	B	A	A	B	B	U	A	B	B	B	B	A	B	B	B	B	B	B	U	B	B	B	B	B	B
supercont1.160	10.147	B	B	U	A	B	A	B	A	A	A	B	B	A	B	B	B	U	B	B	B	A	B	B	B	B	U	B	U	B	B	B
supercont1.1	12.507	A	B	U	A	B	A	B	A	A	A	B	U	A	B	B	B	B	A	U	B	B	A	B	B	B	B	B	B	B	B	B
supercont1.123	12.507	A	B	U	A	B	A	B	A	A	A	B	B	A	B	B	B	B	A	B	U	B	A	B	B	B	B	B	B	B	B	U
supercont1.174	12.507	A	B	U	A	B	A	B	A	A	A	U	B	A	B	B	B	B	A	B	B	B	A	B	B	B	U	B	B	B	B	B
supercont1.234	12.507	A	B	U	A	B	A	B	A	A	A	B	B	A	B	B	B	B	A	B	B	B	A	B	B	B	U	B	U	B	B	B
supercont1.335	12.507	A	B	U	A	B	A	U	A	A	A	B	U	A	B	B	B	B	A	B	B	B	A	B	B	B	U	B	B	B	B	B
supercont1.449	12.507	A	B	U	A	B	U	B	A	A	A	B	B	A	B	B	B	B	A	B	B	B	A	B	B	B	B	B	B	B	B	B
supercont1.508	12.507	A	B	U	A	B	A	B	A	A	A	B	B	A	B	B	B	B	A	U	B	B	A	B	B	B	U	U	B	B	B	B
supercont1.730	12.507	A	B	U	A	B	A	B	A	A	A	B	U	A	B	B	B	B	A	B	B	B	A	B	B	B	B	U	B	B	B	B
supercont1.96	12.507	A	B	U	A	B	A	B	A	A	A	B	U	A	B	B	B	B	U	B	B	B	A	B	B	B	B	B	B	B	B	U
supercont1.1	18.797	A	B	U	A	B	A	B	A	A	A	U	U	A	B	B	B	B	A	B	B	B	A	B	B	A	B	U	B	B	B	B
supercont1.155	18.797	A	B	U	A	B	A	B	A	A	A	B	U	A	B	B	B	B	A	B	B	B	A	B	B	U	B	B	U	B	B	B
supercont1.174	18.797	A	B	U	A	B	A	B	A	A	A	B	B	A	B	B	B	B	A	B	B	B	A	B	B	U	B	B	B	B	B	U
supercont1.270	18.797	A	B	U	U	B	A	B	A	A	A	B	B	A	B	B	B	B	A	U	B	B	A	B	B	U	B	B	B	B	B	B
supercont1.373	18.797	A	B	U	A	B	A	B	A	A	A	B	B	A	B	B	U	B	A	B	B	B	A	B	B	U	B	A	B	B	B	B
supercont1.373	18.797	A	B	U	A	B	A	B	A	A	A	B	B	A	B	B	B	B	A	B	B	B	A	B	B	U	B	A	B	B	B	B

Table S3 *continued*

Linkage group 2

Locus_name	position (cM)	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29	V30	V31
supercont1.416	0	A	U	B	U	A	B	B	B	U	B	B	A	B	B	B	A	U	B	A	B	B	B	A	B	A	B	A	B	B	A	B
supercont1.35	6.19	A	U	B	U	B	B	B	B	B	B	B	B	B	B	B	A	A	B	U	B	A	B	A	B	U	B	A	B	B	B	B
supercont1.126	10.225	U	U	B	U	A	B	B	B	B	B	B	A	B	B	B	A	A	B	U	B	A	B	A	B	A	B	A	B	B	B	B
supercont1.193	10.225	A	U	B	B	A	B	B	B	B	B	B	U	B	B	B	A	A	B	B	B	A	B	A	B	A	B	A	U	U	B	B
supercont1.193	10.225	A	U	B	B	A	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B	A	B	A	B	A	B	A	U	U	U	B
supercont1.247	10.225	A	U	B	U	A	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B	A	B	A	B	A	B	A	B	B	B	B
supercont1.297	10.225	A	U	B	B	A	B	B	B	B	B	B	U	B	B	B	A	A	B	B	B	A	B	A	B	A	B	A	U	U	B	B
supercont1.302	10.225	A	U	B	B	A	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B	A	B	A	B	U	B	A	B	U	B	B
supercont1.349	10.225	A	U	B	B	A	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B	A	B	A	B	A	B	A	U	B	U	B
supercont1.369	10.225	U	U	B	B	A	B	B	B	B	B	B	A	B	B	B	A	A	B	U	B	A	B	A	B	A	B	A	U	B	B	B
supercont1.375	10.225	A	U	B	B	A	B	B	B	B	B	B	A	B	B	B	A	A	B	U	B	A	B	A	B	U	B	A	B	B	B	B
supercont1.404	10.225	A	U	B	U	A	B	B	B	B	B	B	A	B	B	B	A	A	B	U	B	A	B	A	B	U	B	A	B	B	B	B
supercont1.436	10.225	U	U	B	B	A	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B	A	B	A	B	A	B	A	B	U	B	B
supercont1.477	10.225	B	U	U	B	A	B	B	B	B	B	B	A	B	B	B	A	A	B	U	B	A	B	A	B	A	B	A	U	B	B	B
supercont1.565	10.225	A	U	U	B	A	U	B	B	B	B	B	A	B	B	B	A	A	B	B	B	A	B	A	B	A	B	A	U	B	B	B
supercont1.57	10.225	A	U	B	B	A	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B	A	B	A	B	U	B	A	U	U	B	B
supercont1.593	10.225	U	U	B	U	A	B	B	B	B	B	U	A	B	B	B	A	A	B	B	B	A	B	A	B	A	B	A	B	B	B	B
supercont1.61	10.225	A	U	B	B	A	B	B	B	B	B	B	A	B	B	B	A	A	B	U	B	A	B	A	B	A	B	U	U	B	B	B
supercont1.68	10.225	A	U	B	B	A	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B	A	B	A	B	U	B	A	U	B	B	B
supercont1.773	10.225	A	U	B	U	A	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B	A	B	A	B	A	B	U	B	B	B	B
supercont1.102	17.328	A	U	B	U	A	B	B	B	U	B	B	A	B	B	B	A	B	B	B	B	A	B	A	B	A	B	B	B	B	B	U
supercont1.5	17.328	A	U	B	U	A	B	B	B	B	B	B	A	B	B	B	A	B	B	B	B	A	B	B	B	A	B	A	B	U	B	U
supercont1.424	24.258	U	U	B	U	A	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B	A	A	A	B	A	B	A	B	B	B	U
supercont1.567	24.258	A	U	B	U	A	B	B	B	B	B	B	U	B	B	B	A	A	B	B	B	A	A	B	A	B	A	B	A	B	U	B
supercont1.680	24.258	A	U	B	U	A	B	B	B	B	B	B	A	B	B	B	A	A	B	U	B	A	A	B	A	B	A	B	A	B	U	B
supercont1.1037	31	A	U	B	U	A	B	B	B	B	B	B	A	B	B	B	A	B	B	B	B	A	A	A	A	U	B	U	A	A	B	B
supercont1.737	37.231	A	B	B	U	A	U	B	B	B	B	B	A	B	B	B	A	A	A	B	B	A	A	A	A	A	B	A	B	B	B	B
supercont1.519	54.494	A	B	B	U	A	B	A	B	B	B	B	A	B	B	B	B	A	A	B	A	B	A	A	A	A	B	A	B	B	U	U

supercont1.224	54.494	A	B	B	U	A	B	A	B	B	B	B	A	B	B	B	B	A	A	B	A	B	A	A	A	A	B	A	B	B	A	B
supercont1.143	54.494	A	B	B	U	A	B	A	B	B	B	B	A	U	B	B	B	A	A	B	A	B	A	A	A	A	B	U	B	B	B	B
supercont1.143	54.494	A	B	B	U	A	B	A	B	B	B	B	A	B	B	B	B	A	A	B	A	B	A	A	U	B	A	B	B	B	U	
supercont1.221	54.494	A	B	B	U	A	B	A	B	B	B	B	A	B	B	B	B	A	A	B	A	B	A	A	A	U	A	B	U	A	B	
supercont1.224	54.494	U	B	B	U	A	B	A	B	B	B	B	A	B	B	B	B	A	A	B	A	B	A	A	A	B	U	B	B	A	B	
supercont1.224	54.494	A	B	B	U	A	B	A	B	B	B	B	A	B	B	B	B	A	A	B	A	B	A	A	A	A	B	U	A	B		
supercont1.401	54.494	A	B	B	U	A	B	A	B	B	B	B	A	B	B	B	B	A	A	U	A	A	A	A	A	B	A	U	B	B		
supercont1.401	54.494	A	B	B	U	A	B	A	B	B	B	B	A	B	B	B	B	A	A	B	A	B	A	A	A	A	U	B	B	B		
supercont1.401	54.494	A	B	B	U	A	B	A	B	U	B	B	A	B	B	B	B	A	A	B	A	B	A	A	U	A	B	B	B	B		
supercont1.519	54.494	A	B	B	U	A	B	A	B	B	B	B	A	B	B	B	B	A	A	B	A	B	A	A	A	A	U	B	B	A	B	
supercont1.53	54.494	A	B	B	U	A	B	A	B	B	B	B	A	B	B	U	A	A	B	A	B	A	A	A	U	B	A	B	B	A	B	
supercont1.98	54.494	A	B	B	U	A	B	A	B	B	B	B	A	B	B	B	B	A	A	B	A	B	A	A	A	U	B	A	B	B	U	B
supercont1.98	54.494	A	B	B	U	A	B	A	B	B	B	B	A	B	B	B	B	A	A	B	A	B	A	A	A	U	B	A	B	B	U	B
supercont1.287	64.789	A	B	B	U	A	B	A	B	B	B	U	A	B	B	B	B	A	A	A	B	B	U	A	U	B	A	B	B	A	A	
supercont1.10	69.693	A	B	B	U	A	U	A	B	B	B	A	B	B	A	B	A	A	A	B	B	A	A	A	B	A	U	B	A	A		
supercont1.245	69.693	A	B	B	U	A	B	A	B	U	B	A	B	B	A	B	A	A	A	B	A	A	A	B	A	B	U	A	A			
supercont1.245	69.693	A	B	B	U	A	B	A	B	B	B	B	A	B	B	A	B	A	A	A	A	B	A	A	A	U	A	A				
supercont1.245	69.693	A	B	B	U	A	B	A	B	B	B	B	A	B	A	B	U	A	A	A	B	B	A	A	U	B	B	A	A			
supercont1.163	71.414	A	B	B	U	B	A	A	B	B	B	B	A	B	A	A	B	A	A	A	B	B	A	B	A	B	A	B	B	A	A	

Appendix

Table S3 *continued*

Linkage group 3

Locus_name	location (cM)	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29	V30	V31
supercont1.121	0	B	B	U	A	B	A	B	A	A	A	B	U	A	A	A	B	B	B	A	B	U	A	B	B	B	A	B	A	A	B	U
supercont1.172	3.23	B	B	U	A	B	A	A	A	A	A	B	A	A	A	A	B	B	B	A	B	U	B	B	B	B	A	B	A	A	B	B
supercont1.173	3.23	B	B	U	A	B	A	A	A	A	A	B	A	A	A	A	B	B	B	A	B	U	B	B	B	B	A	B	A	A	B	B
supercont1.172	3.23	B	B	U	A	B	A	A	A	A	A	B	A	A	A	A	B	B	B	A	B	U	B	B	B	U	A	B	A	A	B	B
supercont1.172	3.23	B	B	U	A	B	A	A	A	A	A	B	A	A	A	A	B	B	U	A	B	U	B	B	B	B	A	B	A	A	B	U
supercont1.63	8.863	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	B	U	A	B	U	B	B	B	B	A	B	A	A	B	B
supercont1.63	8.863	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	B	B	A	B	U	B	B	B	B	A	B	A	U	B	U
supercont1.239	14.466	B	B	U	A	U	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	B	B	B	A	B	A	U	A	B
supercont1.496	18.81	B	B	U	A	B	A	A	A	A	A	A	U	A	A	A	B	B	B	B	B	U	B	B	B	B	A	B	A	A	B	B
supercont1.25	18.81	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	B	B	B	A	B	U	A	B	B
supercont1.360	18.81	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	B	B	B	A	B	A	A	B	B
supercont1.496	18.81	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	B	B	B	A	B	A	A	B	B
supercont1.244	18.81	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	B	B	B	A	B	A	A	U	U
supercont1.244	18.81	B	B	U	A	B	A	U	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	B	B	B	A	B	A	A	B	B
supercont1.25	18.81	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	B	B	B	A	B	U	A	B	B
supercont1.292	18.81	B	B	U	A	B	U	A	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	B	B	B	A	B	A	A	B	B
supercont1.371	18.81	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	B	B	B	A	U	A	A	B	B
supercont1.371	18.81	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	B	B	U	A	B	A	A	B	B
supercont1.371	18.81	B	B	U	A	B	A	A	A	A	A	U	A	A	A	U	B	B	B	B	B	U	B	B	B	B	A	B	A	A	B	B
supercont1.496	18.81	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	B	B	B	A	U	A	A	U	B
supercont1.63	18.81	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	U	B	U	B	U	B	B	B	B	A	B	A	A	B	B
supercont1.609	26.344	B	B	U	A	B	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	U	B	A	B	U	A	B	A	U	B	B
supercont1.43	31.56	B	B	U	A	B	A	A	A	A	A	B	A	A	A	A	B	B	B	B	B	U	B	A	B	B	A	B	A	A	B	B
supercont1.114	31.56	B	B	U	A	B	A	A	U	A	A	B	A	A	A	A	B	B	B	B	B	U	B	A	B	U	A	B	A	A	B	B
supercont1.114	31.56	B	B	U	A	B	A	A	A	A	A	B	A	A	A	A	B	B	B	B	B	U	B	A	B	B	A	B	A	U	U	B
supercont1.114	31.56	U	B	U	A	B	A	A	A	A	A	B	A	A	A	A	B	B	B	U	B	U	B	A	B	B	A	B	A	A	B	B
supercont1.114	31.56	B	B	U	A	B	A	A	A	A	A	B	A	A	A	B	B	B	B	B	B	U	B	A	B	U	A	B	A	U	B	B

ID	Value
supercont1.168	31.56
supercont1.168	31.56
supercont1.31	31.56
supercont1.43	31.56
supercont1.1046	44.717
supercont1.113	44.717
supercont1.268	44.717
supercont1.268	44.717
supercont1.1175	48.918
supercont1.255	48.918
supercont1.28	48.918
supercont1.28	48.918
supercont1.412	48.918
supercont1.515	48.918
supercont1.559	48.918
supercont1.588	48.918
supercont1.610	48.918
supercont1.610	48.918
supercont1.655	48.918
supercont1.986	48.918
supercont1.879	51.63
supercont1.95	51.762
supercont1.113	57.917
supercont1.1143	57.917
supercont1.1380	57.917
supercont1.157	57.917
supercont1.172	57.917
supercont1.196	57.917
supercont1.196	57.917
supercont1.198	57.917
supercont1.231	57.917
supercont1.242	57.917
supercont1.251	57.917
supercont1.279	57.917

Appendix

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
supercont1.3	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	A	B	B	B	U	A	U	B	B
supercont1.31	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	A	B	B	B	B	A	U	B	B
supercont1.34	57.917	B	B	U	A	B	U	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	A	B	B	U	B	A	A	B	B
supercont1.34	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	A	B	B	B	U	A	A	B	B
supercont1.34	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	U	U	B	B	B	B	B	A	B	B	B	B	A	A	B	B
supercont1.34	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	A	B	B	B	U	A	A	B	B
supercont1.38	57.917	B	B	B	U	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	A	B	B	U	B	A	A	B	B
supercont1.38	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	A	B	B	B	U	A	B	U	
supercont1.380	57.917	U	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	U	B	B	B	A	B	B	B	B	A	A	B	B
supercont1.44	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	A	B	B	U	B	U	A	B	B
supercont1.456	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	A	B	B	B	U	A	A	B	B
supercont1.507	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	A	B	B	B	U	A	A	B	B
supercont1.507	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	A	B	B	B	U	A	U	B	B
supercont1.507	57.917	U	B	B	A	B	A	A	A	A	A	U	B	A	B	A	B	B	B	B	B	B	B	A	B	B	B	B	A	A	B	B
supercont1.507	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	A	A	B	B	B	B	A	A	B	U
supercont1.89	57.917	B	B	B	A	U	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	B	B	U	A	B	B	B	A	B	B	
supercont1.90	57.917	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	U	U	B	B	B	B	B	A	B	B	B	B	A	A	B	B
supercont1.981	57.917	B	B	B	A	B	A	U	A	A	A	A	B	A	B	A	B	U	B	B	U	B	B	A	B	B	B	B	A	A	B	B
supercont1.317	57.917	B	B	B	A	B	U	A	A	A	A	A	B	A	B	A	B	B	B	B	U	A	B	A	B	A	B	B	A	A	U	B
supercont1.684	59.071	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	A	B	A	B	B	U	U	B	A	B	B
supercont1.34	59.546	B	B	B	A	B	A	A	A	A	B	B	A	B	A	B	B	B	B	B	B	A	B	B	B	U	A	U	B	B		
supercont1.963	59.546	B	B	B	A	B	A	A	A	A	A	A	B	A	B	A	B	B	B	B	B	A	B	B	B	A	A	U	A			
supercont1.748	72.864	B	B	B	A	B	A	B	B	A	A	A	B	B	B	A	U	B	B	B	B	B	B	B	B	A	A	U	B			
supercont1.776	73.188	B	B	B	A	U	A	B	B	A	A	A	B	B	B	A	U	B	B	B	B	B	B	U	B	B	A	A	B	A		

Locus_name	location (cM)	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29	V30	V31
Linkage group4																																
supercont1.15	0	B	B	A	B	B	B	A	B	A	B	A	U	A	B	A	B	B	B	U	B	B	A	A	B	B	B	B	A	A	B	A
supercont1.77	1.613	B	B	A	B	B	B	A	B	A	B	A	U	U	A	B	A	B	B	B	B	B	A	B	B	A	B	B	B	A	B	A
supercont1.1047	9.749	B	B	A	B	A	A	A	B	A	B	A	U	A	B	A	B	B	B	B	B	A	B	A	B	B	B	B	U	U	B	A
supercont1.1054	9.749	B	B	A	B	A	A	A	B	A	B	A	U	A	B	A	B	B	U	B	B	B	A	B	B	A	B	B	A	U	B	A
supercont1.124	9.749	B	B	A	B	A	A	A	B	A	B	A	U	A	B	A	B	B	B	B	B	A	B	B	U	B	B	A	B	A	U	A
Linkage group 5																																
supercont1.84	0	B	B	U	B	B	A	B	B	A	A	B	B	A	B	B	B	A	B	A	B	U	A	B	A	B	A	B	A	U	U	A
supercont1.14	11.488	B	B	U	B	B	A	B	B	A	A	B	B	A	B	B	A	B	A	B	U	A	B	A	U	A	B	A	B	B	A	A
Linkage group 6																																
supercont1.14	0	B	B	U	B	B	A	B	A	A	B	A	A	A	B	A	U	A	B	U	A	B	A	B	A	B	A	B	A	A	B	A
Linkage group 7																																
supercont1.145	0	B	B	B	A	A	A	B	U	B	B	A	U	B	A	B	B	B	B	B	A	U	B	B	B	B	U	B	B	A		
Linkage group 8																																
supercont1.576	0	A	A	U	A	B	A	A	A	A	A	B	B	B	B	B	A	A	B	B	B	A	B	B	U	B	A	B	A	B	B	
supercont1.288	1.615	A	A	U	A	B	U	B	A	A	A	B	B	B	B	A	A	U	B	B	A	B	B	B	B	A	B	A	B	A	B	B
supercont1.300	1.615	A	A	U	A	B	A	B	A	A	A	B	B	B	A	B	A	A	A	B	A	B	B	B	B	A	B	A	U	B	B	
supercont1.296	6.469	U	A	U	A	B	A	B	A	A	A	B	B	B	A	B	A	A	A	B	B	A	B	B	A	B	A	B	A	B	U	
supercont1.487	6.469	A	A	U	A	B	A	B	A	A	A	B	B	B	A	B	A	A	A	B	B	A	B	B	U	B	A	U	B	B	
supercont1.970	8.093	A	A	U	A	U	A	B	A	A	A	B	B	B	A	B	A	A	B	B	B	A	A	B	U	B	A	B	A	B	B	
supercont1.22	9.747	A	B	U	A	B	A	B	A	A	B	B	B	A	B	A	A	B	U	A	A	B	A	B	U	B	A	B	B			

Table S3 *continued*

Locus_name	location (cM)	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29	V30	I3A
Linkage group 9																																
supercont1.702	0	A	B	U	A	A	B	B	B	A	A	B	U	A	B	B	B	B	A	B	B	U	A	B	B	U	B	B	A	B	B	B
Linkage group 10																																
supercont1.842	0	B	B	U	A	B	A	B	B	A	A	B	B	B	A	B	B	B	B	B	B	U	A	U	B	U	A	B	A	B	B	A