Improved semiochemical trapping and population genetics of mosquito species vectoring Rift Valley fever in Kenya

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Declaration

I, David P. Tchouassi hereby declare that this thesis which is submitted for the degree of Doctor of Philosophy (Entomology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed: 

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Disclaimer

This thesis consists of a series of chapters that have been prepared as stand-alone papers already published or manuscripts for different scientific journals. Consequently, unavoidable overlaps and/or repetitions may occur and the reference style and format may differ between chapters.

Thesis summary:

The East African region is a major hot bed for old and newly emerging arboviral diseases that are occurring with increasing frequency and magnitude. The lack of effective treatment or preventive vaccinations for most of these infections emphasizes the need for surveillance to monitor circulation, which is critical for informing public health decision for early warning and response. Monitoring mosquito populations and mosquito-borne virus activity are the cornerstones of surveillance programs. As a model, this project focussed on Rift Valley fever (RVF), a mosquito-borne zoonosis, which remains prevalent in most parts of Kenya. Improving mosquito-based arbovirus surveillance by increasing trap captures was identified as a priority for maximizing viral detection probability especially during the inter-epidemic period (IEP) which is characterized by low vector population density and sporadic transmission foci. Initially, mosquitoes' response to color of artificial lights using light-emitting diodes (LEDs) was exploited for improved sampling of important RVF virus mosquito vectors by comparing efficiency of selected LED CDC light traps (red, green, blue, violet, combination of blue-green-red (BGR)) to sample RVF vectors relative to incandescent light (as control) in a CDC light trap in field trapping experiments in two RVF hotspots (Marigat and Ijara districts) in Kenya. Hotspots are defined as areas with RVF epidemic involving higher than normal occurrence of abortions or perinatal mortality in livestock (cattle, sheep, goats) herds, including disease and deaths in humans. Furthermore, the role of host skin odors in attraction of RVF vectors was investigated; the identification of key compounds involved for formulation into attractants (i.e. baiting system) in conjunction with CO_2 to enhance trap captures as a strategy for improved vector surveillance especially during the IEP was established. These involved a series of bioassay-guided field trapping experiments, electrophysiology and chemical analyses. Additionally, as an adjunct to arbovirus surveillance and epidemiology, the population genetics of key RVF vectors (*Aedes* (*Neomelaniconion*) *mcintoshi* Huang and *Ae.* (*Aedimorphus*) *ochraceus* (Theobald)) sampled from RVF-endemic / epidemic / virus-free areas of Kenya was conducted by analysing sequence variation in mitochondrial cytochrome oxidase subunit I (*COI*) gene and nuclear internal transcribed spacer (*ITS*) genome targets. Both genome targets have been used extensively in studies of molecular evolution and have resolved evolutionary relationships among closely related or cryptic mosquito species complexes. Reference data on public databases such as Genbank are therefore readily available, and the *COI* barcoding region,

is well-represented. Although seasonal preference was observed for some species (*Ae. mcintoshi* and *Ae. ochraceus*) to certain coloured lights; generally, higher captures for all species examined were recorded in control traps (incandescent) compared to the other LED traps although this was only significantly different from red and violet. Initial field trapping assays showed that the addition of fur (skin volatile) from sheep, the most susceptible host for RVF virus, to the standard CO_2-baited light trap improves captures of key RVF vectors. As an understanding of interspecific host preferences can reveal new semiochemicals that could be exploited to maximise development of better attractants, the attractiveness of different RVF virus hosts (cow, donkey, goat, human) in addition to sheep to RVF vectors was assessed further in field experiments. An analogous pattern was observed with an increase in mosquito captures recorded following the addition of skin odours from each of these animals to CO_2 traps relative to control traps containing CO_2 alone. Interestingly, a higher proportion of engorged mosquitoes (bloodfed + gravid) were recorded in CO_2 traps containing skin odours from these animal hosts relative to control CO_2 trap alone. Electrophysiology studies to find out which compounds RVFV vectors responded to, revealed a similarity in response profile to the aldehyde components; heptanal, octanal, nonanal and decanal, that were common to all the hosts evaluated. Following field-testing, it was shown that each of these compounds could be exploited as attractants singly and/or blends in a dose-dependent manner. A blend formulated from the optimal attractive dose of each of these compounds synergized with CO_2 significantly increased trap captures over that of control traps baited with CO_2 alone. The four-component blend attracted multiple mosquito vectors under field conditions suggesting that a trapping system based on this formulation offers the opportunity for its use as a tool for RVF mosquito vector surveillance. There was evidence of divergent lineages for *Ae. mcintoshi* that display geographic restriction coinciding with the magnitude of occurrence of RVF in Kenya; both gene loci indicated the presence of four genetic lineages with significant differentiation among them across the study areas as evident from phylogenetic, median-joining network analyses and from analysis of molecular variance (AMOVA). In contrast, a single, relatively homogenous population was evident for *Ae. Ochraceus.* Low mean evolutionary divergence estimates among and within sites and a single lineage was evident in the Neighbor-joining analysis, and in network and TCS parsimony analyses. Interestingly, significant negative neutrality tests of Tajima's *D* and Fu's *Fs* were evident for the *COI* locus only, and supported by a unimodal curve for the mismatch distribution

of frequencies of pairwise differences, indicative of a rapid population expansion for this species. Overall, this study represents the most detailed investigation into the chemical basis of mosquito-host attraction (leading to the development of an improved trapping tool) together with the genetics of the key vectors in explaining RVF transmission and spread in Kenya. This certainly provides an in-depth understanding of the vectoring capability and the means by which the development of improved effective sampling methods can maximize detection probability of the mosquito host which is seminal to effective monitoring of RVF and other arboviral diseases in Kenya and elsewhere.

Key words: Mosquito surveillance, Rift Valley fever; Rift Valley fever vectors, light-emitting diodes, Rift Valley fever hosts; vector sampling; animal odors; CDC light trap; semiochemical attractants; *Aedes mcintoshi*; *Aedes ochraceus*; genetic diversity; cytochrome oxidase subunit I ; internal transcribed spacer; Kenya.

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Chapter 1

General Introduction, Rationale and Key Questions

Arthropod-borne virus (arbovirus) diseases (ABVDs) are primarily zoonotic and transmission typically alternates between blood feeding arthropods (mosquitoes, psychodids, ceratopogonids, and ticks) and, with the exception of dengue and yellow fever, a variety of host species (Kuno & Chang 2005, Weaver, 2005). Human infections are incidental and often dead-end in nature (Taylor *et al.*, 2001) and occur when the zoonotic cycle is interrupted by an infected arthropod taking a human blood meal (Sang & Dunster, 2001).

Arboviral diseases cause a range of clinical syndromes in humans, which depending on the infecting virus, range from a self-limiting, febrile illness to life-threatening encephalitis or hemorrhagic fever (Sang & Dunster, 2001). On a worldwide basis, they take an enormous toll on human health causing mortality, morbidity and loss of productivity, and on food supply due to their direct or indirect effects on humans and domestic animals. Most of these diseases often only receive recognition when they are acute and widespread, making the public lose sight of ongoing transmission, which may have a significant daily impact on the life of people living in endemic countries (Labeaud, 2008). In addition to human and animal illness and potential loss of lives, the economic losses due to zoonotic disease outbreaks can be staggering. Economic consequences can include trade sanctions, travel warnings or restrictions, animal disease control efforts such as animal culling (intentional slaughter), and declining public confidence in animals products (Labeaud *et al.*, 2011a). For example, once Rift Valley fever virus (RVFV), a phlebovirus (Family Bunyaviridae), is known to be circulating in an animal herd, the World Organisation for Animal Health (OIE) places a three-year export embargo on those animals (Labeaud *et al.*, 2011a). Therefore, the political, psychological, and economic implications of reporting arbovirus outbreaks may also contribute to intentional underreporting of these diseases (Labeaud *et al.*, 2011).

Unfortunately, the medical, veterinary and economic impacts of many ABVDs have continued unabated and have in fact increased (Beaty, 2005). This is exemplified in the unexpected but successful establishment of Chikungunya fever in northern Italy (Gould & Higgs, 2009), the

sudden appearance and repeated outbreaks of West Nile virus in North America (Jia et al. 1999; Petersen & Hayes, 2004; Beaty, 2005; Kilpatrick *et al*. 2006), the increasing frequency of Rift Valley fever (RVF) epidemics in various countries in Africa and the Arabian Peninsula (WHO, 2000; Balkhy *et al*., 2003; Gerdes, 2004; WHO, 2008; Sissoko *et al*., 2009; WHO, 2010; Archer *et al.*, 2011), and the emergence of Bluetongue virus in northern Europe (Carpenter *et al*., 2009). Also, epidemics of dengue and dengue haemorrhagic fever-dengue shock syndrome (DHF-DSS) have emerged as a major public health problem in the tropics over the past 20 years (Beaty, 2005). Factors such as climate, demographic and land-use changes and increasing global travel are likely contributors to the upsurge in disease dynamics, presenting an increasing global threat to human and livestock health (Gould & Higgs, 2009; Weaver & Reisen, 2010).

In Kenya and East Africa, the importance of arboviral infections is illustrated by the increasing frequency and magnitude of old and newly emerging arboviral diseases including RVF, Crimean-Congo hemorrhagic fever, West Nile, yellow fever, dengue, Chikungunya and O'nyong nyong viruses (Sang & Dunster, 2001). Most of these diseases are understudied and go unnoticed until outbreaks occur and as such constitute a growing economic burden and significant health threat. There is also the likelihood of the presence and circulation of novel agents, which can only be detected and addressed through intensified research.

Retrospective studies of such epidemics frequently suggest, by association, climate/weather-related factors which could have been responsible for triggering resurgence/emergence, and these associations offer fertile ground for speculative explanations (Reiter, 1988). However, certain outbreaks have been reported in the absence of climatic events (Chevalier *et al*., 2004). Moreover, the timing of epidemics remains enigmatic and notoriously unpredictable and frequently, health or veterinary authorities are unaware of the existence of an epidemic until many weeks after its commencement, and may be unable to implement countermeasures until after the majority of infections have occurred.

As the reasons for the emergence and re-emergence of these arboviral diseases are multifactorial and only partly understood, a comprehensive, in-depth monitoring of the disease process is required, and should not be initiated once transmission has already accelerated, but should be in place and running during the inter-epidemic phase.

Most arbovirus infections have no treatment or preventive vaccinations. Therefore, surveillance to monitor circulation of such agents is critical in informing public health decision for early warning and response. Arbovirus disease surveillance aims to assess existing epidemiological situations for a disease so as to be able to predict the likelihood of human disease outbreaks, and thus permit intervention to obviate such outbreaks (Eldridge, 1987). This objective is based on the concept that various phenomena such as high mosquito population densities precede human disease cases, and that these phenomena can be used as indicators to predict disease outbreaks. As such, the use of these indicators for the purpose of surveillance relies on an understanding of the basic biological components of the disease cycle: pathogen, vectors, and hosts (Eldridge, 1987; Day, 2001).

A thorough understanding of these biological and environmental components associated with any arboviral transmission cycle allows the tracking of these components and the formulation of a prediction about where and when arboviral amplification and transmission might occur (Day & Shaman, 2011). Environmental factors for monitoring and risk mapping systems require the use of a variety of satellite measurements including sea surface temperatures, outgoing long wave radiation, soil data, rainfall, and landscape ecology using the normalized difference vegetation index (NDVI) to identify sites with higher than average abundance of vector mosquitoes, presumably with increased risk of viral activity (Anyamba *et al*., 2002, Brown *et al*., 2008). Biological components include sampling of mosquitoes to estimate population levels, testing of mosquito pools to estimate infection rates, periodic bleeding of sentinel animal flocks and wild life (in the case of RVFV) to detect seroconversions, and monitoring of human cases of disease.

Complementing and integrating both climatic and biological components remains imperative for surveillance programs in order to produce accurate forecasts of human disease activity as the various components are subject to different sources of variation and therefore differ in their sensitivity as predictors, and in the amount of time they provide in advance of human infections (Eldridge, 1987; Moore, 2008; Tabachnick, 2009; Reisen, 2010). Therefore, the integration of comprehensively generated field biological data with environmental data is required to provide potential improvements in the modelling and forecasting framework of an arboviral disease, which in turn is critical for providing a time window for instituting preventive measures in an impending outbreak (WHO, 2009). Because the three biological cycles, pathogen, vector and

host, inform the transmission dynamics of a specific arboviral disease, surveillance protocols have been developed to monitor each of these cycles in order to estimate the local risk of arboviral transmission for a specific region (Day & Shaman, 2011).

Mosquito-based surveillance is one of the cornerstones of arbovirus surveillance which entails systematic collection of mosquito samples and testing pools for arboviruses in order to assess the status of transmission and allow informed decision-making (Gu *et al*., 2008). Mosquito sampling for arbovirus surveillance is done on the assumption that there is a positive correlation between vector density and incidence of human disease (Blackmore *et al*., 1962; Reeves, 1965; van den Hurk, 2012). Entomologic arbovirus surveillance is advantageous because it (i) provides the earliest evidence of transmission in an area, (ii) identifies the potential risk to humans, and (iii) allows emergency control operations to be set in motion in advance of epidemics. Vectors, once infected, remain infected with the virus for the duration of their life. This makes vector surveillance the best option to target for arbovirus activity especially as arbovirus epidemics in susceptible animals, initiated by bites of infected mosquitoes, are also involved in sustaining the diseases.

This project focussed on RVFV vectors as part of an effort to improve prediction of arboviral diseases in Kenya and East Africa with RVF, a mosquito-borne anthropozoonosis affecting livestock and also human, being used as a model. The disease in animals often leads to high mortality among young ruminants and abortions of pregnant females, while human infections are associated with a wide array of syndromes ranging from influenza-like illness to severe symptoms including hemorrhages, encephalitis, hepatitis, ocular complications and fatal outcomes (Laughlin *et al*., 1979).

Because only female mosquitoes feed on humans and other animals and are thus responsible for disease transmission, they have been almost exclusively the target of most monitoring systems (Qiu *et al*., 2007). During the long inter-epidemic periods the virus is maintained silently within the cryptic cycle among vector populations and only sporadic, small and local epidemics may occur (EFSA, 2005, Labeaud *et al*., 2007, Labeaud *et al*., 2011b). Until now, RVF vectors have been monitored using CO_2-baited CDC light traps, which are generally non-specific and trap a wide range of non-target insect species such as beetles and moths, in addition to mosquitoes. Additionally, because of low sensitivity, this trapping system is inadequate for use during the

low intensity inter-epidemic period (IEP) of enzootic virus transmission where viral activity may remain undetected among mosquito species (EFSA, 2005; Labeaud *et al*., 2007, Labeaud *et al*., 2011b). Thus, there is a critical need to develop more sensitive and effective monitoring tools to increase trap captures of mosquito vectors so that maximized detection of virus activity can be achieved.

Kairomones (attractants) originating from the skin or exhaled breath of hosts are the major cues that guide host seeking mosquitoes to find their blood meals (Takken and Knols, 1999). The utilization of host odours as attractants for trapping mosquitoes has received increased interest in the recent years, for population management and surveillance (Kline, 1994). This provides a more active and selective way to attract biting flies, in most cases females, to all kinds of baits including animals and humans (Sharp *et al.*, 1984; Andrade *et al*., 2008), as well as elements of them like clothes, hair/fur/feather, urine, faeces (Kline 1998; Allan *et al*., 2006). Exploiting these host odours as attractants can be used as a strategy for improved vector surveillance through development of an effective lure/bait in a trapping system (Qiu *et al*., 2007; Pickett *et al*., 2010) to enhance trap catches of mosquitoes and map or provide a realistic assessment of the abundance of mosquitoes present in an area (Kline, 2006, Pickett *et al*., 2010).

Besides host odor stimuli, visual and physical stimuli such as variations in skin temperature and moisture are important for mosquitoes to locate their hosts (Day, 2005, Costantini, 1996). Visual cues provided by light, play a significant role in host location and vary considerably among different biting flies depending on their activity patterns (Allan *et al*., 1987). Mosquito eyesight is poor, but sensitivity to light is high (Muir *et al*., 1992b). Such enhanced light sensitivity allows mosquitoes to follow host-odour plumes even at low light intensities. Previous studies have found that mosquitoes are attracted preferentially to specific wavelengths of light (Wilton and Fay, 1972; Burkett & Butler, 2005). A new visual target called a Light Emitting Diode (LED)-CDC trap makes use of specific wavelengths of light to attract insects and can be customised to maximise target species capture. Its effectiveness in increasing captures of medically important Dipterans including mosquitoes has been reported (Cohnstaedt *et al*., 2008).

Arboviral transmission by mosquitoes is controlled by both genetic and environmental factors and varies greatly, both temporally and geographically, between different species of the same genus or even populations of the same species (Hardy & Reeves 1990, Reisen *et al*., 1996; Black

et al., 2002). This therefore necessitates accurate and rapid identification and discrimination of vector mosquito species which are essential components of disease surveillance and epidemiological research studies. For example, even within different climate zones, RVFV transmission may vary considerably as a function of fine-scale differences in local environments (Labeaud *et al*., 2007; Labeaud *et al*., 2011c). The extent to which the observed temporal and spatial differences in RVF transmission are rooted in genetic variation and/or environmental influences is currently unknown. It is possible that part of the genetic differences among populations of vectors may account for differences in viral transmissibility. Therefore, knowledge of how the genetics of vector populations impacts on the spread of arboviral diseases such as RVF is critical for understanding dynamics of disease incidence and for development of risk assessment strategies.

In Kenya, *Aedes mcintoshi* and *Ae. ochraceus* has been implicated as primary vectors of the disease following isolation of the virus from field-collected samples (Davies & Highton, 1980; Linthicum *et al*., 1985; Sang *et al*., 2010). *Aedes mcintoshi* is the most widespread species belonging to the savanna group of the *Aedes* subgenus *Neomelaniconion* comprising of at least seven species including undescribed ones (Zavortink, 1989). Some of the members in this group include *Aedes albicosta* (Edwards), *Ae. aurovenatus* Worth, *Ae. bolensis*, *Ae. circumluteolus*, *Ae. luridus* McIntosh, *Ae. luteolateralis* (Theobald), *Ae. mcintoshi* Huang, *Ae. unidentatus*, and undescribed ones (Zarvotink, 1989). More importantly, the taxonomic status of this group which contains non-vector and vector species involved in the inter-epizootic maintenance and transmission of RVFV (McIntosh *et al*., 1980; Zarvotink, 1989) remains unresolved as the number of constituent taxa in this group and their relationships is unclear. These taxonomic uncertainties are underscored by the earlier misidentification of this species, as *Ae. lineatopennis* (Huang, 1985).

Separation of member species in this group relies solely on male genitalia or morphological analysis of females (Zarvotink, 1989; Jupp, 1996). Males are seldom encountered in the commonly used CDC light traps used for adult mosquito sampling and with respect to females; slight damage to the specimens makes species identification difficult. Therefore, separation of member species in this subgenus has been based on doubtful morphological features (Kengne *et al*., 2009; Sang *et al*., 2010) and the true geographical distribution, relative abundances,

occurrence and ecological characteristics of different member species in this subgenus is severely lacking in the different parts of Kenya. More importantly, it remains unclear if populations, or subpopulations, of a single species of what is currently described as *Ae. mcintoshi* sustain virus maintenance in Kenya or if occurrence of sympatric populations of two or more other vector species in the group are involved.

The biological and phylogenetic species concepts (BSC and PSC) provide the two most widely accepted definitions for a species. The BSC defines species as groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups (Mayr, 1942). In the phylogenetic context, a species is the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent (Cracraft, 1983). The BSC explains why members of a species resemble one another, i.e. form phenetic clusters, and differ from other species. PSC is based on patterns of character distributions and is therefore consistent with the full range of possible evolutionary processes that contribute to species formation (Wheeler, 1999).

Relevance of this study

The worldwide increase in arbovirus activity is unparalleled in East Africa where Yellow fever, Dengue, Rift Valley fever (RVF), Onyong-nyong, Crimean Congo hemorrhagic fever and Chikungunya viruses have caused disease epidemics in humans in the recent past inflicting an unacceptable health and economic burden on humans and animals, including livestock. Most of these diseases are understudied and go unnoticed until outbreaks occur. Whilst most of these arboviral diseases such as RVF are associated with periodic outbreaks in various parts of East Africa, it is still unknown how the virus is sustained between epidemics in hotspot areas and why transmission varies between regions. Vaccines are not available for most of these disease agents. Epidemiologically important changes in patterns of arbovirus transmission, the environment, and human demographics indicate that without well-designed programs for surveillance, prevention and control, the negative effects of arboviral disease threats in Kenya, and the world at large, will continue to grow.

The circulation of these pathogenic agents within a competent population of disease vectors often goes unnoticed until a disease outbreak occurs (Qiu *et al*., 2007). Risk of such outbreaks

could be better predicted and anticipated through regular or continuous intensive monitoring of potential vector populations. Arboviral diseases especially RVF remain prevalent in most parts of Kenya where during the long inter-epidemic periods, the virus is maintained silently within the cryptic cycle and only sporadic, small and local epidemics may occur (EFSA, 2005; Labeaud *et al*., 2007, Labeaud *et al*., 2011c). However, these infections remain difficult to detect unless very sensitive and improved surveillance tools are employed (EFSA, 2005). Therefore, improving mosquito-based arbovirus surveillance by increasing trap captures remains a priority to maximize viral detection probability especially during the inter-epidemic period (IEP) which is characterized by low vector population density, low infection and sporadic transmission foci.

The rationale of this study is thus to exploit the visual cues provided by LED which can produce light of a specific wavelength or colour and also host skin odours as attractants which female adult mosquitoes use in host location as strategies, to increase captures of important RVFV vectors. Additionally, as the capacity of vectors to transmit arboviral pathogens differs greatly between different species of the same genus or even populations of the same species, this study sought to compare the genetic differences among populations of key RVFV vectors (*Aedes mcintoshi* and *Ae. ochraceus*) from virus-endemic, -epidemic and virus-free areas in Kenya. The ultimate goal of this project is thus to develop an efficient trapping tool to enhance trap captures of mosquito vectors of RVFV in addition to investigating the potential role of genetic structure of key vectors in explaining variation in RVFV transmission in Kenya.

Key research questions

The key research questions addressed in the present study include:

Chapter 2 **- Trapping of Rift Valley Fever (RVF) vectors using Light Emitting Diode (LED) CDC traps in two arboviral disease hot spots in Kenya.**

Key research question:

> Q1: Are RVF vectors differentially attracted to light emitting diodes (LED) of different colours or wavelengths and how does the effectiveness in terms of captures compare with the standard incandescent light currently used in the standard CDC light traps?

Chapter 3 **– Improving trap captures of mosquito vectors of Rift Valley Fever using sheep skin odor.**

Key research questions:

Q1: Can skin odor from sheep, a preferred host for RVFV, be exploited to increase captures of RVFV vectors?

***Chapter 4* – Increasing captures of mosquito vectors of Rift Valley fever using common chemical signatures of hosts and the implications for an early warning system.**

Key research question:

Q1: How do skin odors from widely known mammalian hosts (cow, donkey, goat, sheep and human) compare in terms of attractiveness to RVFV vectors?

Q2: What compounds present in the skin host odors account for observed field attraction of RVFV vectors?

Q3: How can synthetic compounds of the identified components be formulated for increased trap captures in the field?

***Chapter 5 – Aedes mcintoshi* genetic diversity and magnitude of Rift Valley fever in Kenya.**

Key research questions:

Q1: What is the diversity and distribution of *Ae. mcintoshi* in Kenya?

Q2: To what extent does vector population genetic structure explain the pattern and incidence of RVFV outbreaks in Kenya?

***Chapter 6* – Genetic diversity of *Aedes ochraceus*, an increasingly important vector of Rift Valley fever virus in Kenya.**

Key research question:

Q1: Is there any correlation between the genetic diversity of *Ae. ochraceus* and increasing spread of RVFV in northeastern Kenya?

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Chapter 2

Trapping of Rift Valley Fever (RVF) vectors using Light Emitting Diode (LED) CDC traps in two arboviral disease hot spots in Kenya

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Abstract

Background: Mosquitoes' response to artificial lights including color has been exploited in trap designs for improved sampling of mosquito vectors. Earlier studies suggest that mosquitoes are attracted to specific wavelengths of light and thus the need to refine techniques to increase mosquito captures following the development of super-bright light-emitting diodes (LEDs) which emit narrow wavelengths of light or very specific colors. Therefore, the study investigated if LEDs can be effective substitutes for incandescent lamps used in CDC light traps for mosquito surveillance, and if so, determine the best color for attraction of important Rift Valley fever (RFV) vectors.

Methods: The efficiency of selected colored LED CDC light traps (red, green, blue, violet, combination of blue-green-red (BGR)) to sample RVF vectors was evaluated relative to incandescent light (as control) in a CDC light trap in two RVF hotspots (Marigat and Ijara districts) in Kenya. In field experiments, traps were baited with dry ice and captures evaluated for *Aedes tricholabis, Ae. mcintoshi*, *Ae. ochraceus, Mansonia uniformis, Mn. africana* and *Culex pipiens*, following Latin square design with days as replicates. Daily mosquito counts per treatment were analyzed using a generalized linear model with Negative Binomial error structure and log link using R. The incidence rate ratios (IRR) that mosquito species chose other treatments instead of the control, were estimated.

Results: Seasonal preference of *Ae.mcintoshi* and *Ae. ochraceus* at Ijara was evident with a bias towards BGR and blue traps respectively in one trapping period but this pattern waned during another period at same site with significantly low numbers recorded in all colored traps except blue relative to the control. Overall results showed that higher captures of all species were recorded in control traps compared to the other LED traps (IRR<1) although only significantly different from red and violet.

Conclusion: Based on our trapping design and color, none of the LEDs outcompeted the standard incandescent light. The data however provides preliminary evidence that a preference might exist for some of these mosquito species based on observed differential attraction to these light colors requiring future studies to compare reflected versus transmitted light and the incorporation of colored light of varying intensities.

Key words: Mosquito surveillance, Rift Valley fever vectors, Light-emitting diodes, Light trap, Kenya.

Background

Mosquitoes are responsible for the transmission of several arboviral pathogens such as Rift Valley fever virus (RVFv), which is associated with periodic outbreaks in domestic animals and humans in Africa and the Arabian Peninsula [1, 2]. Early detection of the vectors and this pathogen is essential to reduce disease risk to humans and animals. Currently, the detection and monitoring of mosquitoes, is performed primarily using Centers for Disease Control and Prevention (CDC) light traps with incandescent bulbs, which are considered the industry standard for mosquito surveillance. However, improving mosquito-based arbovirus surveillance by increasing trap captures remains a priority to maximize viral detection probability especially during the inter-epidemic period (IEP) characterized by low vector population density and sporadic transmission foci.

The impact of the visual cues provided by the incandescent light used in the CDC light trap is important to trapping effectiveness. Earlier studies suggest that insects generally see and show preferences for three specific colors—ultraviolet (UV), blue, and green [3, 4]. As such the incandescent light bulb currently used in mosquito surveillance may have the unintended effect of repelling some mosquito species, and may poorly target them [5] as it emits most strongly in the infrared spectra and weakly in the visible light spectra of blue, green, and red.

Improved trapping of mosquitoes has been achieved by determining mosquito responses to the color and intensity of light sources [4, 6]. Previous studies have found that mosquitoes are attracted preferentially to specific wavelengths of light [7, 8]. With advances in lighting technology, the super-bright light-emitting diodes (LEDs) have recently been developed which can be selected to emit a narrow bandwidth or specific color [9]. This configuration has been shown to work particularly well in enhancing trap catches of disease vectors and thus the need to refine techniques to increase mosquito captures by using more precise light sources.

Similar studies on preferential attraction to specific wavelengths of light have been reported in phlebotomine sand flies [10-12] and *Culicoides* flies [13]. In addition, observed distinct color and pattern preferences employed in trapping technology has been reported in tabanids [14, 15], *Stomoxys* spp [16, 17] and tsetse flies [17-19].

So far there has been no published work pertaining to the evaluation of colored LEDs for improved captures of field populations of mosquitoes in Disease Endemic Countries (DECs) in Africa. In an effort to develop a highly effective visual target for improved surveillance of different arboviral disease vectors, our goal was to determine whether LEDs can serve as effective substitutes for incandescent lamps used in the standard CDC mosquito traps for mosquito surveillance, and if so, to determine the best color for these arboviral disease vectors.

Materials and methods

Study sites

The study sites were Ijara and Marigat districts, which are ecologically distinct and are hot spots for RVF activities in Kenya. In Ijara district located in North Eastern Province of the country, trapping experiments were conducted in two major communities: Sangailu and Kotile. The entire area is semi-arid and normally has two rainy seasons a year: the short rains between October and December and the long rains in March and April. The area is located at an altitude of about 100m above-sea-level (asl) and typical annual rainfall averages between 300 to 500 mm. The people in North Eastern Province are ethnically nearly all Somali pastoralists. Vegetation predominantly consists of shrubs and acacia bushes, while livestock includes cattle, goats, sheep, camels, and donkeys. Livelihoods are primarily dependent on livestock.

In Marigat District located in the Rift Valley Province of the country, trapping experiments were conducted in surrounding villages/communities namely N'gambo, Salabani, Bogoria and Sirata (Figure 1). The vegetation in the low lying arid part of the district consists of northern Acacia-Commiphora bushlands and thickets and has experienced severe land degradation caused by uncontrolled grazing. The local inhabitants mainly agro pastoralists, subsist mainly on limited crop production and livestock rearing. This area located around 3200m asl receives annual rainfall ranging from 300 to 700 mm, with daily temperature variation between 16 and 42°C.

Study design

Trials in Ijara area were run in December 2010 and May-June 2011 which coincided with peak and mild rainy season respectively to take advantage of peak mosquito populations. Experiments in Marigat area were conducted between July and September 2011 when there are rains to ascertain availability of mosquitoes. Mosquito captures in the BioQuip® LED CDC trap with

different color platforms (part number, wavelength) of blue (2770B430, 430 nm), green (2770G570, 570 nm), red (2770R660, 660 nm), violet (2770UV, 390nm) BGR –were compared against a 1.5 watt incandescent light (control) in a standard CDC light trap (John Hock). Each LED assayed had 8 LEDs of the same color (arranged in a circular alignment) to provide 360-degree coverage in the horizontal plane with each LED having a viewing angle of 45 degrees.

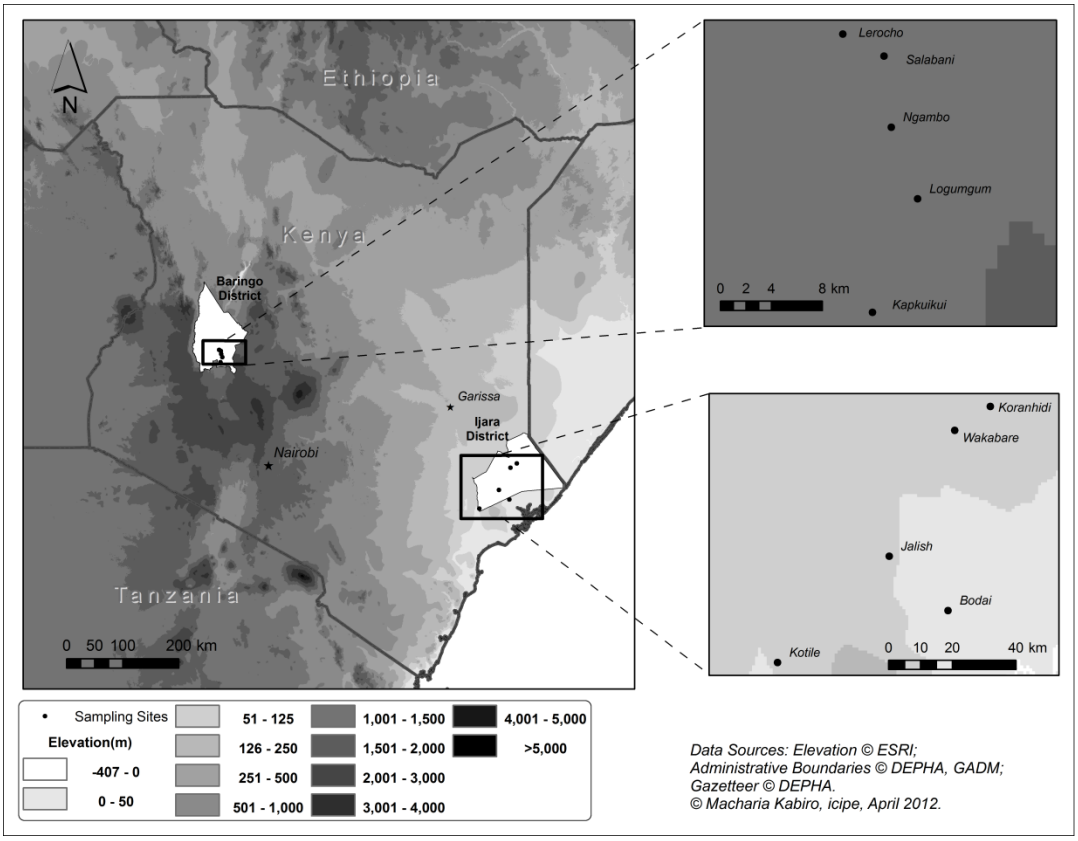

Figure 1 Map showing trapping sites in Kenya.

One array consisted of the combinations of several of different-colored LEDS and contained three green, 3 blue and two red LEDS (BGR). Super bright LED arrays typically produce 1-2 watts although non-superbright ultraviolet array produce about 800 mmW. The incandescent bulb has a typical spectrum of an incandescent bulb with 95% of the energy emitted as heat. With an inter-trap distance of at least 40 m, all traps set following a Latin square design with days as replicates were activated 30 min before sunset and collected between 6:30-7:00, shortly after sunrise. All traps were baited with CO_2 supplied in the form of dry ice to maximize collections.

Data Analyses

Daily count of each mosquito species recorded in the various trap treatments were analyzed using a generalized linear model with Negative Binomial error structure and log link using R 2.11.0 software [20]. Using the treatment incandescent light (control) as the reference category, the incidence rate ratios (IRR) that mosquito species chose other LED treatments colors instead of the control were estimated. The IRR for the control is 1 (unity) and values above this indicates better performance and values below under performance of the treatments relative to the control. Observed differences in the abundance and composition of mosquito species during the different trapping periods and districts/sites were analyzed independently of trapping period and districts/sites. Analyses were limited only to mosquito species that occurred in significant numbers to allow for discrimination across the different trap treatments. In Ijara, analysis was limited to flood water mosquitoes which are primary vectors of Rift Valley Fever (RVF) namely *Ae. mcintoshi, Ae. tricholabis, Ae. ochraceus* and/or *Cx. pipiens* s.l. (secondary vector) which were abundant at these sites but completely absent or occurred in extremely low numbers at Marigat except for *Cx. pipiens* sl. Data was analyzed in Marigat for *Mn. uniformis, Mn. africana* and *Cx. pipiens* s.l., all secondary RVF vectors.

Results

Aedes tricholabis Higher captures of this species were recorded in control traps compared to other treatments (Table 1). Overall, order of performance was control> BGR> violet then followed by the other colors with red performing least. There was a highly significant effect of treatments on this species at Ijara both during the experimental period of December 2010 (χ^2 = 154.913, d.f. = 5, p = 0.003) and May-June 2011 (χ^2 = 74.893, d.f. = 5, p = 0.0000). When compared to the control trap during December 2010, significantly fewer captures for this species were recorded in blue, green and red colors. Equally, higher captures were recorded in the control relative to BGR and violet colors, although the differences were not significantly different (Table 2). However, analysis of the results during the low density period of May 2011 revealed significantly lower numbers of this species were recorded in all the colored traps compared to the control incandescent light (Table 3).

Aedes mcintoshi A similar significant effect of treatments on this species capture during December 2010 (χ^2 = 174.128, d.f. = 5, p = 0.0492) was observed but only after taking into account the effect of replicates and site (Kotile and Sangailu) but with a marked effect of the treatments on captures during May-June 2011 (χ^2 = 76.765, d.f. = 5, p = 0000). In December 2010, apart of BGR treatment which recorded a 44% increase in captures compared to the control [IRR=1.44, CI (0.60-3.44)] all the other treatments recorded lower captures relative to the control (IRR<1) which were however only significantly different from those recorded in violet (Table 2). Surprisingly, this pattern dwindled during the low period of mosquito population density in May 2011 where significantly lower capture numbers were recorded in all the colored light treatments when compared to the control (IRR<1) except blue (Table 2).

Aedes ochraceus An analogous effect of treatment on the captures of this species was evident during both trapping periods of December 2010 (χ^2 = 415.93, d.f. = 5, p = 0.0002) and May-June 2011 (χ^2 = 90.398, d.f. = 5, p = 000000). In December 2010, a slight preference for blue-green colors was apparent with increases in captures of 24 and 7% recorded in blue [IRR=1.24, CI (0.29-5.32)] and green IRR=1.07, CI (0.25-4.60)] colored traps respectively compared to the control which were not significantly different. During this period, fewer *Ae. ochraceus* captures were recorded in the remaining colored traps relative to the control (IRR<1) which was only significantly different from violet (Table 2). The trend dwindled during the trial in May 2011 where significantly fewer were observed in all the colored traps compared to the control incandescent light (Table 3).

Culex pipiens sl Treatment significant effect were observed both during May-June trial at Ijara 2011 (χ^2 = 75.284, d.f. = 5, p = 0.00001) and Marigat (χ^2=118.10, d.f. = 5, p = 0.02064) on the captures of this species. Significantly fewer were captured in all the colored traps compared to incandescent light (IRR<1) except for BGR light in Ijara 2011 and BGR and blue at Marigat (Tables 3 and 4).

Mansonia uniformis There was no overall significant effect of treatments on the species trap captures across treatment replicates (χ^2 = 116.05, d.f. = 5, p = 0.1896). Although fewer were captured in light traps compared to the control incandescent light, it was only significantly so for violet and red light but not for BGR, blue and green (Table 4).

Mansonia africana and (χ^2 = 76.765, d.f. = 5, p = 0.0000) Analogous response patterns were observed for *Ma. africana* with no overall significant effect of treatments on the species trap captures across treatment replicates (χ^2 = 118.70, d.f. = 5, p = 0.08368). However, captures were all significantly less in all colored lights relative to incandescent except blue (Table 4).

Table 1 Composition of RVF mosquito species collected in traps with different lights at three time intervals and two locations

Experimental period	N	Species	Treatment Control (incandescent light)	BGR	Blue	Green	Red	Violet
Ijara December 2010	14	*Ae. tricholabis*	3464	2245	1141	1338	895	1395
		Ae.mcintoshi	198	255	137	119	184	121
		Ae.ochraceus	734	732	913	789	582	192
IjaraMay-June 2011	11	*Ae. tricholabis*	2755	969	604	314	267	781
		Ae.mcintoshi	196	84	108	64	38	43
		Ae.ochraceus	162	54	61	23	31	40
		Cx. pipiens	244	86	81	26	26	78
Marigat July-September 2011	17	*Ma. uniformis*	1195	941	853	747	580	566
		Ma. africana	1134	504	538	531	464	438
		Cx. pipiens	682	385	377	242	227	331

N= No. of replicates.

Table 2 Comparisons of colored LED collections relative to the control (incandescent light) for trapping experiment at Ijara district, December 2010

Vector species	Treatment comparison relative to the control	IRR (95% CI)	P-value
	BGR	0.78 (0.36-1.67)	0.511
	blue	0.37 (0.17-0.81)	0.0113*
	green	0.41 (0.19-0.89)	0.0220*
	red	0.44 (0.20-0.97)	0.0336*
Ae. tricholabis	violet	0.47 (0.22-1.02)	0.0539
	BGR	1.44 (0.60-3.44)	0.4031
	blue	0.46 (0.19-1.13)	0.0878
	green	0.63 (0.26-1.55)	0.3093
	red	0.72 (0.30-1.74)	0.4552
Ae. mcintoshi	violet	0.33 (0.13-0.83)	0.0182*
	BGR	0.99 (0.23-4.27)	0.1699
	blue	1.24 (0.29-5.32)	0.2292
	green	1.07 (0.25-4.60)	0.2294
	red	0.79 (0.19-3.39)	0.1181
Ae. ochraceus	violet	0.27 (0.12-0.57)	0.0004***

Estimated incidence rate ratio (IRR); Confidence interval (CI) and corresponding P-values; Asterisks indicate that the index is significantly different from unity at the P,0.05 (*), P,0.01 (**) , P,0.001 (***) levels of probability.

Table 3 Comparisons of colored LED collections relative to the control (incandescent light) for trapping experiment at Ijara district, May-June 2011

Vector species	Treatment comparison relative to the control	IRR (95% CI)	P-value
	BGR	0.36 (0.17-0.78)	0.0091**
	blue	0.20 (0.09-0.45)	< 0.0001***
	green	0.11 (0.05-0.25)	< 0.0001***
	red	0.10 (0.05-0.22)	< 0.0001***
Ae. tricholabis	violet	0.27 (0.12-0.59)	0.0007***
	BGR	0.45 (0.22-0.92)	0.0259*
	blue	0.56 (0.28-1.13)	0.1031
	green	0.33 (0.16-0.68)	0.0026**
	red	0.19 (0.09-0.40)	< 0.0001***
Ae. mcintoshi	violet	0.24(0.11-0.50)	0.0001***
	BGR	0.35 (0.16-0.77)	0.0096**
	blue	0.44 (0.20-0.98)	0.0421*
	green	0.18 (0.08-0.44)	< 0.0001***
	red	0.20 (0.08-0.45)	0.0001***
Ae. ochraceus	violet	0.25 (0.11-0.56)	0.0009***
	BGR	0.41 (0.15-1.10)	0.0567
	blue	0.34 (0.13-0.86)	0.0214*
	green	0.11 (0.04-0.30)	< 0.0001***
	red	0.11 (0.04-0.31)	< 0.0001***
Cx. pipiens	violet	0.35 (0.13-0.91)	0.0253*

Estimated incidence rate ratio (IRR); Confidence interval (CI) and corresponding P-values; Asterisks indicate that the index is significantly different from unity at the P, 0.05 (*), P, 0.01 (**), P, 0.001 (***) levels of probability.

Table 4 Comparisons of colored LED collections relative to the control (incandescent light) for trapping experiment at Marigat district, July-September 2011

Vector species	Treatment comparison relative to the control	IRR (95% CI)	P-value
	BGR	0.79 (0.41-1.53)	0.4757
	blue	0.71 (0.37-1.38)	0.3145
	green	0.63 (0.32-1.21)	0.1613
	red	0.49 (0.25-0.94)	0.0314*
Ma. uniformis	violet	0.47 (0.24-0.92)	0.0262*
	BGR	0.44 (0.21-0.95)	0.0340*
	blue	0.47 (0.22-1.02)	0.0512
	green	0.47 (0.22-0.99)	0.0472*
	red	0.41 (0.19-0.87)	0.0196*
Ma. africana	violet	0.39 (0.18-0.82)	0.0130*
	BGR	0.56 (0.28-1.13)	0.1054
	blue	0.55 (0.27- 1.11)	0.0933
	green	0.35 (0.18- 0.71)	0.0035**
	red	0.33 (0.16-0.67)	0.0020**
Cx. pipiens	violet	0.49 (0.24-0.98)	0.0410*

Estimated incidence rate ratio (IRR); Confidence interval (CI) and corresponding P-values; Asterisks indicate that the index is significantly different from unity at P, 0.05 (*), P, 0.01 (**), P, 0.001 (***) levels of probability.

Discussion

The observed variation in trap captures recorded in the different colored configurations suggests that mosquito species vary in attractiveness to light-baited traps [21, 22]. As such it is logical to expect that individual species wavelength preference will vary although such behavioral wavelength preferences may or may not correspond to spectral sensitivities [8].

Following the study design employed, the incandescent light recorded an overall higher capture of mosquitoes compared to any other LED colored traps (red, blue, green, violet, BGR). This was followed by BGR, blue, green, violet and red in the order of performance for most of the mosquito species examined. The results of field trials by Burkett et al. [4] with LED-modified CDC traps observed color preferences for some species of *Anopheles*, *Culex*, *Culiseta*, *Ochlerotatus*, and *Psorophora*. With a significant effect of light color on capture numbers, blue or green light was particularly preferred in most instances, with incandescent light most often performing nearly as well as blue and green light and generally better than red, orange, or yellow

light. A similar order of effectiveness of green> incandescent >blue>red light, in trapping mosquitoes from three genera (*Anopheles*, *Culex* and *Aedes*) was reported by Hoel et al. [11]. This contrasts with the findings in this study where incandescent light proved to be superior in a majority of instances compared to the LED colors used in our experiment although in terms of performance were followed by BGR or blue and green in this order. This difference might be related to the lighting design used in our experiment. The LEDs used in our design produce only transmitted light (direct line of sight) at very specific frequencies as opposed to reflected light (off of aluminum rain shields) used in the abovementioned previous studies. Transmitted light might not be as scattered as reflected light thereby reducing visual contrast and target size [11].

A related work by Burkett comparing blue and green light LED-modified CDC traps to incandescent light traps in north Florida demonstrated that, with the exception of *Culex* (*Melanoconion*) spp., mosquitoes showed no preferences between incandescent, blue, or green light as either transmitted light or reflected light (Doug Burkett, personal communication)(cf: [11]. In this regard, the lack of consistency in trap performance to colored light remains a challenge. Microhabitat/ecological and seasonal differences might interplay as evident in our data where a clear bias for BGR and blue lights was observed for *Ae.mcintoshi* and *Ae. ochraceus* respectively during one trapping season but dwindling effect recorded in another season. Although the reason for this is unclear; perhaps, environmental changes such as dust storms or vegetation changes could lead to reduced brightness of the LEDs and therefore attraction to mosquitoes. As such, the intensity of the light produced by the LEDs needs to be considered. The traps were all baited with dry ice to enhance trap captures. Using CO_2 is important because it is a long range attractant and the light color is a short range attractant. Therefore, to bring in statistically significant numbers of mosquitoes to compare trap captures, a long range attractant is needed to bring the mosquitoes into closer proximity at which time their photo attraction will supersede the chemo attraction. A standardized amount of dry ice (1 Kg) was used for this purpose and it is unlikely that the addition of CO_2 may have affected the trapping experiments.

In a previous study using several colored light bulbs of different intensities to capture mosquitoes (*Anopheles* and *Aedes* species), Barr et al. [23] determined that color had little effect on trap capture and that light intensity played a significant role with higher intensity lights (100 W

lamps) being more attractive than lower intensity light (60 W and 25 W lamps). Similarly, Breyev [24] reported significant attraction of *Ae. vexans* with one 220 W mercury lamp than with two 109 W incandescent lamps. However, evaluation of six different colors (white, yellow, green, orange, blue, and red) of varying intensities on mosquito captures by Ali et al. [25] found that five predominate species (*Psorophora columbiae*, *Ps. ciliata*, *Culex salinarius*, *Cx. nigripalpus*, and *Cx. erraticus*) were much more strongly affected by color than by light intensity. A similar pattern was established by Gjullin et al. [26] who found no evidence of importance of light intensity over color for mosquito attraction. The above results therefore suggest confounding findings regarding the relative importance of light color and intensity in mosquito attraction.

Stacking 2 LED lighting chips (16 LED bulbs) can provide an equal measure of light intensity of each colored LED trap platform compared to the incandescent light used in our experiment (Cohnstaedt, personal communication) although this was not possible with the trap designs we used. However with sufficient evidence that light color and intensity affect trap attractiveness to mosquitoes, and that mosquito species appear able to discern color and in cases prefer some colors to others [27-29] it may be worthwhile to consider in future studies, colored light of varying intensities.

Mosquitoes response to artificial light in many field and laboratory studies have reported a dominant spectral sensitivity to light in the ultraviolet-blue and green light and incandescent light spectrum [4, 30, 31]. Although recorded lower captures compared to incandescent, BGR and Blue colors performed better than violet and red for most species including *Ma. uniformis*, *Ma. africana* and *Cx. pipiens*. This concurs with findings that blue and green light is often more attractive than light in the yellow-orange and red regions of the visible spectrum. Many insects are insensitive to red spectrum frequencies as noted by Breyev [24]. This may account for lower captures recorded in the red colored light compared to the others for most of the mosquito species. This observation however, may not pertain to sandfly species. In fact Hoel et al. [11] in a field study in southern Egypt found that over half (55.13%) of all sand flies were collected from red light traps with significantly more recorded than in blue, green, or incandescent light traps.

The data in this study suggest clearly that irrespective of trapping period, most of the mosquito species were far more attracted to multi-spectrum light (incandescent light) as compared to monochromatic light. It is possible that the high intensity incandescent light was favored over the lower intensity monochromatic lights due to superior luminosity/intensity even though further studies are required to ascertain this.

Conclusions

Based on color alone, the data suggest that none of the colored lights is an effective substitute for standard incandescent light currently being used for surveillance of mosquitoes. This work notwithstanding presents preliminary evidence that a preference might exist for some of these mosquito species to light colors, therefore more studies to determine optimal color preferences of medically important mosquitoes are desirable and worth evaluating across a range of microhabitats in diverse ecologies. Future studies should consider comparing reflected versus transmitted light and incorporation of colored light of varying intensities.

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Chapter 3

Sheep Skin Odor Improves Trap Captures of Mosquito Vectors of Rift Valley Fever

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Abstract

In recent years, the East African region has seen an increase in arboviral diseases transmitted by blood-feeding arthropods. Effective surveillance to monitor and reduce incidence of these infections requires the use of appropriate vector sampling tools. Here, trapped skin volatiles on fur from sheep, a known preferred host of mosquito vectors of Rift Valley fever virus (RVFV), were used with a standard CDC light trap to improve catches of mosquito vectors. We tested the standard CDC light trap alone (L), and baited with (a) CO_2 (LC), (b) animal volatiles (LF), and (c) CO_2 plus animal volatiles (LCF) in two highly endemic areas for RVF in Kenya (Marigat and Ijara districts) from March-June and September-December 2010. The incidence rate ratios (IRR) that mosquito species chose traps baited with treatments (LCF, LC and LF) instead of the control (L) were estimated. Marigat was dominated by secondary vectors and host-seeking mosquitoes were 3-4 times more likely to enter LC and LCF traps [IRR=3.1 and IRR=3.8 respectively] than the L only trap. The LCF trap captured a greater number of mosquitoes than the LC trap (IRR=1.23) although the difference was not significant. Analogous results were observed at Ijara, where species were dominated by key primary and primary RVFV vectors, with 1.6-, 6.5-, and 8.5-fold increases in trap captures recorded in LF, LC and LCF baited traps respectively, relative to the control. These catches all differed significantly from those trapped in L only. Further, there was a significant increase in trap captures in LCF compared to LC (IRR=1.63). Mosquito species composition and trap counts differed between the RVF sites. However, within each site, catches differed in abundance only and no species preferences were noted in the different baited-traps. Identifying the attractive components present in these natural odors should lead to development of an effective odor-bait trapping system for population density-monitoring and result in improved RVF surveillance especially during the inter-epidemic period.

Author Summary

The East African region is a major epizootic center for endemic and emerging mosquito borne-arboviruses such as Rift Valley fever virus (RVFV), as evidenced by the increasing frequency and magnitude of this disease. The absence of vaccines or prophylactic drugs for most of these diseases emphasizes the need for accurate sampling of mosquito vector populations and testing for arboviruses. Accurate surveillance is crucial for early warning of potential or assessing mitigation of existing outbreaks. However, it is a challenge to sample mosquitoes in adequate numbers during the inter-epidemic periods (IEP) because this period is characterized by low

mosquito population densities, sporadic transmission foci and low mosquito infection rates. Therefore more efficient tools are needed to increase capture rates so maximized virus detection probability in the mosquitoes can be achieved for assessing risk and outbreak predictions. This can be accomplished by exploiting the host-seeking behavior of adult female mosquitoes and the olfactory cues used to locate a potential host. Here, odors emanating from fur of sheep, a susceptible host for RVFV, is shown to improve trap capture rates of mosquito vectors of RVF in a standard surveillance trap. These data provide for future investigations to identify attractive components present in these natural odors, so that they can be incorporated into existing traps to serve as a population density-monitoring tool for improved arbovirus disease surveillance during IEP.

Introduction

Rift Valley fever virus (RVFV) is transmitted primarily by mosquitoes and there are periodic outbreaks of this disease in humans and domestic animals in Africa and the Arabian Peninsula [1,2]. Key mosquito vectors involved in the enzootic transmission include flood water *Aedes* spp. as the primary vectors, and other epizootic culicine vectors such as *Mansonia*, *Culex* and *Anopheles* spp. as the secondary vectors [3]. In Kenya, the number of suspected vectors continues to rise with increasing isolation of the virus from additional species [3]. Since human vaccines and therapeutic treatments are not available for RVFV, surveillance is essential for early warning to ensure that devastating outbreaks and/or sporadic infections are prevented.

Efficient surveillance is essential for early detection of increased vector abundance and detection of pathogens in trapped mosquitoes. This requires a systematic collection of mosquito samples and routine testing of mosquito pools for arboviruses in order to assess the status of transmission and to allow for informed decision-making [4]. However, fluctuations in mosquito abundance and arboviral infections pose a challenge for mosquito based surveillance programs, since different surveillance strategies are required to detect different arboviral vectors and infection rates and potential and transmission rates. This is particularly problematic in the case of early detection and during the inter-epidemic periods (IEP), when transmission foci are sporadic and mosquito infection rates are low. Therefore, detection of mosquito infections when there is low transmission requires the collection of large samples of mosquitoes. For West Nile virus, 700 mosquitoes are needed for a modest detection probability of 0.5 when the natural infection rate is 0.1% for mosquito surveillance programs in the early season or in areas of low transmission [5].

Trapping large numbers of mosquitoes for detection of RVFV can be accomplished by improving the efficiency of existing surveillance traps, such as the standard CO_2-baited CDC light trap. One way to improve trapping efficiency is by exploiting the host-seeking behavior of female mosquito vectors. Adult female mosquitoes use host-emitted olfactory cues to locate hosts to obtain blood meals [6]. Domestic animals including cattle, sheep, camels and goats serve as hosts for these vectors of RVFV. However, sheep appear to be more susceptible to RVF infections than cattle or camels [7,8]. Whether or not animal susceptibility is associated with increased attraction is unclear; however, it is clear that sheep are preferred hosts of these vectors. We hypothesized that body odors from sheep are important cues used by RVF mosquitoes. The present study was carried out to investigate the response of mosquito vectors of RVFV to the CO_2-baited CDC light trap combined with sheep skin odors, in a field setting.

Materials and Methods

Study sites

All experiments were conducted at two ecologically distinct sites: Ijara and Marigat districts, which are highly endemic areas for epidemic Rift Valley fever (RVF) in Kenya [3,9,10] and are currently under active surveillance for arbovirus activities.

Ijara District is located in the North Eastern Province of Kenya and is characterized by a semi-arid to arid climate. Mosquitoes were sampled at Kotile (1.97˚S, 40.19˚E) (near Masalani) and Sangailu (1.31˚S, 40.71˚E), which is around 60 m above sea level. The average annual rainfall is 540 mm with bimodal peaks recorded from March-June and September-December each year. However, the interannual rainfall variability is very high and reaches abnormal levels leading to floods during El Niño years. Minimum temperatures are always above 20°C, and maximum temperatures reach 30°C to 34°C with a high seasonal and interannual variability.

The predominant vegetation is Acacia-Commiphora deciduous bushland and thicket (Savannah, Shrubland, open to very open shrubs), which is much degraded due to overgrazing around the settlement areas. The road leading from Masalani to Sangailu demarcates the boundary between these semi-arid landscapes and the more moist Tana River delta and Boni Forest towards the coast. Boni Forest is an indigenous open canopy forest that forms part of the Northern Zanzibar-Inhamdare Coastal Forest Mosaic.

The second study site is Marigat district, located in the Kenyan Rift Valley 250 km northwest of Nairobi where traps were set in surrounding villages/communities of N'gambo (0.50˚N, 36.06˚E) and Salabani (0.55˚N, 36.06˚E). The study site covers the basin between Lake Baringo and Lake Bogoria with the town of Marigat as an economic center and lies about 1000 m above sea level. The climate is hot and dry with high rainfall variability, both annually and inter-annually. The average annual rainfall is 650 mm with weak bimodal peaks recorded from March-May and June-August. Temperatures vary from 30 to 35°C, but can rise to 37°C in some months.

The low lying arid part of the Baringo basin consists of northern Acacia-Commiphora bushlands and thickets but it has experienced severe land degradation caused by uncontrolled grazing and deforestation. *Prosopis juliflora* (Sw.) DC, locally called mathenge, was introduced to Baringo in the early 1980s for fuelwood production and reforestation as a mitigation measure to stop desertification. The plant was introduced at two sites but now covers large areas, i.e. N'gambo village, one of the vector sampling sites.

Three indigenous human communities live in this area, the Ilchamus, Pokot and Tugen. They earn their living through pastoralism and agro-pastoralism keeping large numbers of cattle and small livestock such as sheep and goats. The Perkerra irrigation scheme (growing of vegetables, maize seed production), fishing and tourism provide additional income to these communities.

Choice of animal

Sheep are the most susceptible among livestock hosts afflicted by RVFV [1,8,11], and the living animal has been exploited as a lure in trapping mosquito vectors [12]. Its role in the enzootic maintenance of the RVFV [13] is the reason why it is the preferred domestic animal currently being used as sentinels in an ongoing surveillance program for RVF at the two study sites.

Ethics Statement

The study was conducted with the approval of the national ethics review committee based at the Kenya Medical Research Institute (KEMRI) and is renewed on an annual basis after a scientific audit. The Animal use component was also given approval by the KEMRI Animal Use and Care committee (KEMRI-AUCC). KEMRI-AUCC complies with the national guidelines for care and use of laboratory animals in Kenya developed by the Kenya Veterinary Association and the Kenya lab animal technicians association 1989. The KEMRI-AUCC which approved the study

protocol has an assurance identification number A5879-01 from the Office of Laboratory Animal Welfare (OLAW) under the Kenyan department of health and human services. For purposes of livestock use, funds from the project were used to purchase animals to monitor RVFV seroprevalence and used for all experimental activities described in this study. These animals were owned and maintained for the study by the project. The project bought 492 animals comprising 5 sentinel herds; two in Marigat, three in Ijara district (one in Kotile and 2 in Sangailu). The animals were left with the owners as part of their flocks but they were not allowed to sell or slaughter them because the project was monitoring the animals. The animals were reverted back to the owner at the end of the project activity. Any newborns born out of the tagged animals belonged to the farmers. We worked in collaboration with the department of veterinary services and veterinary doctors mandated by the government to do livestock sampling and research. The above terms were stipulated well in an agreement between the farmers and the International Centre of Insect Physiology and Ecology (*icipe*), the hosting institution for the AVID Project Consortium.

Experimental design

Experiments were conducted in October and December 2010 during the rains to ascertain the presence of mosquitoes. This comprised 10 replicates of 4 treatments per district. The treatment-trap combinations consisted of the standard CDC light trap alone (L) and baited with (a) animal volatiles (LF), (b) CO_2 (LC), or (c) CO_2 and animal skin volatiles (LCF) using fur obtained from living sheep. The animal volatiles consisted of fresh sheep (*Ovis aries* Linnæus) hair samples shaved from the belly and back areas of the animals (avoiding the head and anal regions) daily. The animal fur was wrapped in five layers of aluminum foil, kept in a cold box (10^{o}C) and immediately transported to the trapping site (located between 2 to 5 km). Once at the trapping site, approximately 19 g of the animal fur were placed in each canister (cylindrical in shape with a diameter of 9.5 cm and height 22.5 cm) designed from Brass mesh wire (mesh size, 0.15 mm, McNichols Co, Tampa FLA). With an inter-trap distance of 40±2 m, the traps were hung in trees 1.5±0.2 m off the ground and activated within 30 min of sunset (1800-1830) and trap contents collected within 30 min after sunrise (0600-0630 hours). Treatments and control were assigned to a predetermined similar area following a Latin square design with days as replicates. Traps were rotated on every trapping day to minimize variability due to trap placement. Dry ice (1 kg)

was used as the CO_2 source, which was delivered in Igloo thermos containers (~2 L) (J.W. Hock, Gainesville, FL) with a 13-mm hole in the bottom center. Treatments with the canisters containing fur (which released skin volatiles) were hung at the base of the standard CDC trap (battery-powered model 512, John W Hock Co., Gainesville, FL) and when in the presence of CO_2 directly in the air flow. All bait canisters were boiled in l0% bleach solution after each nightly trapping to eliminate any residual odor.

Mosquito processing

Mosquitoes caught daily from each of the treatments were anesthetized using triethylamine and identified morphologically to species using taxonomic keys [14-16]. When large numbers of mosquitoes were trapped, they were anesthetized, sorted from other insects and immediately stored in 15 or 50 mL centrifuge tubes, and transported in a liquid nitrogen shipper to the laboratory where they were later identified and the total number by species for each treatment-trap were recorded.

Data analyses

Trap count data were analyzed per district and were also subdivided into four categories (i.e., key primary vectors, primary vectors, secondary vectors and non-vectors) based on the relative importance and involvement of member species in RVFV transmission [2,3]. The four main categories of trapped mosquitoes recorded in the different treatments were further categorized as follows: flood water *Aedes* species (key primary vectors; *Aedes mcintoshi* and *Aedes ochraceus*); primary vectors (*Aedes sudanensis*/*Aedes tricholabis*); secondary vectors (*Mansonia* and *Culex* spp.) and non-vectors, which do not fall into any of these categories (Table 1). Analysis of key primary and primary RVFV vectors was limited to Ijara district where they were mainly encountered and secondary vectors limited to Marigat district where they occurred in substantial numbers (Table 1). Daily count of mosquitoes recorded in the various trap treatments were analyzed using a generalized linear model with negative binomial error structure and log link using R 2.11.0 software [17]. Using the treatment L only (control) as the reference category, the incidence rate ratios (IRR) that mosquito species chose other treatments (LCF, LC and LF), instead of the control, were estimated. The IRR for the control is 1 (unity) and values above this

indicates better performance and values below under performance of the treatments relative to the control.

Results

Species abundance and composition

The distribution of RVFV mosquitoes captured per treatment-trap combination for the two districts are contained in Table 1. Mosquito species composition and trap captures differed markedly between the two districts which might suggest varied habitat preferences for each mosquito species. Differences in abundance were observed between the treatments with no clear pattern of preference of any species for a particular trap treatment. Some species were not caught in all replicates, and it is unclear if such variability was due to overall low population densities or the mosquitoes failing to enter (or to respond to) the traps. In general, traps baited with CO_2 (LCF and LC) captured more mosquitoes than those without (LF and L) (Table 1 and Figures 1 and 2).

Effect of treatment on overall mosquito captures

There was a significant effect of treatments compared to the unbaited CDC trap on overall mosquito captures from Marigat (χ^2=20.68, df=3, p<0.001) and from Ijara (χ^2=37.51, df=3, p<0.001). Trap catches from Marigat indicate that, compared to L only, LC and LCF traps caught 3-4 times more host-seeking mosquitoes [IRR=3.1 for LC and IRR =3.8 for LCF]. LCF traps recorded higher mosquito catches compared to LC traps (IRR=1.23) although the difference was not statistically significant (Figure 1). Similarly, the LF trap caught slightly more mosquitoes (IRR=1.03) than L only but was not significantly different (Figure 1). Similar findings were observed at Ijara where there was a significant treatment effect on mosquito catches (χ^2=37.51, df=3, p<0.001). Carbon dioxide (LC), CO_2 + fur (LCF) significantly increased trap captures by 6.5 and 8.5 times, respectively, compared to the control. The LF caught more than the control, L, but this was not statistically significant (Figure 1).

Treatment effect per vector category per district

Trap catches at Ijara were dominated by flood water aedine mosquitoes categorized as key and primary RVFV vectors; these species were sparse or absent at Marigat. There was a highly

significant effect of treatments on key primary RVFV vectors (χ^2=199.99, df=3, p<0.001). For this group, relative to the control, there was a 4.0- and 6.5-fold significant increase in captures recorded in LC and LCF traps, respectively (Figure 2). Additionally, LCF capture rates were significantly higher than LC capture rates (IRR=1.63).

A significant effect of treatments on RVFV primary vectors was also evident (χ^2=74.24, df=3, p<0.001). Compared to the control, the treatments LF, LC and LCF caught 2.9, 31 and 42 times as many primary vectors. Interestingly, for this group, there was a 34% significant increase in captures for traps baited with LCF compared to LC (IRR=1.34) (Figure 2).

Marigat yielded very low catches for key primary vectors and there was a total absence of primary vectors. Therefore results are only presented for secondary vectors. For secondary vectors at Marigat, there was a highly significant effect of treatments on the mosquito catches (χ^2=22.94, df=3, p<0.001). Relative to the control, there were 3 to 4-fold increases in captures for LC and LCF traps, respectively (Figure 2). Comparable captures were recorded for LF and L traps with only a slight increase recorded in LF baited traps relative to the control (IRR=1.04). Captures rates, although not significant were higher for LCF traps than LC traps (IRR=1.24) (Figure 2). Mosquito collections within this category at Ijara were low and dominated by *Cx. pipiens* s.l. with an observed increase in captures in the other treatments compared to L.

The non-vectors category included species of the genera *Ficalbia*, *Coquilettidia*, *Anopheles* and *Aedes* (Stegomyia). Members of these genera occurred in low numbers in both districts, especially at Ijara (Table 1). However, data for Marigat suggest a bias in trap captures in LCF and LC, compared to L although there were no significant differences in the captures between these treatments, while similar trap captures were observed for the LF and L-baited traps. Non-mosquito species notably beetles and moths were trapped in addition to mosquitoes but were not included in our data.

Table 1. Number of each mosquito species captured by baited and unbaited CDC light traps at two districts in Kenya.

	Marigat district				Ijara district			
RVFV vector group	L	LC	LCF	LF	L	LC	LCF	LF
Key primary vectors								
Ae. mcintoshi	5	7	8	3	20	141	↑208	27
Ae. ochraceus	0	0	0	0	470	856	↑1034	648
Primary vectors								
Ae. sudanensis	0	0	0	0	4	18	30	4
Ae. tricholabis	0	0	0	0	85	2,794	↑3745	251
Secondary vectors								
Culex poicilipes	445	5,154	↓3522	696	1	9	24	3
Cx. ethiopicus	0	5	7	1	0	0	1	1
Cx. bitaenorrhynchus	5	14	38	2	0	0	0	0
Cx. pipiens	49	457	↑657	53	7	53	34	10
Cx. tigripes	0	4	4	2	0	0	0	0
Cx. univittatus	30	181	234	37	6	14	9	12
Cx. vansomereni	0	6	2	0	0	0	0	0
Mansonia africana	6,223	17,521	↑24254	6,484	0	2	0	0
Ma. uniformis	2,124	5,563	↑7334	1,912	1	0	2	0
Non-vectors								
Aedes furcifer	0	0	0	0	0	1	2	0
Ae. hirsutus	0	3	2	1	0	0	0	0
Ae. metallicus	0	0	0	0	1	2	2	0
Ficalbia splendens	376	368	354	265	0	0	0	0
Aedomyia furfurea	0	0	0	0	0	4	5	0
An. coustani	744	1,324	↓1315	826	1	3	2	1
An. funestus	0	1	1	2	0	0	0	0
An. pharoensis	27	18	51	20	0	0	0	0
An. squamosus	0	9	5	1	1	3	3	2
An. gambiae s.l.	20	45	66	46	0	0	0	0
Coquilettidia aurites	0	2	6	0	0	0	0	0
Cq. metallicus	0	1	1	0	0	0	0	0

L, light only; LF, light+animal odor; LC, light+CO_2; LCF, light+CO_2+animal odor;↑, increase in captures in LCF traps relative to LC; ↓, decrease in captures in LCF traps relative to LC.

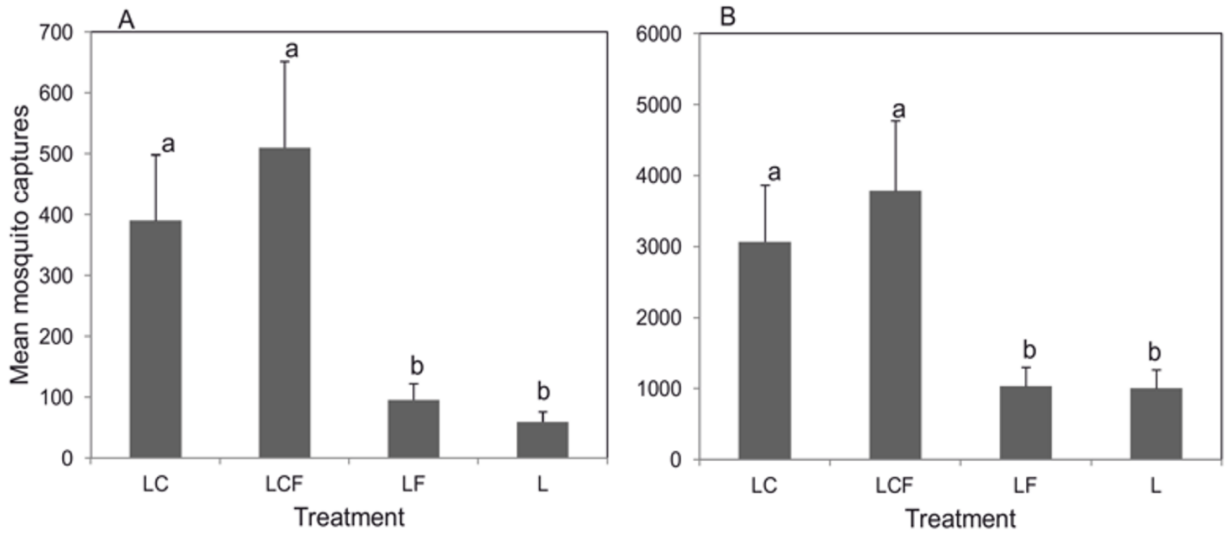

Figure 1. Mean mosquito captures in 10 replicate trials per treatment at the two districts in Kenya. A) Ijara district; B) Marigat district. Bars followed by similar letters are not significantly different at P=0.05. L, light only; LF, light+sheep odor; LC, LC, light+CO_2; LCF, light+CO_2+sheep odor.

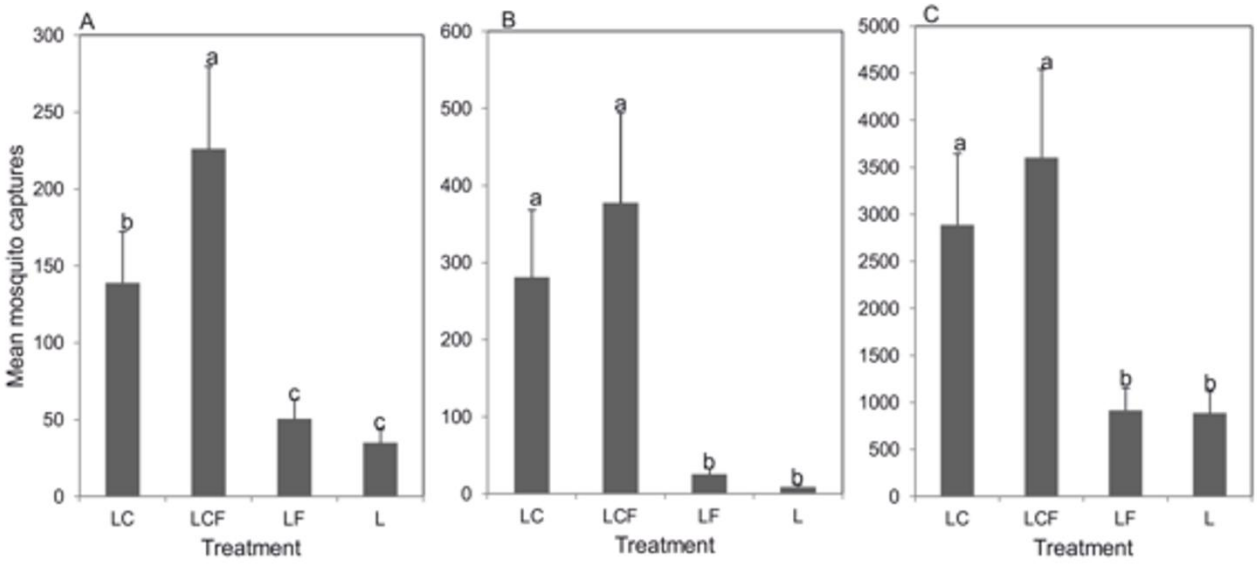

Figure 2. Mean mosquito captures/trap/night for different RVFV vector groups in 10 replicate trials/district in Kenya. A) Key primary vectors; B) Primary vectors; C) Secondary vectors. Bars followed by similar letters are not significantly different at P=0.05. L, light only; LF, light+sheep odor; LC, light+CO_2; LCF, light+CO_2+sheep odor.

Discussion

Effect of sheep fur on trap captures

The results demonstrate that more mosquitoes were caught in traps that contained a release of the combination of sheep odors+CO_2 and were in most cases the most attractive bait compared to the conventional CO_2-baited light trap. This confirms that odors emanating from sheep fur play a role in host-location by these mosquitoes. The attractive effect was highly evident in captures of flood water aedines comprising key and primary RVFV vectors as well as secondary vectors. The effectiveness of sheep is supported by a study on blood meal patterns during a RVF outbreak where widespread feeding on sheep was observed [18]. Moreover, most mosquitoes belonging to the *Culex, Mansonia* and *Aedes* genera have been reported to feed opportunistically and readily on mammals [19-21].

The entire animal body emanations comprising breath and skin volatiles influence the outcome of mosquito host-seeking process [22]. Research has indicated that animal skin emanations have a kairomonal (attractive) effect on mosquitoes while breath volatiles have an allomonal or repellent effect [23]. Skin body odor may be the primary factor for mosquito attraction and discrimination when mosquitoes are in close proximity of a host. It is therefore not surprising that addition of skin volatiles captured in sheep fur enhanced captures of mosquitoes attracted to sheep hosts when combined with the conventional CO_2-baited light trap.

The effect of sheep skin odors emanating from fur was not evaluated alone but in combination with CO_2 and/or light which are known attractants for mosquitoes and other biting flies. Although animal odors enhanced trap captures when added to either CO_2-baited light trap or light trap only, the captures were greater in the CO_2-based blend than in the combination without CO_2. However, the crude animal skin odor in traps is imperfect because of possible loss of volatile attractive components over time compared to the dynamic production from live animals. Therefore it would be beneficial to identify the attractive compounds and develop a synthetic blend.

In some replicates there was a decrease in trap catches when host odor was added to light or to CO_2. This could be attributed to variation in attractiveness of the batches of animal fur used in the daily trapping experiment as odors used were not from the same animal; low occurrence of targeted mosquitoes, as observed at the districts for certain vector categories; a difference in

preferred host other than sheep e.g. *Cx. poicilipes* between LCF and LC baited traps (Table 1); and volatiles from fur are a static system and most volatile compounds evaporate first and therefore the odor profile changes. The effect of host odors did not markedly influence trap catches of the non-vector category. The low abundance of mosquitoes was insufficient to observe a significant preference in trap catches for the different treatments used; even though there was an increase in trap catches for those baited with host odors compared to light only.

The effect of CO_2 on trap catches was not evaluated independently; however, its effect was evidenced in the difference in trap catches between LC and L baited traps. The data support its role in enhancing trap captures [24], especially for RVFV vectors. Our experimental setup excluded landing response as a measurement, instead focussing solely on trap catch. The goal was to evaluate animal fur containing skin emanations that provided attractive stimuli. However, the large response of these mosquitoes to CO_2, suggests that it can serve as a good positive control for evaluating candidate synthetic attractants of skin origin for this group of arbovirus vectors of medical and veterinary importance.

Preliminary trials (data not shown) and earlier studies highlighted the importance of these attractants in flight activation of mosquitoes towards host odors [25,26]. This justifies the inclusion of these well-known long-range attractants in trap design. Our data suggest that host skin odors other than CO_2 are important in enhancing mosquito trap captures in concurrence with studies reporting enhanced effect of mosquito attraction to animal skin volatiles in the presence of CO_2 or light [22,27,28].

Role of Carbon dioxide (CO_2) and Light

It is well-known that many nocturnally-active hematophagous insects are attracted to light [29,30]. In conformity with earlier findings [31], our results show that light as a visual cue is enhanced by sheep skin odors and CO_2. Besides being non-specific, previous studies have argued that CO_2 activates mosquitoes to initiate host-finding, but may not necessarily attract it and at close range, can actually act as a deterrent [26] and be of limited use in host discrimination [32]. Although this was not the subject of our study, CO_2 increased trap captures in the presence of host skin odors, in agreement with previous research [24,25,33].

The observed trap captures recorded in the LCF traps were generally higher compared to those caught in the LC traps. Nonetheless, among the mosquito species trapped, differences in capture rate were not observed between the LCF and LC traps. Therefore, CO_2-baited light traps may be adequate for monitoring and surveillance of these species. However, for effective arbovirus disease surveillance, an improved sampling method is vital especially during the inter-epidemic period where transmission foci are sporadic and infected vectors are rare. Emphasis needs to be placed on increasing the collections with an additional advantage of depicting the dynamics of populations.

Less attractive, unattractive or just different?

Beyond the already described finding that animal odor inclusion increases trap catch with CO_2 present, there were some cases where it suppressed trap catch. In some cases, lower catches of the LCF trap were noted on days with light showers; therefore, precipitation may have interfered and reduced mosquito attraction to skin odor baits as observed before by Olanga *et al*. [34]. However there was no record of variation in weather patterns during the study period. Another possibility is the variation from fur samples used in this study. Samples were obtained from various animals without prior assessments of their degree of attractiveness. Animals in a herd are known to vary greatly in their attractiveness to mosquitoes [35,36]. Reduced attraction due to loss of important volatile compounds during the fur extraction process remains plausible.

A higher number of *Cx. poicilipes* were collected in CO_2-baited light traps than in similar traps baited only with host skin odor, although the difference in trap captures was not significant. This suggests that sheep are not preferred hosts for this species. However, the effect of CO_2 in the presence of host-related odors may be variable and a strong attraction response may be observed with often different responses between species [37,38]. This observation might emphasize the importance of trap placement in the sampling process, though it is not certain if this species would be attracted to the host that emanates the greatest amount of CO_2 in nature.

Differential catches of *Cx. pipiens* s.l. and *An. gambiae* s.l. to odors from sheep fur were recorded at the different sites (Table 1). Among the species complexes captured, there are known marked differences in olfactory responses between members of the complexes [39,40]. *Culex pipiens* preferentially feed on birds [41] although they can adapt and readily feed on mammals in proportions possibly based on host abundance [42]. The observed differences in trap responses

in the highest trap treatments (i.e., LC and LCF) at both districts may indicate different spatial feeding preferences in geographically separate populations. Related response patterns of discrete populations of mosquito species to host odors has been reported [42,43], as such, it may be worthwhile to include a preference test involving odors from other livestock hosts in field bioassays.

Only volatiles from skin emanations captured in the fur were tested in this study. Studies on other volatile sources involved in host attraction to hematophagous flies have been reported from feces [44] and urine [45,46]. In this regard, other sources of attractive odors might contribute to the attraction of mosquitoes and combination using these odors may be worth investigating.

Laboratory bioassays have commonly been used to evaluate the effect of semiochemicals on mosquito behavior whilst minimizing other environmental variables. However, such an approach is inadequate for predicting effects on natural populations and on ecosystem-level features [47]. Alternatively, insect behaviors have been assessed in the field by baiting traps with extracts of animal volatiles [42,48]. Use of whole animals provides another approach but it becomes difficult to delineate individual contributions of attractants from breath or skin emanations or other exogenous compounds to the overall trap catches. Our design followed a field-based approach to evaluate the role of skin emanations on mosquito trap catches. The design can account and provide for an understanding of heterogeneities which dramatically influence dynamics of natural systems. This is similar to the trapping design employed by Njiru *et al*. [49] and Jawara *et al*. [50] to investigate mosquito captures in conventional traps baited with human foot odors trapped on nylon stockings.

Although the contribution of geographical variability to the total variance was not estimated, possible experimental confounders such as time, location and environmental influence are unlikely to affect the overall observed results as the present experiments were performed at a variety of sites with different animals of the same species and treatment traps treated alike. As such, it is likely that many of the mosquitoes approaching the trap had the opportunity to sample more than one of the treatment-traps, and may have made a choice between them. Albeit the above mentioned challenges, the use of crude volatiles in the field approach presented in this paper can contribute to the evaluation of the effect of host volatiles in the standard CDC light trap.

In conclusion, the addition of sheep skin odor to the CO_2–baited light trap improved trap catches of RVFV vectors in line with similar findings reporting enhanced effect of animal skin odors and other cues such as CO_2 [27,28,44]. The results in this study indicate host skin olfactory cues are important signals in mediating mosquito host location. The finding is also in accordance with the consensus that additional compounds other than CO_2 from animal skin may be exploited by mosquitoes in host location [51-53]. Sheep skin odor contributes to the attraction of host-seeking RVFV mosquito vectors. Identification of chemicals emanated by sheep might provide the basis for the development of improved devices to sample these vectors. However, refinements into an effective monitoring tool requires identifying and understanding the specific behavioral effects of the attractive components present in these skin odors which is currently underway.

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Chapter 4

Common Host-derived Chemicals Increase Catches of Disease-transmitting Mosquitoes and can Improve Early Warning Systems for Rift Valley Fever Virus

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Abstract

Rift Valley fever (RVF), a mosquito-borne zoonosis, is a major public health and veterinary problem in sub-Saharan Africa. Surveillance to monitor mosquito populations during the inter-epidemic period (IEP) and viral activity in these vectors is critical to informing public health decisions for early warning and control of the disease. Using a combination of field bioassays, electrophysiological and chemical analyses we demonstrated that skin-derived aldehydes (heptanal, octanal, nonanal, decanal) common to RVF virus (RVFV) hosts including sheep, cow, donkey, goat and human serve as potent attractants for RVFV mosquito vectors. Furthermore, a blend formulated from the four aldehydes and combined with CO_2-baited CDC trap without a light bulb doubled to tripled trap captures compared to control traps baited with CO_2 alone. Our results reveal that (a) because of the commonality of the host chemical signature required for attraction, the host-vector interaction appears to favor the mosquito vector allowing it to find and opportunistically feed on a wide range of mammalian hosts of the disease, and (b) the sensitivity, specificity and superiority of this trapping system offers the potential for its wider use in surveillance programs for RVFV mosquito vectors especially during the IEP.

Author Summary

Enzootic transmission of arboviral diseases such as Rift Valley Fever (RVF) continues to occur at a low intensity among mosquito vectors in Kenya, which may remain undetected by most monitoring programs unless very sensitive tools are employed to detect virus activity before an outbreak occurs. Here, we report a more sensitive and mosquito-specific surveillance trapping system for RVF virus (RVFV) mosquito vectors based on mammalian-skin derived semiochemicals. We show that RVFV mosquito vectors detect similar components (heptanal, octanal, nonanal, decanal) in the skin of RVFV mammalian hosts. In field trials, each of these compounds when combined with CO_2 increased captures of these mosquito vectors in a dose-dependent manner. Additionally, a blend formulated from optimal attractive dose of each of these compounds combined with CO_2 significantly increased trap captures compared to control traps baited with CO_2 alone. The four-component blend attracted multiple mosquito vectors of the disease under field conditions suggesting that a trapping system based on this formulation offers opportunity for its use as a tool for RVFV vector surveillance.

Introduction

Rift Valley fever (RVF) is a mosquito-borne zoonosis which is of major public health and veterinary concern in sub-Saharan Africa (SSA). In the last 20 years, epidemics of the disease have occurred at irregular intervals with hundreds of thousands of infections in humans and livestock. The 1997–1998 RVF outbreak in East Africa including Kenya, Somalia, and Tanzania represents the largest outbreak of RVF infection ever recorded in SSA that affected over 100,000 humans with over 450 deaths in Kenya alone [1]. The emergence and re-emergence of the disease especially in East Africa, poses not only a huge threat to livestock, and human health, but it also represents a looming health threat likely to spread beyond Africa due to global environmental, demographic and societal changes and trade [2]. Additionally, the economic losses due to zoonotic disease outbreaks can be staggering including trade sanctions, travel warnings or restrictions, animal disease control efforts such as animal culling (intentional slaughter), and declining public confidence in animal products [3]. For example, once RVF is known to be circulating in an animal herd, the World Organization for Animal Health (OIE) places a three-year export embargo on those animals.

Mosquito bites are the most important transmission mechanisms of the disease to mammalian hosts including humans [4,5]. Although several mosquito species in diverse genera have been implicated as vectors following isolation of the RVF virus (RVFV) [5-8], there is strong evidence that in Kenya, *Aedes mcintoshi* and *Ae. ochraceus* play key roles in the transmission of the disease [8-10]. During the 2007/2008 RVF outbreak in Kenya, these two species were identified as primary RVFV vectors, accounting for over 77% of positive pools of mosquitoes sampled in the field [8] which occur predominantly in North-Eastern Kenya. In addition, *Mansonia* and other *Culex* mosquitoes are important RVFV vectors in Marigat District of Rift-Valley area, a highly endemic area of the disease [8].

In spite of the apparent emergence and re-emergence of arboviral diseases and especially RVF in East Africa, sensitive surveillance programs to actively monitor vector populations to provide an early warning system are lacking. Entomologic arbovirus surveillance is advantageous because it (i) provides the earliest evidence of transmission in an area, (ii) identifies the potential risk to humans, and (iii) allows emergency control operations to be set in motion in advance of epidemics. Vectors once infected, remain infected with the virus for the duration of their life,

unlike in humans and other vertebrates which are only transiently infected [11]. The option of serologic surveillance in animals is complicated by problems of cross reactivity among arbovirus groups [12-14]. Moreover, other challenges that may compromise the efficacy of animal hosts as a surveillance tool include ethical issues associated with using animals; challenges of bleeding larger animals which represents an occupational health and safety issue [12]; and reduced sensitivity due to the development of herd immunity [13,14] which may dampen seroconversion. This makes vector surveillance the best option to target for arbovirus activity especially as RVF epidemics in these susceptible animals, initiated by bites of infected mosquitoes, are also involved in sustaining the disease. Until now, RVFV vectors have been monitored using CO_2-baited CDC light traps, which are generally non-specific and trap a wide range of non-target insect species such as beetles and moths, in addition to mosquitoes. Additionally, because of low sensitivity, this trapping system is inadequate for use during the low intensity inter-epidemic period (IEP) of enzootic virus transmission where viral activity may remain undetected among mosquito species [4,12,15]. Thus, there is a critical need to develop more sensitive and effective monitoring tools to increase trap captures of mosquito vectors so as to maximize detection of virus activity.

Like most hematophagous insects, RVFV vectors use olfactory cues to locate their hosts for a blood meal [16,17] which may involve more than mammalian breath odors such as CO_2, a non-specific semiochemical, commonly used in the CDC light trap. We therefore refined the sensitivity of the existing trapping system for RVFV vectors by combining it with known mammalian host skin-derived semiochemicals in order to target only mosquitoes. Here we report the identification of key kairomones responsible for attraction of RVFV vectors which demonstrate the commonality of mammalian host skin-derived attractants for mosquito vectors of the disease, and development of a highly efficient monitoring tool for RVFV vectors which exploits a semiochemical lure, developed from skin odors of these mammals that can potentially impact RVFV mosquito surveillance during the IEP.

Materials and Methods

Study sites

All experiments were carried out at two ecologically distinct sites, i.e. Ijara and Marigat districts of Kenya (Figure 1), both highly endemic areas for epidemic RVF [8] and which are currently under active surveillance for arbovirus activities.

Ijara District is located in the North Eastern Province of Kenya, where traps were set out in two major locations: Sangailu (01.31˚S, 40.71˚E) and Kotile (1.97˚S, 40.19˚E). The entire district is semi-arid and normally experiences two rainy seasons a year which frequently fail: the so-called short rains between October and December and the long rains in March and April. The area is located at an altitude of about 60 m above sea level (asl) and typical annual rainfall averages between 300 to 500 mm. The people in North Eastern Province are predominantly ethnic Somali and practice pastoralism, keeping livestock including cattle, goats, sheep, camels, and donkeys. Vegetation predominantly consists of shrubs and acacia bushes.

In Marigat District located in the Rift Valley Province of Kenya, traps were set in surrounding villages/communities namely N'gambo (0.50˚N, 36.06˚E), Salabani (0.55˚N, 36.06˚E), Lerocho (0.56˚N, 36.01˚E), Bogoria (0.37˚N, 36.05˚E) and Sirata (0.46˚N, 36.10˚E). The vegetation in the low lying arid part of Marigat district consists of northern Acacia-Commiphora bushlands and thickets and has experienced severe land degradation caused by uncontrolled grazing. The local inhabitants who are mainly agro-pastoralists subsist on limited crop production and livestock rearing. This district located around 1000 m asl receives annual rainfall ranging from 300 to 700 mm.

Odor collection and field evaluation of crude skin odors of RVFV hosts on mosquito captures

Skin odors from cow, donkey, goat and sheep were collected by rubbing stockinette cotton material (Clinitex, FL Orthopedics, USA, latex free with antimicrobial protection) on the belly and back areas avoiding the head and anal regions for 12-15 minutes using 4 pieces of the material per animal each measuring 10 cm x 26.5 cm. The stockinette material was handled with latex gloved hands in order to minimize contamination from human skin. Human odor used in the experiment consisted of four pieces of worn stockinette (of same material and sizes as used for the animals) containing trapped foot odors from a 30-year old African male volunteer by wearing them for 20 hours (21:00 – 17:00 the following day) prior to the start of the experiments.

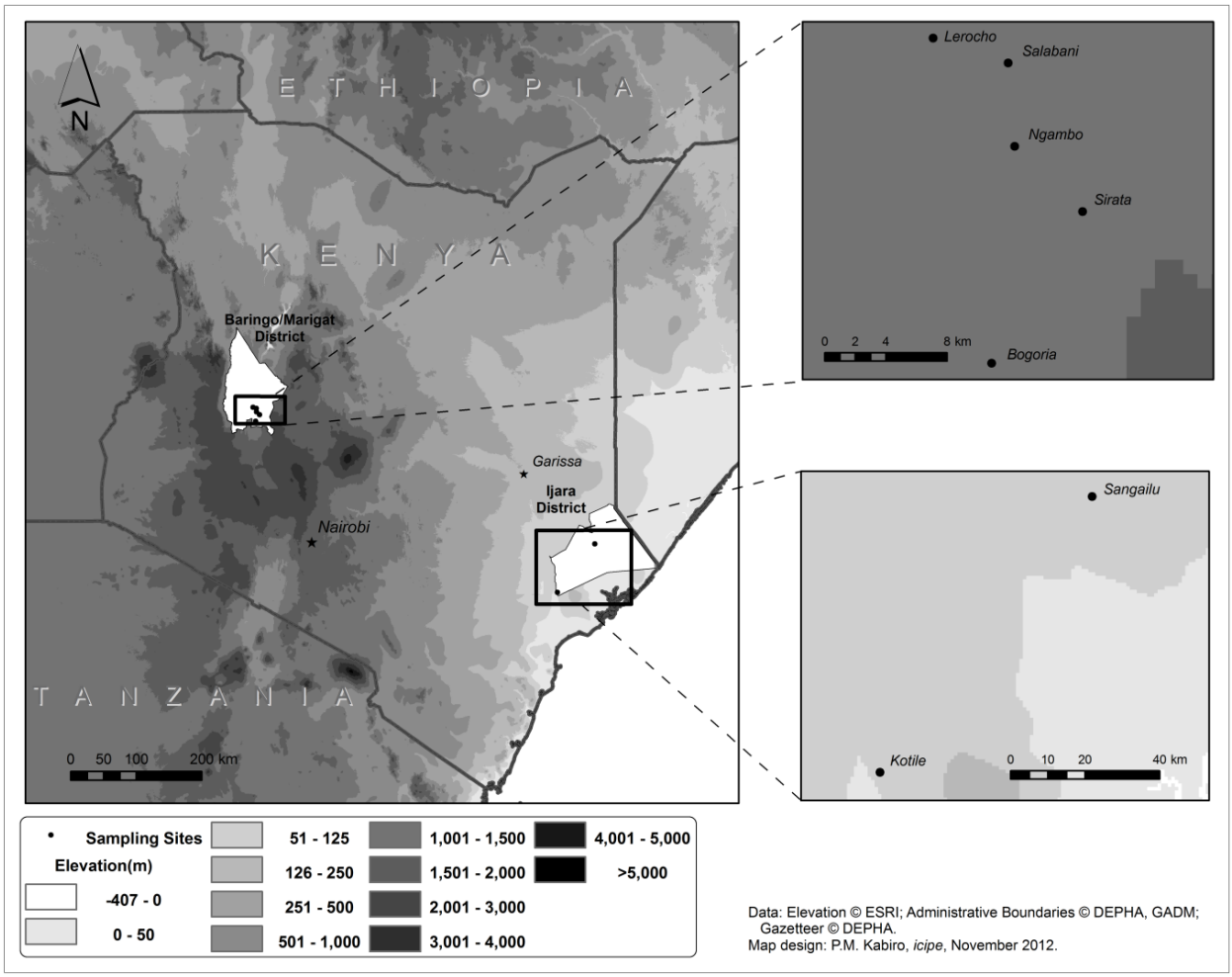

Figure 1. Map of Kenya showing the location of the study sites.

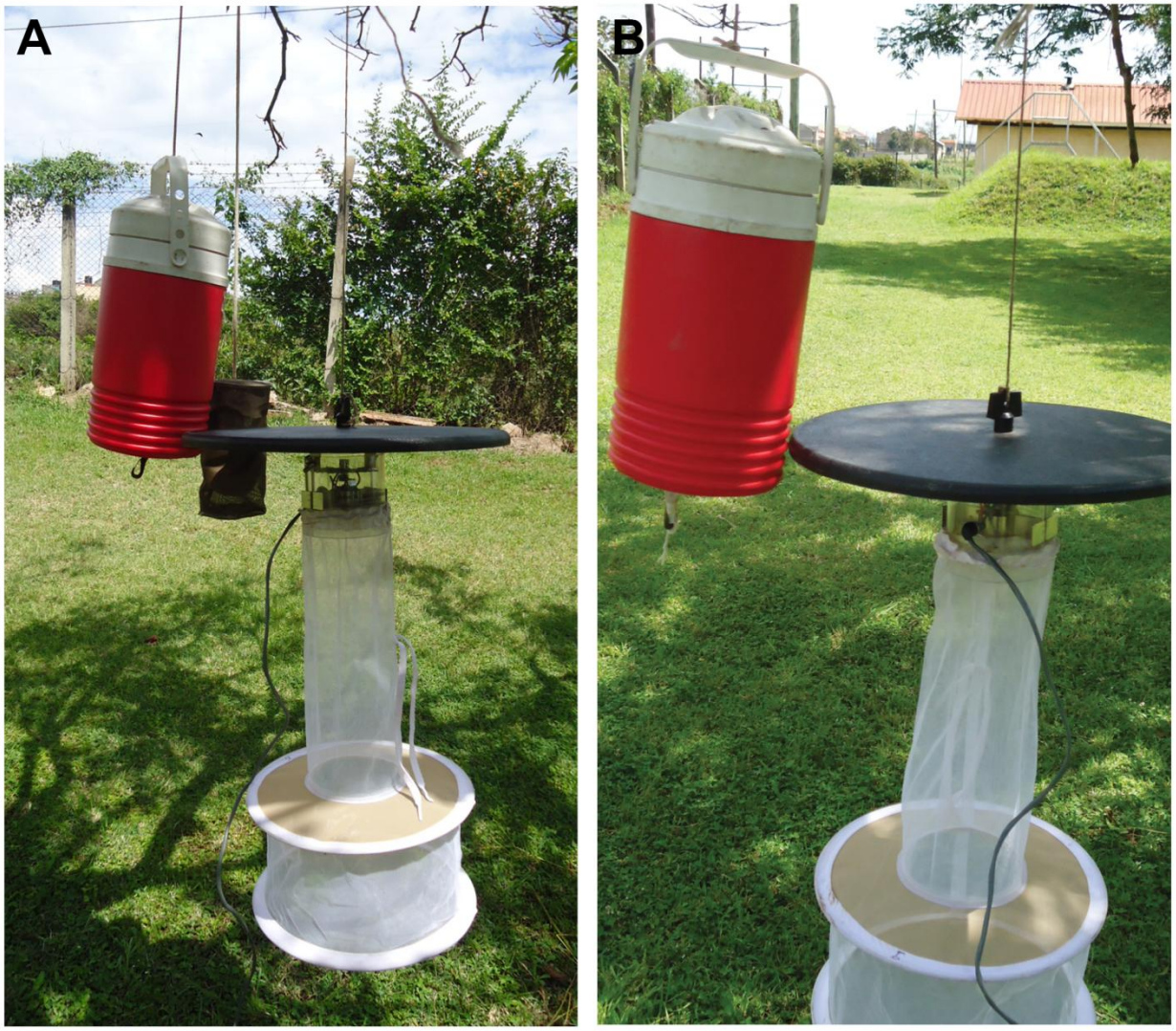

Figure 2. Trap design using CDC trap without a light bulb for field evaluation. (A) crude animal skin odors: arrangement of canister and CO2 released at the bottom of the Igloo container all placed close to the fan of the trap; (B) synthetic compounds released from a 0.5 ml tube placed under the Igloo close to the air flow of CO_2.

Stockinette materials with animal odors were wrapped in at least four layers of aluminium foil, kept in a cold box (10^{o}C) and immediately transported to the trapping site. Once at the trapping site, the stockinette human and animal odors were placed in separate canisters (cylindrical in shape with diameter 9.5 cm and height 22.5 cm) designed from Brass mesh wire (mesh size, 0.006 Inch, McNichols, Tampa, FL) and hung close to the air flow of CO_2 released at the bottom of an Igloo thermos container both mounted close to the fan of the CDC trap without a light bulb (Figure 2). All canisters made from the same material and of similar size, were boiled in l0%

bleach solution after each night's trapping to eliminate any residual odor. The stockinette material containing collected odors were replaced each day for a repeat of the experiment. In total, six treatments were tested consisting of animal skin odor of each animal type + CO_2 (five treatments) and CO_2 only. Carbon dioxide was added nightly and delivered by placing 1 kg dry ice in Igloo thermos containers (2 L) (John W Hock, Gainesville, FL) with a 13-mm hole in the bottom center. With an inter-trap distance of 40 m, the treatments and control were randomly assigned to a predetermined similar area following a Latin square design with days as replicates. Traps were activated within 30 min of sunset (1800-1830 hr) and trap contents collected within 30 min after sunrise (0600-0630 hr). Traps were rotated on every trapping day to minimize variability due to trap placement. The field experiments were conducted at Ijara and Marigat districts which are two highly endemic areas for RVFV activities in Kenya. The 'attraction' of animal/human odor was estimated by the number of mosquitoes collected from the CO_2-baited CDC trap (model 512, John W Hock, Gainesville, FL) without a light bulb containing the bait odor from that animal compared to the control (CO_2 alone without a light bulb only) in several replicate exposures. Mosquitoes were morphologically identified to species using taxonomic keys [18-20]. Mosquitoes were categorized as engorged when blood fed or gravid based on observation of their abdominal condition as illustrated in the WHO Manual [21]. Daily counts of number of mosquitoes per treatment were analyzed using a generalized linear model (GLM) with negative binomial error structure and log link in R 2.11.0 software [22]. Using the CO_2 baited CDC trap (control) as the reference category, the incidence rate ratios (IRR), a likelihood measure, that mosquito species chose other treatments instead of the control were estimated including Confidence Interval (CI) and corresponding P-values. The IRR for the control is 1 (unity) and values above this indicates better performance and values below under performance of the treatments relative to the control. Chi-square goodness-of-fit was used to analyze the effects of odors on the proportion of total engorged mosquito (i.e., total counts of blood fed + gravid mosquitoes in the total captures) recorded for each trap treatment. Also, a pair-wise test of significant differences in the proportions of engorged mosquitoes between each of the combined CO_2+animal odor treatments relative to the control (CO_2 only) was performed using chi-square goodness-of-fit. Another measure was derived based on the ratio of these proportions for each of the animal odors relative to the control. This measure is termed the catch index; odors which, say, double or halve the catch from a trap would have catch indices of 2 and 0.5, respectively.

Collection of stockinette trapped odors

We collected headspace odors (24hrs) from stockinette samples brought from the field under cold storage on Super Q adsorbent (30 mg, Alltech, Nicholasville, KY) and eluted filters with 200µl dichloromethane (DCM)/hexane mixture (50:50). Headspace trapping was performed using the Volatile entrainment system using the trapped odors on 4 pieces of stockinette material of equal size taken per animal. All the eluents were concentrated under nitrogen to 100µl and followed by GC-EAD and GC-MS analyses.

Coupled gas chromatography electroantennographic detection (GC-EAD) and coupled gas chromatography mass spectrometry (GC-MS) analyses of odors

Mosquito head excised with a scalpel from adult female mosquitoes (aged 5-8 days, for laboratory-reared mosquitoes) were used for electrophysiological recordings. Similar preparations were made for wild caught adult mosquitoes (age unknown for field collected insects) trapped with CO_2-baited CDC miniature light trap and maintained on 6% glucose solution. The excised head was mounted between two glass capillary (1.1 mm I.D.) electrodes filled with Ringer solution (prepared by dissolving 6.5g NaCl, 0.42g KCl, 0.25g $CaCl_2$ and 0.1g $NaHCO_3$ in one liter of distilled water). Silver–silver chloride junctions were used to maintain electrical contact between the electrodes and input of preamplifier (10A; Syntech, Hilversum, The Netherlands). The grounded reference (indifferent electrode) was connected to the base of the antenna, and the tip connected to the recording electrode. The analog signal was detected through a probe (INR-II, Syntech, Hilversum, The Netherlands), captured and processed with a data acquisition controller (IDAC-4, Syntech, The Netherlands) and into a personal computer. Recordings were later analyzed with software (EAG 2000, Syntech, Hilversum, The Netherlands).

In GC-EAD analysis, 2µl of the odor extract was injected into a GC linked to the antenna recording setup. Injections of the extracts were conducted on a HP 5890 gas chromatograph fitted with a splitless injector (220°C) and flame ionization detector (FID) (280°C). Compounds were separated on a nonpolar capillary column HP-1 (30 m x 0.25 mm x 0.25µm film thickness) with nitrogen as the carrier gas. The oven temperature was held at 35°C for 5 min and then increased at 10°C/min to a final temperature of 280°C, which was held for 10 min. The GC was fitted with a split at the end of the column, delivering half the column effluent to the flame ionization detector (FID) and the other half to a humidified airstream (1ml/min) flushing over the

antenna via a heated transfer line (260°C) (Syntech). Commercial authentic standards were used to confirm EAG activity of tentatively identified components following similar procedures.

For GC-MS analysis, 1µl of volatile extract from the different animals were analyzed on an Agilent system consisting of a model HP 6890A gas chromatograph, a model 5973 mass selective detector (EIMS, electron energy, 70 eV), and an Agilent ChemStation data system. The GC column was an HP-5 ms fused silica capillary with a (5% phenyl)-methylpolysiloxane stationary phase (30 m x 0.25 mm x 0.25µm film thickness). The carrier gas was helium with a column head pressure of 7.07 psi and flow rate of 1.0 ml/min. Inlet temperature was 200^{o}C and MSD detector temperature was 280^{o}C. The oven temperature was held at 35°C for 2 min and then increased at 10°C min^{-1} to a final temperature of 280^{o}C, which was held for 10min. The identity of EAG-active compounds was determined by comparison with references from mass spectral libraries (NIST05, Agilent Technologies [NIST database, G1036A, revision D.01.00, ChemStation data system (G1701CA, version C.00.01.08). Final confirmation of identity was achieved by coinjection with synthetic reference compounds. Additionally, trapped odors from unused cotton material were included as control in all GC/MS analysis. Solvent blanks (hexane or DCM) were concentrated and analyzed to identify contaminants. The blanks were analyzed as described for the samples. Compounds present in the blank analyses were excluded from the composition percentages of compounds in the samples. To estimate ratio of abundance of aldehyde components, in each GC-MS run, the percent composition of each of the aldehyde components in the overall chemical profile of headspace odor from each animal was recorded. The percent abundances based on peak areas from an integrated chromatogram were then used to establish mean ratio of one component in relation to the other for each animal after 3 replicate runs (Table S1). The ratios of synthetic blends were also compared to the ratios in the naturally occurring blends. Similar chromatographic data were used to estimate release rates of the constituent aldehydes from each of the animals (Table S2) by recording the peak area of each constituent obtained from an integrated chromatogram. External quantification was done using authentic sample of nonanal. Peak areas were recorded for different known concentrations of nonanal covering the expected analyte concentration range and a calibration curve and subsequent linear equation obtained which was then used to estimate the amount or quantity of each component produced following GC-MS analysis of crude animal volatiles. Estimated

release rate was calculated taken into consideration the trapping duration, total volume of sample eluted in solvent (dichloromethane) and quantity analyzed by GC-MS (1µl).

Field evaluation of identified components on mosquito captures

Heptanal, octanal, nonanal, decanal (>98% pure, Sigma-Aldrich) were formulated in hexane at different concentrations with antioxidant, 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene, BHT, Aldrich) added for field evaluation. Initial field assessments of lures at *icipe*'s Duduville Campus, followed by GC-MS analysis showed that the aldehydes oxidized to their corresponding fatty acids after at least 18 hr exposure. Therefore for long term assay, we formulated our lures using this anti-oxidant. Two milligrams (10% of individual component) of antioxidant was added to 20 mg of each component in 1ml of hexane to obtain a stock concentration and this was serially diluted to obtain various concentrations. Blends were constituted by mixing equal amounts of the respective components. All lures either singly or blends were released by diffusion from 0.5 ml polyethylene tubes with a pin hole in the center of the cap. Preliminary trials to determine possible range of attractive doses of compounds were conducted at *icipe*'*s* Nairobi campus (Table S3). No mosquitoes were trapped in the control CDC trap with the light bulb removed. Informed by similar response profile in all the RVFV mosquitoes, it was clear that likely attractive doses would be at most 5 mg/ml for the individual compounds. Consequently, concentrations of individual compounds, including 0.1, 0.5, 1, 2 and 5 mg/ml were evaluated in preliminary field assessments to determine optimal attractive doses of each compound in three replicate trials (Figure S1). We also conducted an analysis of the amount of aldehyde released from the different animals per hour (Tables S2 and S3). Representative blends simulating ratio of occurrence of the compounds in each of the animal odors were also evaluated. Details of these blends are shown in Table S4. Nominal release rates were measured in the laboratory at 20^{o}C and 0.5 m/sec airflow (Table S5) by loading 0.5ml of each compound tested in replicated dispensers and then measuring weight loss every 12 hours. Final weight loss measurements were recalculated as micrograms of compound lost per hour.

Lures were attached underneath in the airflow of CO_2 (dry ice) released from an Igloo cooler. These were mounted close to the fan of the CDC trap without a light bulb suspended 1.5 m off the ground on a tree. Effect of individual components and blends were evaluated for mosquito

trap captures in field experiments conducted in January 2012 at Ijara district following a randomized experiment in the same predetermined similar area with days as replicates. Traps were rotated on every trapping day to minimize variability due to trap placement. Mosquito captures in CDC traps without a light bulb baited with a combination of CO_2 and different doses of individual components/blends and were compared with captures to control trap with CO_2 alone. Traps were activated within 30 min of sunset (1800-1830 hr) and trap contents collected within 30 min after sunrise (0600-0630 hr). Daily counts of number of mosquitoes in the different trap treatments were recorded and analyzed using negative binomial regression following GLM procedures in R as described previously. Also proportion of engorged mosquitoes and catch indices for each treatment were analyzed as described previously. Data were analyzed for total mosquito captures of primary RVFV vectors (*Ae. mcintoshi* and *Ae. ochraceus*) and for total mosquito collections including other important RVFV *Culex* vectors such as *Cx. pipiens quinquefasciatus*, *Cx. univittatus*, *Cx. poicilipes*.

Ethics Statement

The study was conducted with the approval of the national ethics review committee based at the Kenya Medical Research Institute (KEMRI) and is renewed on an annual basis after a scientific audit. The Animal use component was also given approval by the KEMRI Animal Use and Care committee (KEMRI-AUCC). KEMRI-AUCC complies with the national guidelines for care and use of laboratory animals in Kenya developed by the Kenya Veterinary Association and the Kenya lab animal technicians association 1989. The KEMRI-AUCC which approved the study protocol has an assurance identification number A5879-01 from the Office of Laboratory Animal Welfare (OLAW) under the Kenyan department of health and human services. For purposes of livestock use, funds from the project were used to purchase animals to monitor RVFV seroprevalence and used for all experimental activities described in this study. The animal owners consented to the use of their animals. These animals were owned and maintained for the study by the project. The project bought 492 animals comprising 5 sentinel herds; two in Marigat, three in Ijara district (one in Kotile and 2 in Sangailu). The animals were left with the owners as part of their flocks but they were not allowed to sell or slaughter them because the project was monitoring the animals. The animals were reverted back to the owner at the end of the project activity. Any new borns born out of the tagged animals belonged to the farmers. We worked in collaboration with the department of veterinary services and veterinary doctors

mandated by the government to do livestock sampling and research. The above terms were stipulated well in an agreement between the farmers and the international Centre of Insect Physiology and Ecology (*icipe*), the hosting institution for the AVID Project Consortium. Human odor was collected from one of the authors, DPT, on worn stockinette and the mosquitoes are the subject of the experiment which responded to the stimuli on the stockinette. Entomological surveys were conducted away from homesteads and on community land as authorized by Community Elders after explaining the purpose of the study to them.

Results

Mosquito sampling with animal skin odors

Compared to the control CO_2-baited trap, the addition of mammalian skin odors in all the treatment traps not only selectively targeted mosquitoes, but also significantly increased trap captures of primary RVFV vectors (*Ae. mcintoshi*/*Ae. ochraceus*) (p=0.043); cow [IRR=2.01, CI (1.14-3.57)], donkey [IRR=1.95, CI (1.10-3.45)], goat [IRR=2.12, CI (1.20-3.75)] and sheep [IRR=1.66, CI (1.03-2.95)] (Figure 3). Notably, addition of human skin odors did not significantly increase mosquito captures over the control [IRR=1.33 CI (0.74-2.38)]. A similar pattern of mosquito captures was observed for secondary vectors of RVFV, mainly *Culex* and *Mansonia* species in the mammalian skin-baited traps with a combination of CO_2 and skin odors of these hosts although no significant differences were found compared to CO_2 only (p=0.872 for *Culex* spp. and p=0.964 for *Mansonia* spp). Performance on captures of total *Culex* spp. (*Culex. pipiens*, *Cx. univittatus*, *Cx. poicilipes* and *Cx. bitaenorryhnchus*) were: cow [IRR=1.13, CI (0.70-1.82)], donkey [IRR=1.18 CI (0.74-1.91)], goat [IRR=1.09, CI (0.68-1.76)], human [IRR=1.34, CI (0.83-2.15)], sheep [IRR=1.26 , CI (0.78-2.02)] and for total *Mansonia* spp. (*Mansonia. uniformis* and *Mn. africana*): cow [IRR=1.30, CI (0.62-2.75)], donkey [IRR=1.37, CI (0.65-2.90)], goat [IRR=1.09, CI (0.52-2.30)], human [IRR=1.26, CI (0.60-2.66)], sheep [IRR=1.18 CI (0.56-2.48)]. A significant difference in the proportion of engorged mosquito was also observed (i.e., blood fed +gravid) recorded in the different treatments for both primary vectors (χ^2=28.838, df=5, p<0.001) and secondary vectors (χ^2=122.897, df=5, p<0.001). A higher proportion of engorged mosquito in baited traps containing CO_2 plus animal odor relative to the control CO_2 trap alone was evident (Table 1).

Chemical identification of animal skin odors

By comparing GC-EAD patterns, four identical peaks were observed in volatiles from each host that consistently elicited antennal responses from the different mosquito species (Figure 4). Using GC-MS, the components representing these peaks were identified as heptanal, octanal, nonanal and decanal and confirmed their identities by comparing retention times and fragmentation patterns with authentic standards. The total amount of these aldehydes in the volatiles varied with the host *viz*: cow, 29-43%; goat, 45-56%; donkey, 35-63%; sheep, 26-44 %; and human, 18-40%.

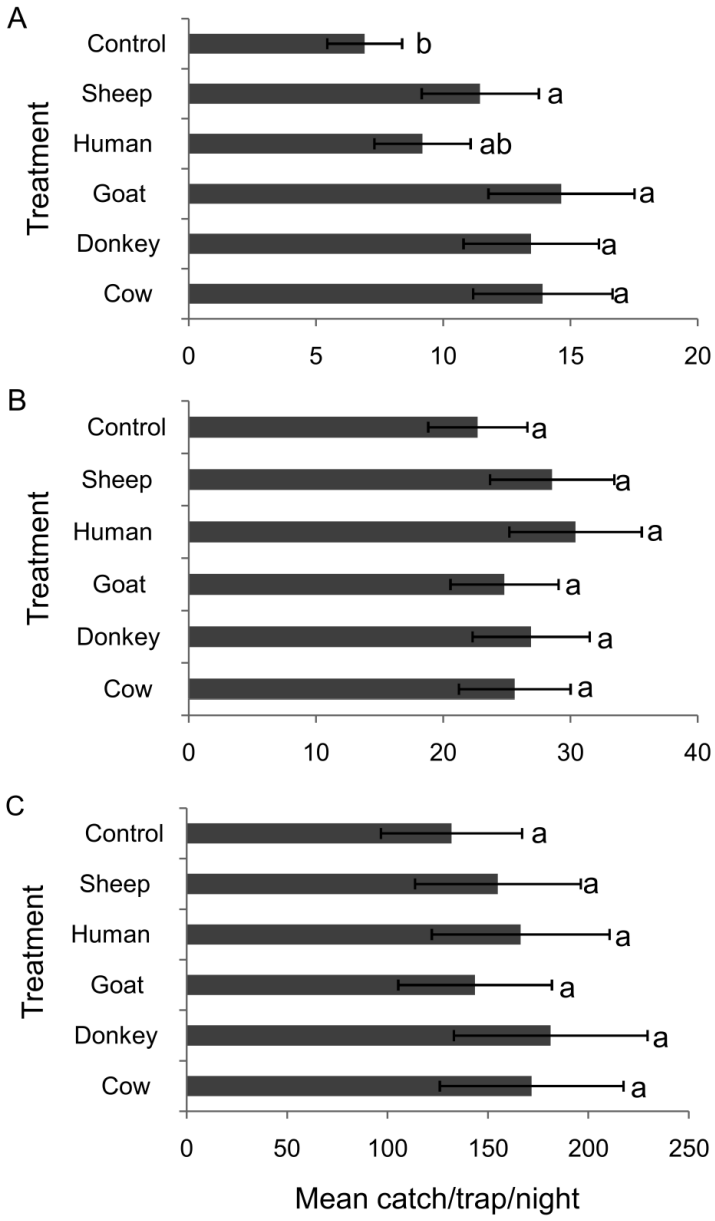

Figure 3. Mean daily captures of RVFV vectors in the different trap treatments in 11 replicate trials. (A) primary vectors (Ae. mcintoshi and Ae. ochraceus); secondary vectors comprising (B) total Culex spp.; (C) total Mansonia spp. Control, CO2-baited traps only; host treatments represent skin odors from each host type combined with CO_2. Treatments followed by the same letters are not significantly different at P=0.05 following generalized linear model (GLM) with negative binomial error structure and log link in R 2.11.0 software; Error bars indicate standard error of the mean.

Table 1. Proportion of engorged RVFV mosquito (blood fed + gravid) of total captures recorded in control traps (CO2 only) and combined lures having CO2 and crude skin odors from the different hosts evaluated with corresponding catch indices.

Treatment	Primary vectors (n)	Catch index	P-values	Secondary vectors (n)	Catch index	P-values
Control (CO_2 only)	0.21 (76)	1	-	0.06 (3209)	1	-
Cow	0.46 (153)	2.17	<0.001	0.12 (4057)	2.15	<0.001
Donkey	0.24 (148)	1.16	0.703	0.09 (4277)	1.62	<0.001
Goat	0.30 (161)	1.45	0.207	0.12 (3497)	2.15	<0.001
Sheep	0.34 (126)	1.62	0.069	0.10 (3829)	1.79	<0.001
Human	0.20 (101)	0.94	0.987	0.08 (4101)	1.44	0.001

Primary vectors (*Ae. mcintoshi* and *Ae. ochraceus*); secondary vectors (total counts of *Culex* and *Mansonia* spp.); n in parenthesis represents total mosquito captures recorded per treatment. P-values based on pair-wise comparison to CO_2 following chi-square goodness-of-fit in R 2.11.0 software.

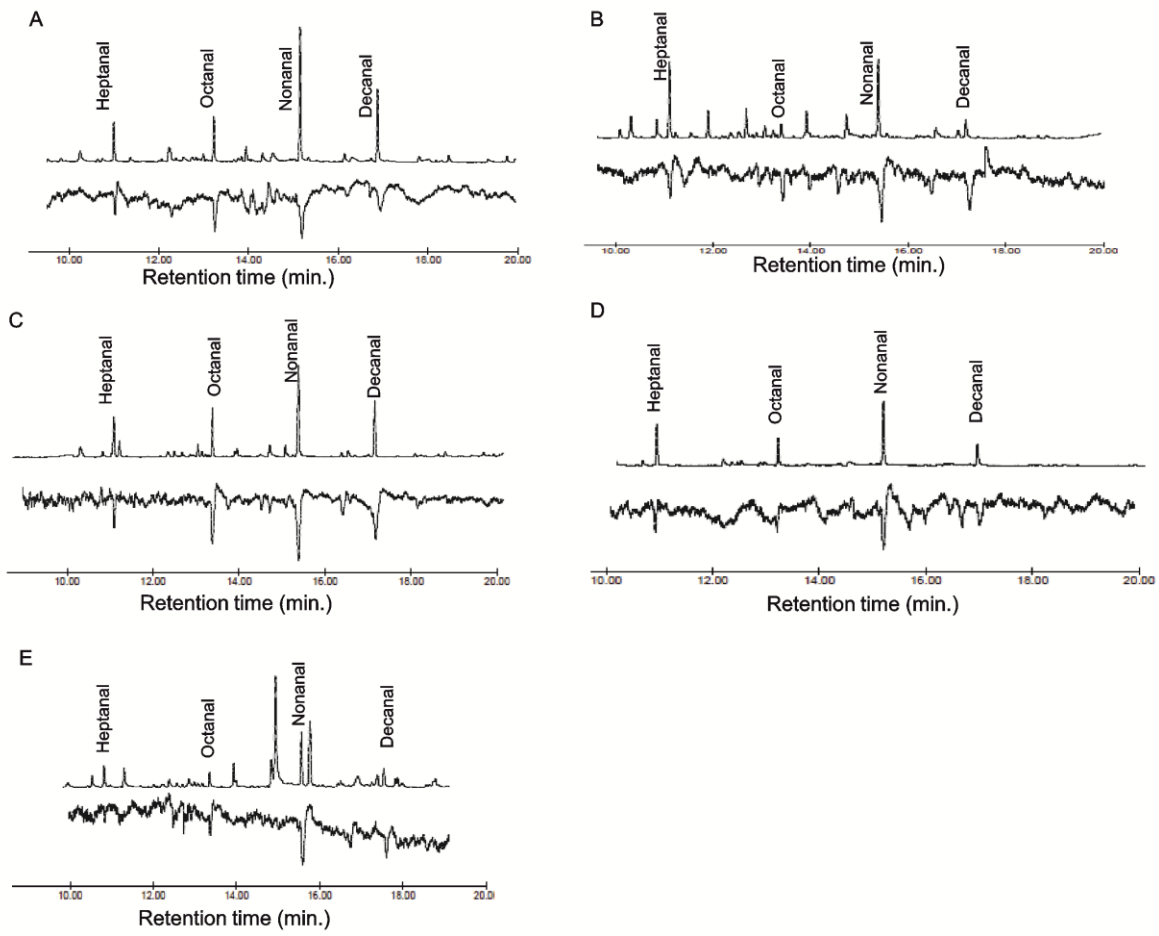

Figure 4. Representative GC-EAD profiles using wild caught adult female Ae. mcintoshi to the different host odors. (A) Cow (B) Donkey (C) Human (D) Goat (E) Sheep. Upper traces are FID (chemical profile) of the respective host odor and lower traces are EAD responses. Regardless of host type, similar responses to the four aldehydes (whose peaks are labeled in the uppermost trace) were reproducibly recorded not only in this species but in *Ae. Ochraceus* and diverse species of *Culex* and *Mansonia* which are secondary RVFV vectors (n=3). (Scale bar in all the GC-EAD runs, 1 mV.)

Development and evaluation of an improved RVFV mosquito vector trapping system

To maximize sensitivity of the trapping system to target selectively RVFV mosquito vectors, mosquito trap captures were first compared in preliminary field dose-response assays using CO_2-baited CDC trap without a light bulb combined with individual synthetic EAG-active compounds to the control trap baited with CO_2 alone. Since chemical analysis showed that the total amount of aldehydes varied within and between replicates of individual host odors, a blend (Blend F) was therefore formulated from the four aldehydes based on the doses of individual components that elicited optimal attraction in the preliminary field assays (Figure S1). These were heptanal, 2mg/ml; octanal, 0.5 mg/ml; nonanal, 0.1 mg/ml; and decanal, 0.1 mg/ml (Figure S1). Representative blends were also constituted reflecting the mean ratio of occurrence of these aldehydes in each of the animals: Blend A (cow); Blend B (human); Blend C (goat); Blend D (sheep); Blend E (donkey) (Table S4). In subsequent dose-response field assays, the attractiveness of these blends (A-F) were then compared to these individual components at their respective optimal doses. Overall for individual components, heptanal recorded the highest captures (61% increase at 2 mg/ml), followed by nonanal (44% increase at 0.1 mg/ml, decanal (36% increase at 0.1 mg/ml and octanal (34% increase at 0.5 mg/ml) (Table 2). However, these increases were not significantly different from the control captures (Table 2).

There was significant treatment effect on mosquito captures of primary RVFV vectors (*Ae. mcintoshi* and *Ae. ochraceus*), (χ^2=104.81, df=10, p=0.003) and for total mosquito captures (χ^2=107.28, df=10, p=0.01). Expectedly, there was an observed increased captures of total primary RVFV vectors in traps baited with CO_2 combined with the optimal doses of each component and blends representing the mammalian odors (Blends A-E) although these captures were not significantly different from the control (Table 2). Interestingly, Blend F formulated from the optimal attractive doses of individual components performed far better than any of the representative mammalian blends (A-E) and the individual components (Table 2), trapping significantly three-fold more of the primary RVFV vectors than the control trap [(IRR=3.23, CI (1.76-5.91)] (Table 2). Equally interesting, there was a significant increase in total mosquito captures including *Culex* RVFV secondary vectors relative to the control [IRR=2.35, CI (1.33-4.18)]. An equally interesting finding observed, was a significant difference in the proportion of engorged mosquito in the total mosquito captures for the optimal compounds and blends tested

(χ^2=56.174, df=10, p<0.001) relative to the control (Table 3). Similarly as found for animal crude odors, a clear pattern of a higher proportion of engorged mosquitoes in all traps containing CO_2 plus single compounds/blends relative to the control was observed (Table 3).

Table 2. Captures of primary RVFV mosquito vectors (*Ae. mcintoshi* and *Ae. ochraceus*) recorded at different optimal synthetic component doses and a blend in 9 replicate field trials.

Aldehyde treatments	Number caught	IRR (95% CI)	P-values
Blend A	104	1.30 (0.70-2.43)	0.408
Blend B	85	1.06 (0.57-2.00)	0.850
Blend C	96	1.20 (0.64-2.24)	0.567
Blend D	88	1.10 (0.59-2.06)	0.766
Blend E	91	1.14 (0.61-2.13)	0.686
Blend F	258	3.23 (1.76-5.91)	<0.001
2 Heptanal	129	1.61 (0.87-2.99)	0.128
0.5 Octanal	107	1.34 (0.72-2.49)	0.358
0.1 Nonanal	115	1.44 (0.77-2.67)	0.250
0.1 Decanal	109	1.36 (0.73-2.54)	0.328

Incidence rate ratio (IRR) and corresponding 95% confidence interval (CI) and P-values; P-values based on comparison to CO_2 following generalized linear model (GLM) with negative binomial error structure and log link in R 2.11.0 software; number in front of each compound represents the optimal dose of each component evaluated in mg/ml.

Table 3. Proportion of total engorged RVFV mosquito (blood fed +gravid) of total captures recorded in control traps (CO2 only) and combined lures having CO2 and optimal single compounds/blends evaluated and corresponding catch indices.

Treatment	Optimal dose (mg/ml)	Proportion (n)	Catch index	P-values
Control (CO_2 only)		0.05 (210)	1	-
Heptanal	2	0.19 (325)	3.58	<0.001
Octanal	0.5	0.14 (291)	2.69	0.002
Nonanal	0.1	0.08 (320)	1.43	0.271
Decanal	0.1	0.09 (299)	1.66	0.152
Blend A		0.08 (237)	1.45	0.326
Blend B		0.07 (268)	1.28	0.523
Blend C		0.13 (243)	2.51	0.007
Blend D		0.07 (237)	1.29	0.518
Blend E		0.15 (212)	2.88	0.001
Blend F		0.14 (494)	2.63	0.001

n in parenthesis represents total mosquito captures recorded per treatment. P-values based on pair-wise comparison to CO_2 following chi-square goodness-of-fit in R 2.11.0 software.

Discussion

RVF represents a looming health threat to various parts of the world [2]. The virus continues to circulate among animals and humans in many areas, both during intermittent epidemics/epizootics and IEPs [4,23]. Thus, the presence and intimate association of capable vectors and susceptible hosts such as livestock, humans in the same ecosystem as in Marigat district and Ijara district of North-Eastern Kenya, may help sustain this virus. As such, low numbers of vectors may be required for virus maintenance in a population of large susceptible vertebrate hosts in the environment as in the IEP. Spread and introduction of infection to new areas over long distances via movement of infected vectors remains plausible.

This studydemonstrated overwhelmingly through robust field-guided and chemical analyses that mammalian skin odors attract RVFV vectors. The findings concur with published literature highlighting the importance of animal skin odors in mosquito attraction [17,24-26]. Among the animal hosts examined, primary RVFV vectors (comprising *Ae. mcintoshi* and *Ae. ochraceus*) showed a bias towards skin odors of animal hosts compared to humans in agreement with previous observations showing that during the 2007/2008 RVF outbreak in North Eastern Kenya these two mosquito species accounted for approximately 80% of positive pools of mosquitoes sampled in the field [8]. Furthermore, this observation supports the epidemiology of RVF as a zoonosis with circulation mainly among vertebrate animals which serve as efficient amplifiers of the virus and only incidental transmission to humans [10,27]. For the secondary *Culex* and *Mansonia* vector species, although there was a marked effect of odors on mosquito captures, there was no clear pattern of preference in their attraction among the animal host odors examined. This may suggest a more widespread feeding pattern of these vectors and cement their role as bridge vectors in the extension of the disease to humans.

The results show that the amounts of host skin-derived aldehydes varied within and between hosts, and further that given the commonality of the host skin-derived volatiles; these profiles may extend to related mammals attractive to RVFV mosquito vectors including wildlife. Host skin-derived aldehydes seem to play an important role in the attractiveness of RVFV mosquitoes, but better as a blend rather than as individual components. This pattern has been observed for other mosquito species and tsetse flies in combination with other chemical compounds [28-30]. The data show that blends of synthetic aldehydes representing different host animals worked in combination with CO_2 to attract RVFV mosquito vectors differentially. Because these captures were comparable to those we obtained with the crude skin volatiles, our data lends strong support for the role of aldehydes in mosquito attraction to host animals. However, a striking feature of our evaluation of aldehyde blends is the higher attractiveness for the altered blend formulated from doses of the individual aldehydes that elicited optimal attraction of vectors, clearly demonstrating its potential for practical use in monitoring RVFV mosquito vector populations. From this observation there is strong conviction that the ratios and release rates of aldehydes from an individual animal, irrespective of the host, determine its relative attractiveness in a herd. As such, some individuals in a herd would be relatively more attractive and serve as a sponge for RVFV mosquito vectors than others as demonstrated in this study when addition of individual

compounds, at certain doses, reduced mosquito trap capture while other blends of components significantly increased trap capture. Nonetheless, because of the commonality of the host chemical signature required for attraction, the host-vector interaction would appear to favor the mosquito vector allowing it to find and opportunistically feed on a wide range of mammalian RVFV hosts. Hence, the natural survival of this arboviral pathogen is tightly bound to the success of this olfactory-based activity which these mosquito vectors use in seeking and feeding on multiple hosts. It is possible that microbial endogenous breakdown products of surface lipids may be the likely origin of these compounds on animal skin or from exogenous deposition via contact with foreign substances [31], which would require additional research. Furthermore, the influence of skin bacteria on mosquito attraction has recently been highlighted [32].

A number of studies have reported the importance of aldehydes in the sensory ecology of mosquitoes [26,33,34] and various blood feeding arthropods, including ticks [35], triatomine bugs [36], and tsetse flies [37,38]. For mosquitoes in particular, their roles in the balance of attraction and inhibition have been suggested [39,40] mainly in laboratory assays and with limited efforts in field settings. Nonetheless, in a recent study CO_2 was reported to synergize nonanal to increase trap captures of *Culex* mosquito vectors of West Nile Virus [26]. Clearly, the data in this study stress a fascinating dose-dependent behavioral blend effect of four aldehydes as kairomones which in combination with CO_2 significantly increases trap captures for RVFV mosquito vectors. Furthermore, the data also suggest that individually, heptanal, octanal and decanal can also be exploited in a similar manner to increase field captures of RVFV mosquito vectors.

Surveillance to monitor virus movement among vectors and hosts is crucial in informing public health decision makers for early warning and rapid response. Although trapping of adult female mosquito vectors remains a cornerstone of this strategy, efficient trapping tools for most of these RVFV vector species remain wanting especially during the IEP due to low sensitivity and non-specificity of currently available CO_2-baited light trap. Moreover, enzootic transmission of arboviral diseases continues to occur at a low intensity among mosquito vectors in Kenya [23] and remain undetected. The development of the attractant blend described here circumvents the challenges by increasing captures not only for key RVFV vectors but also diverse mosquito species. This constitutes an important landmark as a practical effective population monitoring

tool especially during the IEP so as to maximize trap captures for viral isolation in order to reveal the true burden of arbovirus circulating in affected communities. Furthermore, once mosquito vectors have been trapped and identified, their populations can be tracked to reveal important epidemiological parameters such as, population age structure, infection status, blood feeding patterns all culminating in assessing disease transmission risk. Improved arboviral vector surveillance equally requires knowledge of the mosquito population being sampled. Blood fed and gravid mosquito cohort because of their previous host encounter can be advantageous during surveillance as testing this cohort increases the likelihood of viral detections. The results clearly indicate that traps baited with CO_2 and animal skin odors both crude and synthetic compounds captured a higher proportion of engorged mosquitoes (blood-fed + gravid) than control traps. Our findings therefore, suggest the inclusion of attractive skin odors to CO_2-baited traps for improved entomological surveillance.

The blend developed requires combination with CO_2 supplied in the form of dry ice. This commercial source of CO_2 is not only expensive but may readily be unavailable in remote areas, which may hamper mosquito collection. However, alternative forms of generating CO_2 as an attractant using yeast have been evaluated [41,42]. However, the efficacy of CO_2 supplied as dry ice has been shown to increase mosquito captures significantly over that generated using yeast [43]. As the search for a suitable substitute for CO_2 continues, a recent study identified 2-butanone as a mimic of CO_2 activity following similar activation patterns in the odorant receptor neurons of mosquitoes [44]. The behavioral significance in terms of attraction in the natural habitat of mosquito vectors remains to be evaluated towards developing economical lures for use in trap-based mosquito surveillance especially in remote settings.

In summary, this work is the first comprehensive report of translational research utilizing chemical ecology to generate better tools for surveillance of RVFV vectors. The study employed field bioassay guided experiments in combination with conventional chemical ecology approaches to identify four compounds, heptanal, octanal, nonanal and decanal, which when tested in the field, singly and in blends, increased capture of a number of RVFV mosquito vectors. The most effective blend (Blend F), significantly improves attraction when used in conjunction with CO_2 over that of the CO_2-baited CDC traps alone, the latter currently used for

surveillance and provides a clear improvement in the ability to monitor mosquito vectors especially during the IEP.

Acknowledgments

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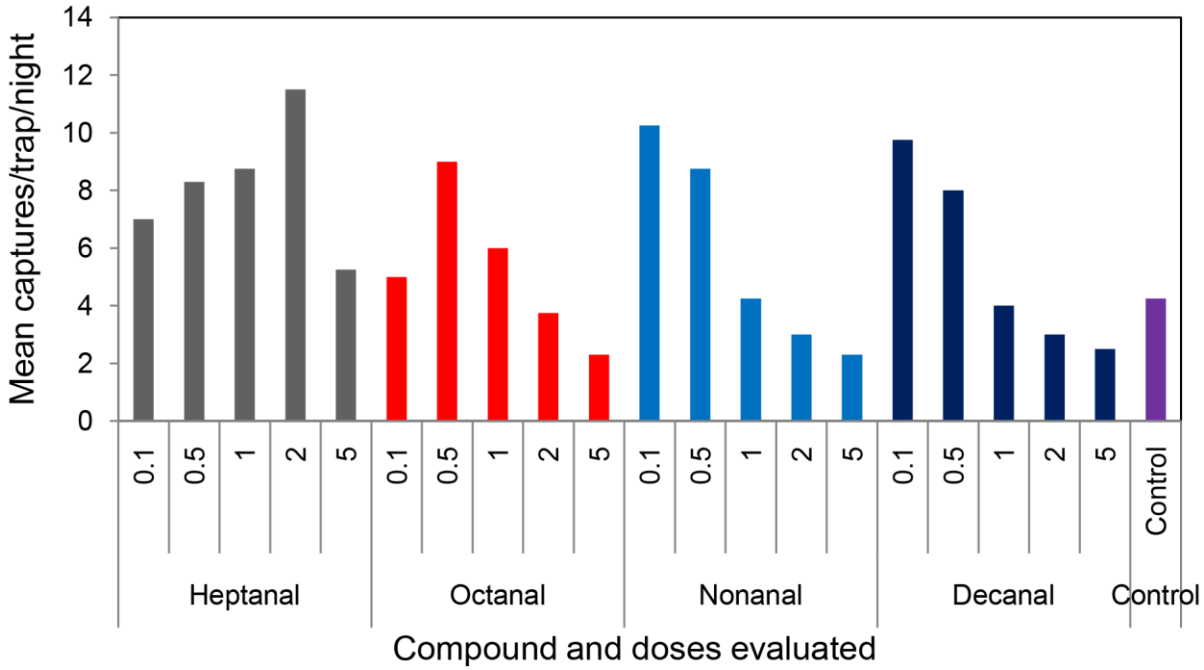

Figure S1. Mean captures of primary RVFV vectors to different doses of compounds in preliminary field trials to establish optimal doses. Numbers represent doses of each compound in mg/ml tested in combination with CO2; control, CDC trap without a light bulb baited with CO_2 only; number of replicates, n=3.

Table S1. Approximate mean ratio of aldehyde components in the host odor profiles in GC-MS runs.

	Compound			
Host	Heptanal	Octanal	Nonanal	Decanal
Human	1	1	4	2
Goat	1	1	3	2
Cow	1	1	3	2
Donkey	4	1	4	2.7
Sheep	1	1	2	1.5

Table 5. Estimated mean amounts of aldehyde components released from the skin host volatiles.

	Compound			
Host	Heptanal	Octanal	Nonanal	Decanal
Human	1	1	4	2
Goat	1	1	3	2
Cow	1	1	3	2
Donkey	4	1	4	2.7
Sheep	1	1	2	1.5

Table 6. Preliminary trials conducted at icipe Duduville campus, Nairobi.

Compound	Dose (mg/ml)	Mosquito captures[1] (n=6)
Heptanal	1	10
	5	8
	10	0
Octanal	1	7
	5	5
	10	0
Nonanal	1	9
	5	0
	10	0
	20	7
Decanal	1	0
	5	0
	10	0
Control		

[1]Trap captures mainly *Culex quinquefasciatus* compared in CDC traps without a light bulb baited with individual doses of the compounds.

Table S4. Blend composition.

	Compound (mg/ml)			
Blend	Heptanal	Octanal	Nonanal	Decanal
Blend A	0.125	0.165	0.5	0.3
Blend B	0.25	0.33	1	0.6
Blend C	0.2	0.2	0.5	0.375
Blend D	0.5	0.125	0.5	0.5
Blend E	0.75	0.25	1	0.375
Blend F	2	0.5	0.1	0.1

Table S5. Optimal dose of single compounds and estimated release rates together with blends evaluated

Compound	Optimal dose (mg/ml)	Release rate (µg/hr)
Heptanal	2	0.14
Octanal	0.5	0.12
Nonanal	0.1	0.13
Decanal	0.1	0.15
Blend A		0.16
Blend B		0.05
Blend C		0.12
Blend D		0.13
Blend E		0.15
Blend F		0.14

Chapter 5

Aedes mcintoshi genetic diversity and magnitude of Rift Valley fever in Kenya

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Abstract

The complex interplay of virus-host-vector cycle driven by climate change has been attributed to the spread and sporadic outbreak of many arboviral diseases such as Rift Valley fever (RVF). However, the genetics of vectors in conditioning their maintenance and spread is least appreciated despite variation which may be evident in the pattern of outbreak occurrence. In this study the *COI* mitochondrial gene (1448 bp inclusive of the barcode region) and *ITS* nuclear marker (1065 bp) were used to characterize the genetic structure of *Aedes mcintoshi* populations, a key vector of Rift Valley fever (RVF) virus, from 14 sites including virus-endemic/free areas and epidemic prone areas of Kenya. Neighbor-joining trees, Bayesian inference, median joining network and analysis of molecular variance (AMOVA) confirmed the presence of four discrete genetic lineages and high levels of genetic diversity among these lineages across the study areas. The lineages displayed distributional differences correlating with the magnitude of RVF occurrence in Kenya possibly influenced by prevailing environmental conditions at these locations. Broadly for both markers, lineage I was restricted to Central, Rift Valley and Western areas and lineages III and IV were restricted to localities in North Eastern Kenya. However within North Eastern Kenya, the epicenter of RVF epidemics in Kenya, these two lineages (III and IV) occurred in sympatry at most of the localities sampled and had relatively lower between-lineage mean evolutionary distances suggestive of the presence of two variants. The disproportionate abundance of these two genetically distinct lineages within the RVF epicenter locality may drive differential transmission and outbreak patterns of the disease in different communities of the North Eastern Province of Kenya. Of interest is that on the basis of the *COI* barcoding region, lineages III and IV constitute one of three species of what is currently defined as *Ae. mcintoshi* on the basis of morphology, with lineages I and II each corresponding to the other two species. The identification of three genetically diverse, monophyletic lineages paves the way for investigating RVFV competence among individuals representative of the three putative species, and the two North Eastern province variants and signals the intensification of efforts to resolve taxonomic relationships within this important RVF species complex.

Introduction

Rift Valley fever (RVF) is a mosquito-borne viral infection that affects humans, livestock and many other animals [1-4]. Outbreaks caused by multiple lineages of the virus have occurred at irregular intervals especially in sub-Saharan Africa [5]. In Kenya, RVF activity has been detected in animals and mosquitoes across a wide area [3,6-8]. Even within different climate zones, RVF virus (RVFV) transmission may vary considerably as a function of fine-scale differences in local environment [6,7,9]. Such microgeographic variation in habitat types is known to promote significant genetic diversity within and between populations of vectors [10]. Moreover, arboviral transmission is controlled by both environment and genetic factors and varies both temporally and geographically between different vector species or among individuals and even populations of the same species [11-13]. However, the extent to which the observed temporal and spatial differences in RVFV transmission are hinged on vector genetic variation is currently unknown.

In Kenya, *Aedes mcintoshi* has been implicated as a primary vector of RVFV following isolation of the virus from field collected samples [8,14,15]. *Aedes mcintoshi* is the most widespread species belonging to the savanna group of the *Aedes* subgenus *Neomelaniconion* comprising of at least seven species including undescribed ones [16]. More importantly, the taxonomic status of this group which contains non-vector and vector species involved in the inter-epizootic maintenance and transmission of RVF [14,16-20] remains unresolved as the number of constituent taxa in this group and their relationships is unclear [16]. These taxonomic uncertainties are underscored by the earlier misidentification of this species, the major RVF vector in Kenya, as *Ae. lineatopennis* [21].

Separation of member species in this group relies solely on male genitalia or morphological analysis of females [16,22]. Males are seldom encountered in the commonly used CDC light traps used for adult mosquito sampling and with respect to females; slight damage to the specimens makes species identification difficult. Therefore, separation of member species in this subgenus has been based on doubtful morphological features [8,23] and the true geographical distribution, relative abundances, occurrence and ecological characteristics of different member species in this subgenus is severely lacking in the different parts of Kenya. More importantly, it remains unclear if populations, or subpopulations, of a single species of what is currently

described as *Ae. mcintoshi* sustain virus maintenance in Kenya or if occurrence of sympatric populations of two or more other vector species in the group are involved.

Genetic analysis plays an increasingly important role in identifying changes in population structure, elucidating taxonomic status and phylogenetic relationships [10,24-27]. Such analyses provide an important framework for understanding the actual vector populations and genetic divergence that contribute to the spread and differential disease transmission patterns [10,28]. Both nuclear and mitochondrial genome targets have been used extensively in studies of molecular evolution [29-32] and these gene markers have resolved evolutionary relationships among closely related or cryptic mosquito species complexes [27,28,31,33]. Furthermore, the discovery of an increased number of sibling species among mosquitoes and other arthropods using genetic approaches highlights the taxonomic unreliability of anatomical features for species identity [10].

Epidemiological diversity of RVF activity in Kenya has been observed based on the level of animal and human exposure and outbreak patterns of the disease in defined ecological areas which include: North Eastern Province; Central Province (Ruiru and Muranga), Rift Valley Province (Marigat and Naivasha) etc. Differences in selection pressures due to differing degrees of disease endemicity or microclimatic heterogeneities at these sites can impact on the diversity and population structure of vectors. This study was undertaken to assess the population genetic structure of what is currently described as *Ae. mcintoshi* against the backdrop of variable ecological and epidemiological factors present in Kenya. To ensure broad geographical coverage, 14 sampling sites inclusive of RVF endemic, free and epidemic areas were included. Using mitochondrial and nuclear sequence data, we sought to address the following questions: 1) what is the diversity and distribution of *Ae. mcintoshi* in Kenya? and 2) To what extent does vector population genetic structure explain the pattern and incidence of RVFV outbreaks in Kenya? Use of both markers was justified as confirmation of monophyly with a nuclear gene marker for monophyletic lineages identified with the more rapidly evolving mitochondrial gene marker, confirms complete lineage sorting and lends support when assigning species. Moreover, as the *COI* target incorporates the barcoding region that guides species delineation and as this mitochondrial gene and the nuclear *ITS* region are both highly represented in the Genbank database, the availability of reference data for other *Aedes* species is ensured.

Results

Phylogeny

A total of 165 and 79 sequences were analysed for the *COI* and *ITS* loci, respectively. The principal component analysis (PCA) based on the larger *COI* dataset yielded four components; the first two principal axes contributed 81.2 % (the first axis 65.1 %, and the second axis 16.1 %) of the total variance and showed partial separation of these populations into four clusters (Figure 1).

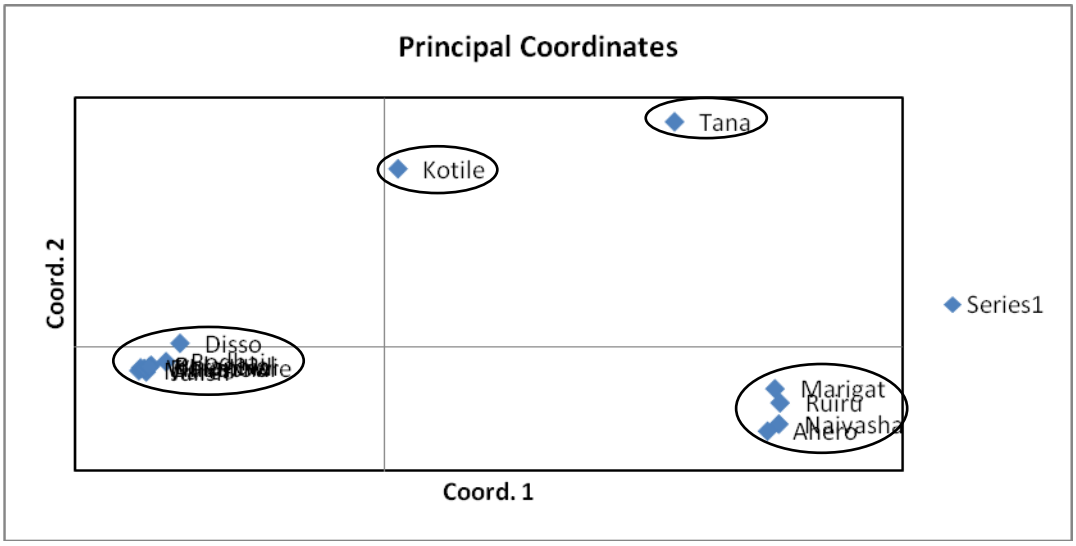

Figure 1. Plot of the principal coordinate analysis (PCA) generated using GenAlEx for *Aedes mcintoshi* samples from the different localities.

The clustering observed by PCA using the genetic distances was confirmed in the NJ tree. Four *Ae. mcintoshi* lineages (denoted I-IV) were detected with both gene loci, and all, with the exception of lineage III, in the *COI* phylogeny, were well supported (Figures 2a and 2b). Furthermore, the same four lineages were recovered when the concatenated (*COI+ITS*) dataset was used for phylogenetic inference, although Bayesian posterior probability (BPP) values were low for lineages III and IV (Fig. 3) for this dataset. On the basis of these results, the four lineages were defined and treated as different populations. However, it should be noted that analyses based on the *COI* barcoding region alone, suggest that these four lineages may in fact represent

three discrete species, tentatively denoted species A-C (Fig. 4). In particular, the mean between-lineage distances for the barcoding region suggest that lineage I corresponds to 'species A', lineage II to 'species B' and lineages III and IV together, to 'species C' (Fig. 4). Despite this, haplotype clustering patterns for both gene loci in a median-joining network confirmed the presence of four discrete genetic clusters of haplotypes (Figures 5a and 5b) corresponding to lineages I-IV. For both gene loci, lineage I (BS of 99 % and 98 % for *COI* and *ITS*, respectively) was restricted to Marigat, Ruiru, Naivasha and Ahero. Samples from Tana were exclusive to lineage II (BS 100 % for both *COI* and *ITS*) for both loci, although distribution of this lineage was much wider for the *COI* locus which included samples from Kotile, and a few from Marigat, Ruiru and Disso. Notably, lineages III (BS 64 % and 100 % for *COI* and *ITS*, respectively) had a wider distribution and together with lineage IV (BS 75 % and 98 % for *COI* and *ITS*, respectively, for both loci) were restricted to localities in the North Eastern Province of Kenya. Thus, there was an extensive area of overlap in the distribution of lineages III and IV within North Eastern Province of Kenya, involving samples from seven of the eight localities sampled in that region (Figures 5a and 5b). A slight incongruence in the distribution of samples among these lineages between both loci was evident. For the *ITS* locus, all sequences from Disso and Elhumow were resolved in lineage IV with those from Bodhai were exclusive to lineage III; a pattern which showed overlapping distribution from these sites in lineages III and IV for the *COI* locus. However for both markers, a higher proportion of samples from the North Eastern localities were represented in lineage IV relative to lineage III again suggesting sympatry in occurrence of these lineages at most of the localities. With respect to the *COI* locus, Kotile can be considered the most diverse having individuals from lineages II, III and IV present.

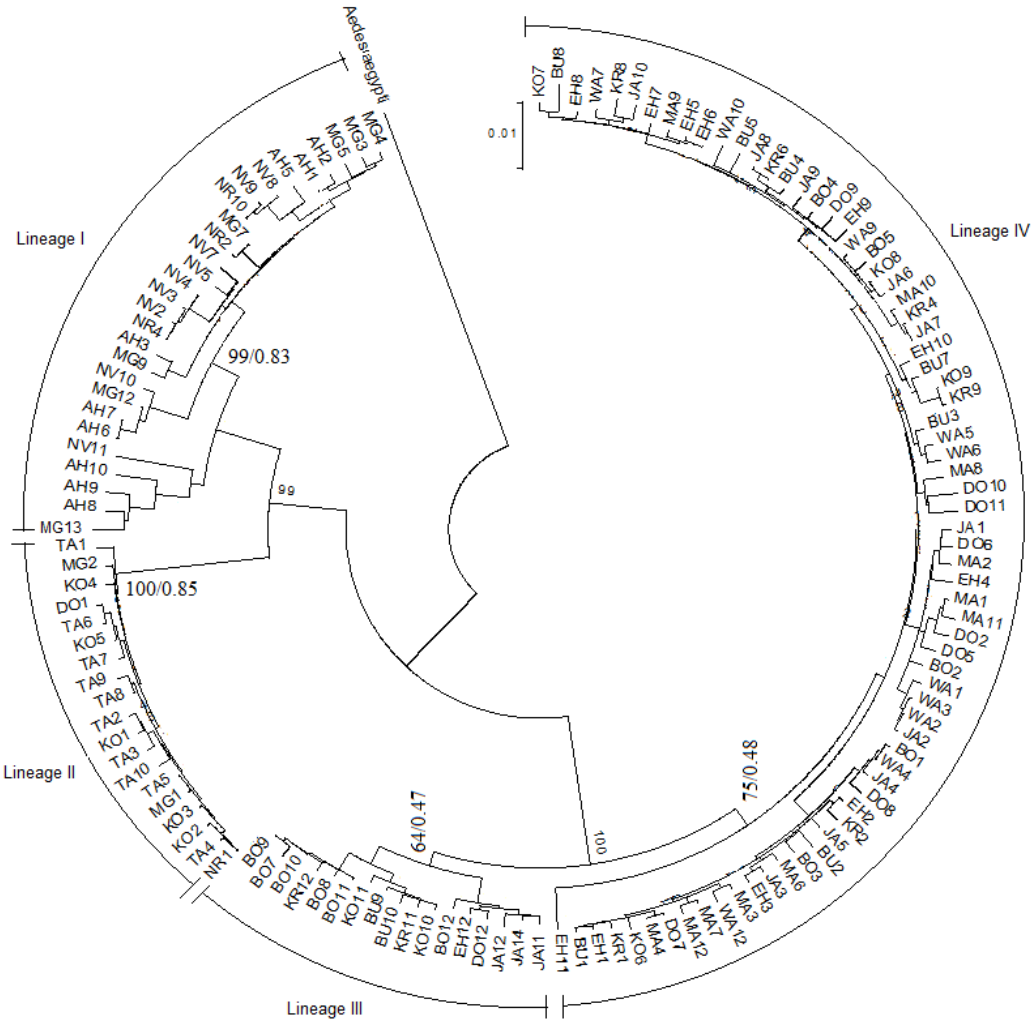

Figure 2a. *COI* gene relationships between *Aedes mcintoshi* from Kenya represented by a neighbor-joining tree with posterior probabilities from Bayesian inference transferred onto the relevant nodes. Taxon abbreviations follow those provided in Table 7.

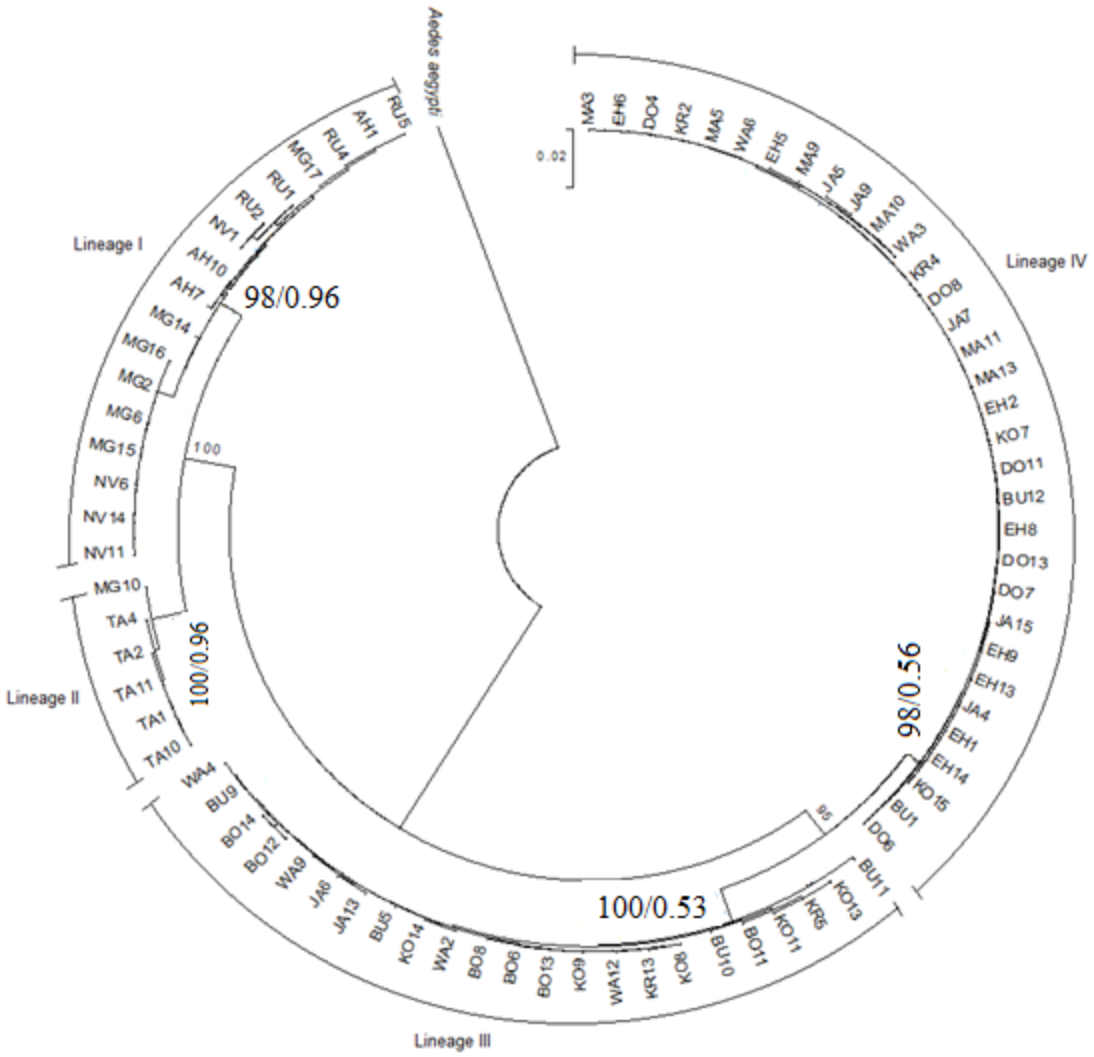

Figure 2b. *ITS* gene relationships between *Aedes mcintoshi* from Kenya represented by a neighbor-joining tree with posterior probabilities >70% from Bayesian inference transferred to the relevant nodes. Taxon codes follow those provided in Table 7.

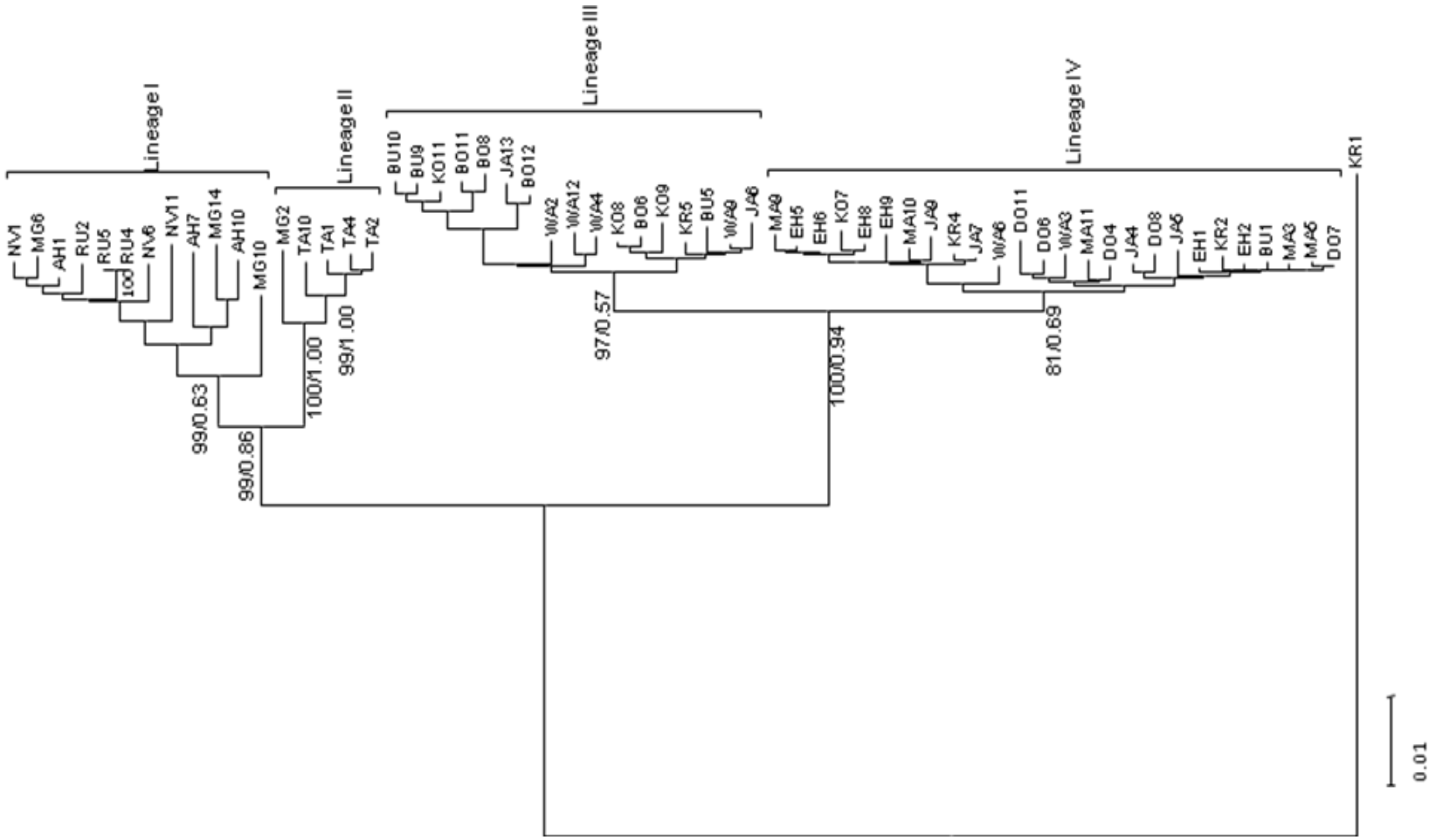

Figure 3. Neighbor-Joining p-distance tree for concatenated (*COI* and *ITS*) dataset with pairwise deletion of gaps and missing data. Nodal support values are indicated NJ/BPP next to the relevant nodes. Taxon codes follow those provided in Table 7.

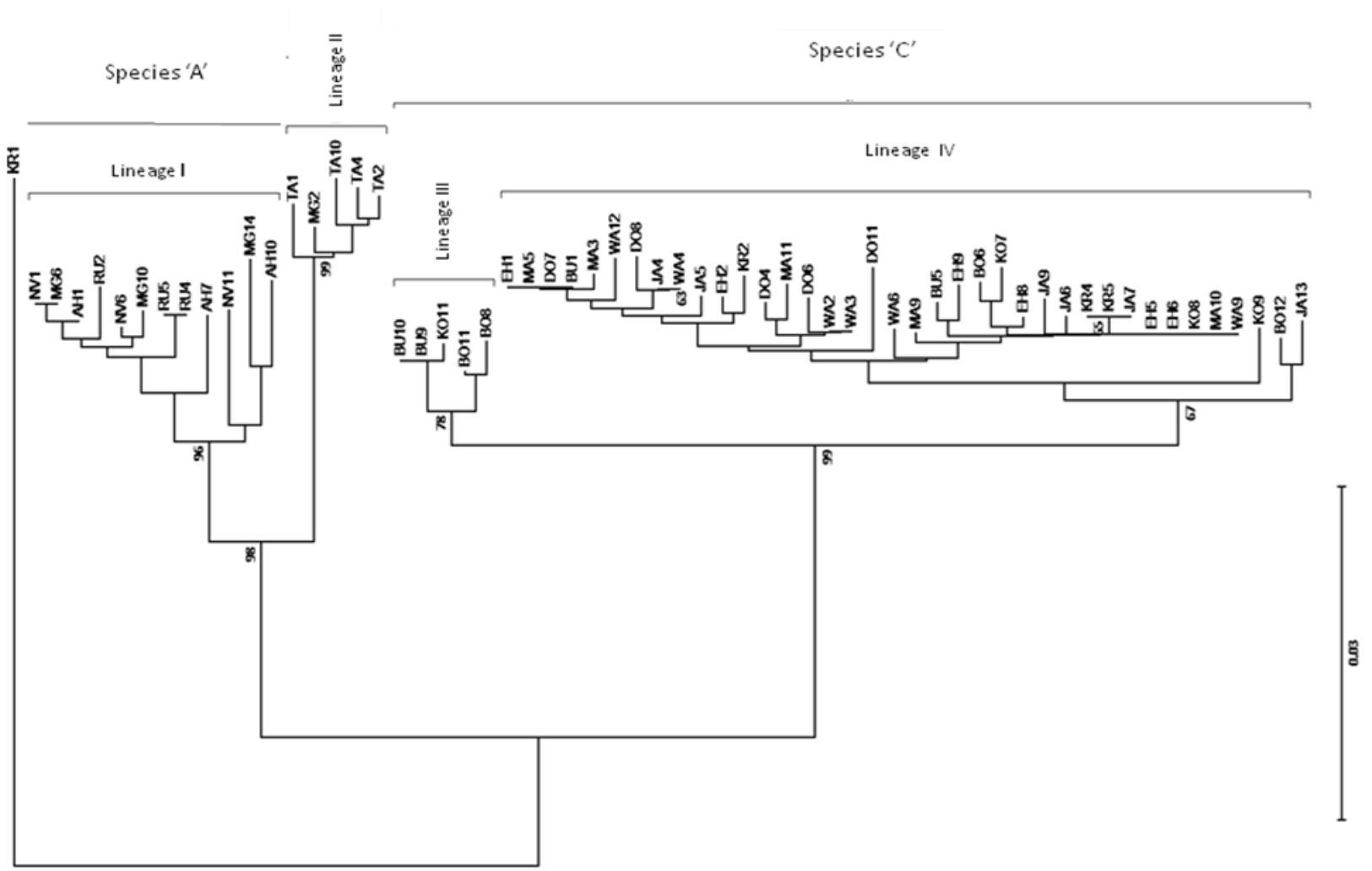

Figure 4. Neighbor-Joining p-distance tree for concatenated dataset of DNA barcoding region of *Ae. mcintoshi*.

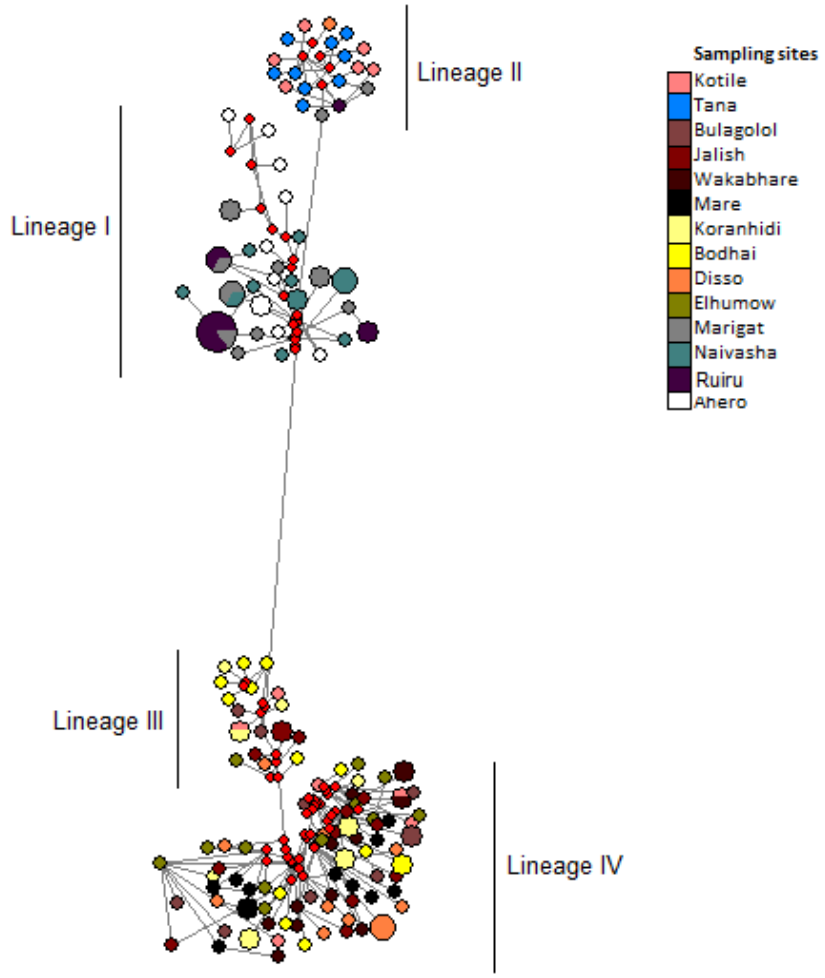

Figure 5a. Median-joining network showing mutational differences among 136 cytochrome oxidase subunit I (*COI*) haplotypes (1448 bp) representing 165 *Aedes mcintoshi* specimens from Kenya. Each circle represents a haplotype and the color depicts the origin in terms of sampling site; size of each circle indicative of the frequency of each haplotype. The smallest circles denote unique haplotypes. Labels correspond to the four lineages identified and each very small red square represents the mutational steps. The area of circles is proportional to the frequency of the haplotypes.

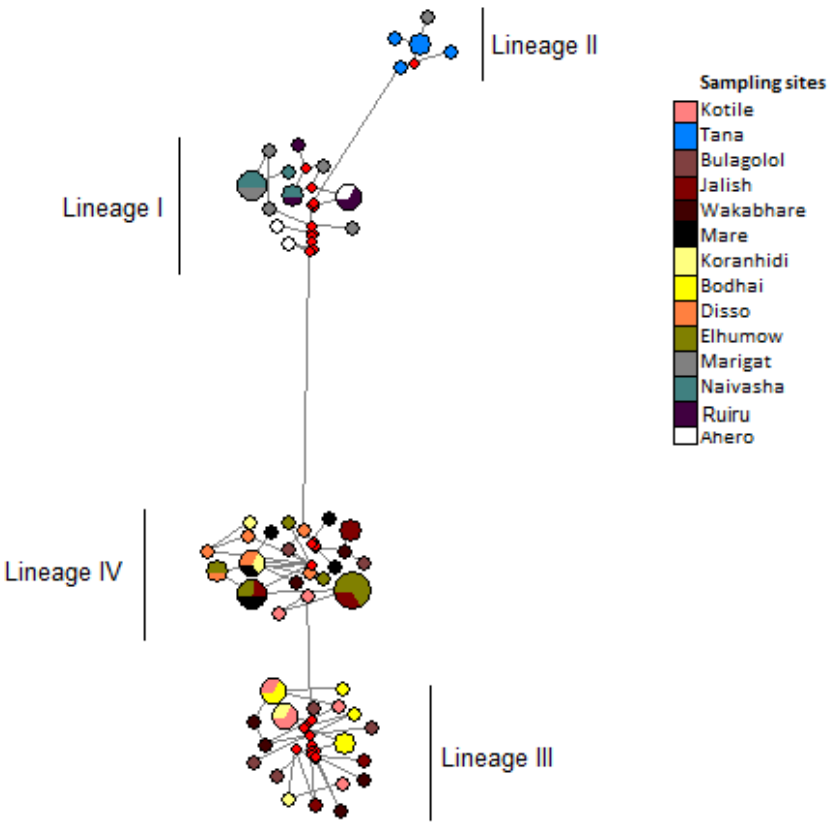

Figure 5b. Median-joining network showing mutational differences among 55 internal transcribed spacer (*ITS*) haplotypes (1065 bp) representing 79 *Aedes mcintoshi* specimens from Kenya. Each circle represents a haplotype and the color depicts the origin in terms of sampling site; size of each circle indicative of the frequency of each haplotype. The smallest circles denote unique haplotypes with labels corresponding to the four lineages identified and each small very red square representing the mutational steps. The area of circles is proportional to the frequency of the haplotypes.

Polymorphism and diversity

Haplotype diversity was high across both loci, with 136 haplotypes identified from *COI* and 55 from *ITS* (Table 1). There was a high frequency of single haplotypes with most of the shared haplotypes being within sites; however, few were found in geographically diverse localities. For the *COI* locus, there were shared haplotypes between Marigat and Naivasha; Ruiru and Marigat;

Kotile and Wakabhare and Kotile and Koranhidi (Figure 5a). There were shared haplotypes between Kotile and Koranhidi; Kotile and Bodhai; Elhumow and Jalish; Mare, Jalish and Elhumow; Mare, Disso and Koranhidi; Elhumow and Disso; Marigat and Naivasha; Ruiru and Ahero; Ruiru and Naivasha for the *ITS* locus (Figure 5b). This pattern of haplotype diversity was also consistent throughout the four lineages with haplotype diversity ranging from 0.80 to 1.00 (Table 1). Nucleotide diversity, for each lineage was higher within the *COI* locus when compared to *ITS* locus (Table 2). For the *COI* locus, nucleotide diversity was highest in lineage I and higher for lineage III in the *ITS* locus. In addition, the number of polymorphic sites was consistently higher in lineages IV and I for both loci followed by lineages III and II, respectively (Table 1). The neutrality tests of Tajima's *D* and Fu's *Fs* revealed significant negative values in lineage IV for *COI* locus and lineage III for *ITS* locus, suggesting past occurrence of demographic expansion within these lineages (Table 1). All other tests of neutrality within lineages were positive or negative and non-significant (Table 2). Overall neutrality tests of Tajima's *D* and Fu's *Fs* were non-significant for *COI* (Tajima's *D*: 0.84718, $P > 0.1$; Fu's *Fs*: -49.565, $P > 0.1$) but highly significant for the *ITS* locus (Tajima's *D*: 2.26747, $P < 0.05$; Fu's *Fs*: 11.669, $P < 0.02$).

Genetic structure

Mean between-lineage divergence ranged from 1.9 % to 7.0 % for *COI* and from 2.2 % to 5.3 % for *ITS* (Table 2). For the barcoding region it ranged from 2.0 % to 8.8 % with lowest divergence being recorded between linages III and IV (Table 3). The evolutionary divergence within *Ae. mcintoshi* at the *COI* locus was highest between lineages II and IV (7.0%) and lowest between lineages III and IV (1.9%) (Table 2). Genetic divergence at the *COI* locus between lineages I and II, lineages I and III, lineages I and IV, and II and III was 4.8 %, 6.5 %, 6.7 % and 6.9 %, respectively (Table 2). A similar pattern was observed in the *ITS* locus, with the maximum mean genetic divergence being 5.3 % between lineage II and lineage III (Table 2). Estimates of intra-lineage diversity for both gene loci were highest in lineage I followed by lineage IV for the *COI* locus and highest for lineage III for the *ITS* locus (Table 1). However, consistently lower divergence estimates were detected between lineages III and IV for both loci (Table 2).

The divergence estimates from the *COI* barcoding region (Table 3) indicate the likely presence of at least three species of what is currently described as *Ae. mcintoshi*, a finding that is

supported by the results of the *COI* barcoding region phylogeny (Fig. 4), which confirms the presence of well-supported monophyletic lineages. Significant genetic differentiation within *Ae. mcintoshi* was identified by both loci, with F_{ST} values between the four lineages ranging from 0.50 to 0.91 for *COI* and om 0.84 to 0.98 for *ITS* (Table 5). The genetic structure identified in both the *COI* and *ITS* loci are supported by the AMOVA. The AMOVA showed there was significantly more variation between the lineages for the *ITS* locus but significantly higher variation within the lineages for the *COI* locus (Table 6). Marked genetic structure was observed using AMOVA, indicating that 81.61 % of the variation occurred between lineages with 18.39 % of the variance within lineages (Table 4) in the *COI* locus. An analogous pattern was observed for *ITS*, with among and within variation in lineages being 95.3 % and 4.7 % respectively (Table 4).

Table 1. Total genetic diversity of *Aedes mcintoshi* and genetic diversity within each of the four lineages resolved in the phylogenetic analyses using *COI* and *ITS* sequences.

	Mean intra-genetic distance (%)	**N**	**H**	**Hd±SD**	**Pi±SD**	**S**	**Tajima's *D***	**Fu's F_S**
COI								
Lineage I	1.52	45	28	0.965±0.015	0.015±0.008	108	-0.5024	-1.402
Lineage II	0.39	19	19	1.000±0.017	0.004±0.002	33	-1.6269	-16.673
Lineage III	0.87	19	17	0.988±0.021	0.009±0.005	43	0.085	-4.995
Lineage IV	0.99	82	72	0.997±0.003	0.010±0.005	114	-1.2578	−61.91*
ITS								
Lineage I	0.6	17	11	0.816±0.079	0.006±0.003	46	1.1049	0.443
Lineage II	0.1	6	5	0.600±0.215	0.001±0.0005	15	-1.132	-0.858
Lineage III	0.3	23	18	0.917±0.034	0.003±0.0002	22	0.7313	-5.088 *
Lineage IV	0.1	33	21	0.593±0.081	0.001±0.0001	28	0.5033	-0.801

*P < 0.05; N, number of sequences per lineage; H, number of haplotypes; Hd, haplotype diversity; SD, standard deviation; Pi, nucleotide diversity; S, number of polymorphic sites.

Table 2. Estimates of average evolutionary divergence (%) over sequence pairs between each of the four lineages of *Aedes mcintoshi* resolved in the phylogenetic analyses using *COI* and *ITS* sequences.

	Lineage I	**Lineage II**	**Lineage III**
COI			
Lineage I	–		
Lineage II	4.8	–	
Lineage III	6.5	6.9	–
Lineage IV	6.7	7	1.9
ITS			
Lineage I	–		
Lineage II	2.6	–	
Lineage III	5.1	5.3	–
Lineage IV	5.1	5.1	2.2

Table 3. Between-lineage nucleotide sequence divergence (%) based on *COI* barcode region (639 bp) for the morphologically defined *Aedes mcintoshi* taxa that were included in the concatenated dataset.

	Lineage I	**Lineage II**	**Lineage IIII**
Lineage I	--	--	--
Lineage II	5.34	--	--
Lineage IIII	6.98	8.26	--
Lineage IV	7.77	8.72	2.02

Table 4. Between-species nucleotide sequence divergence (%) based on *COI* barcode region (639 bp) of morphologically defined *Aedes mcintoshi* in Kenya.

	Species A	**Species B**
Species A	--	--
Species B	5.34	--
Species C	7.68	8.67

Table 5. Genetic differentiation in *Aedes mcintoshi* as measured by F_{ST}, between the four lineages resolved in the phylogenetic analyses using *COI* and *ITS* gene sequences.

	Lineage I	**Lineage II**	**Lineage III**
COI			
Lineage I	−		
Lineage II	0.76*	−	
Lineage III	0.8*	0.91*	−
Lineage IV	0.82*	0.88*	0.5*
ITS			
Lineage I	−		
Lineage II	0.84*	−	
Lineage III	0.95*	0.970.94*	−
Lineage IV	0.96*	0.980.95*	0.94*

*P < 0.05.

Table 6. Summary of the analysis of molecular variance (AMOVA) for *COI* and *ITS* loci.

	d.f.	**% variation**	**Fixation index**
COI			
Among lineages	3	81.61	
Within lineages	161	18.39	
Total	164	100	0.82*
ITS			
Among lineages	3	95.5	
Within lineages	75	4.7	
Total	78	100	0.95*

*P < 0.05; Populations correspond to the four distinct lineages identified in the *Aedes mcintoshi* phylogeny.

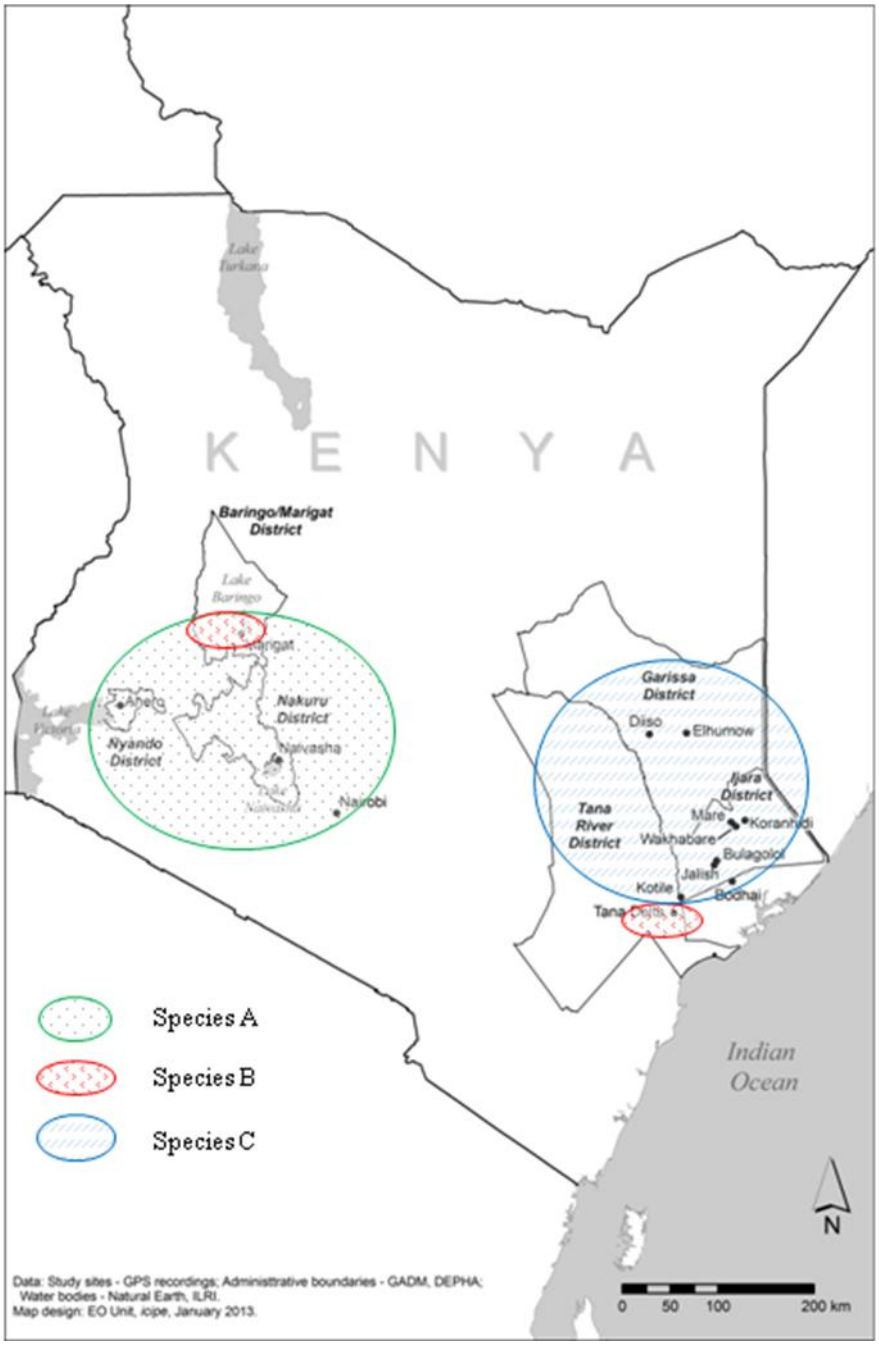

Figure 6. Map of Kenya indicating the geographical distribution of the three putative species within what is called *Ae. mcintoshi* in Kenya, delineated on the basis of the *COI* barcoding region. These braod sampling areas are color-coded as follows: green (species A); red (species B); blue (species C).

Discussion

This is the first study using DNA-based markers to explore the level of diversity and genetic differentiation of *Ae. mcintoshi* from RVF endemic and epidemic areas of Kenya. Based on mitochondrial and nuclear molecular data we have shown that *Ae. mcintoshi*, as it is currently described, is composed of at least four distinct lineages that coincide with geographic location reflecting the magnitude of RVF in Kenya possibly influenced by prevailing environmental conditions in these locations; samples of *Ae. mcintoshi* from North Eastern Province of Kenya form two distinct lineages (III and IV), samples from Rift Valley, Central and Western Provinces form a single lineage (I). Most of the variation resides among lineages implying that most of the variation within samples happens within local scales. However, within each of the sampling sites, there appears to be population substructuring especially in the *COI* locus. This observation supports the existence of substantial within-species genetic divergence even among local populations [34].

North Eastern Province of Kenya is an arid to semi-arid area where sudden, dramatic epidemics of RVF occur at intervals of approximately 10 years, associated with widespread flooding that result in swarms of mosquitoes. Because of the scale of livestock involvement in this area, these outbreaks are usually associated with explosive outbreaks in humans. In this zone, the rains are rarely reported and during long drought intervals when there is low or no virus activity, there is a huge buildup of susceptible animals which serve to amplify the virus in vectors and human population when heavy persistent rains with flooding occur. Rift Valley and Central areas are wet grassland areas, where sporadic disease is frequently reported in livestock in form of abortions and deaths in young animals during the rains, which gets confirmed by diagnosis but these sporadic occurrences in livestock is not usually associated with reports of human cases. The rains here are more frequent and hence more frequent virus activity which results in low susceptibility in animals, possibly the reason for low human exposure.

The species showed limited variability within lineages, but strong differentiation among lineages. Analysis of molecular variance also revealed that more than 90% of the variation resided among lineages for *ITS* locus and about 80 % for *COI* locus further suggesting very sharp interpopulational differences. We observed a similar pattern of population differentiation for both gene loci; however, there was subtle discordance in the resolution of samples from certain

localities within some lineages. For example, lineage II distribution was limited to Tana for *ITS* but with a wider distribution based on the *COI* locus. Although the reason for this difference in resolution is unclear; it could be related to efficacy of the different marker types in recovering lineages at different taxonomic levels or differences in sample sizes used for both markers. Overlapping distribution of lineages III and IV was evident for both gene loci from our analyses spanning most of the localities of North Eastern Province of Kenya. These localities are also within the flood prone arid to semi-arid ecozones of Kenya highlighting an environmental dimension likely influencing the pattern of population structuring in this vector. If *ITS* is considered a better marker of deeper phylogeny [35], then it is clear certain lineages like IV are predominant in certain localities and absent in some which can help explain differential RVF pattern even among different localities in North Eastern Kenya.

The clustering of samples from endemic RVF sites precludes any possible interplay of genetic differentiation due to isolation by distance. Naivasha, Ahero and Marigat are all located on lake basins of Lake Naivasha, L. Victoria and L. Baringo and Bogoria respectively (Figure 4). These could influence the general environment and physiology of the vector as well. Although there are more frequent rains in this zone, reduced or poor quality breeding parameters at these sites could negatively affect populations of this species resulting in reduced potential for population expansion. Flood water *Aedes* principally *Aedes mcintoshi* form the highest proportion of mosquitoes in North Eastern Province of Kenya compared to the other sites outside this area where it constitutes less than 5% of the mosquito fauna (Lutomiah et al, unpublished data). Small population sizes affect fitness and consequently evolutionary potential for expansion. Such low numbers or populations of this vector in endemic areas are often characterized by a high degree of relatedness, and reduced potential for expansion as evidenced by the non-significant neutrality tests among lineage I encompassing all the endemic sites (Table 1).

Although historically the first outbreak of RVF was reported in Naivasha, Rift Valley Province of Kenya [36,37], there seem to be a shift in the pattern and frequency of recent outbreaks of the disease to North Eastern Province. Wind, human activity and trade/animal movement might play an important role in the spread of mosquitoes to other areas [38-41]. Thus, favorable micro-habitats and physico-chemical parameters of breeding sites in more humid areas together with

availability of abundant hosts might have contributed to the abundance and possibly survival of this species in North Eastern Province of Kenya.

Rainfall and excessive flooding has been associated with the precursor of past RVF epidemics in Kenya with North Eastern Province as its epicenter. This Province is an arid area prone to severe drought and dependent on rainfall only for flooding of depressions (i.e. dambos), the preferred breeding sites for this RVFV vector [15]. However, areas such as Kotile and Tana Delta because of their proximity to the Tana River provide conditions for more frequent breeding due to the riverine effect. Such expansion of vectors into new aquatic niches may increase population density and longevity with ensuing effect on population structure and diversity with local adaptation impacting on important phenotypic differences between geographic locations [42-44].

Areas, notably Kotile could provide a refugial population from which active dispersal of females would have originated in the other localities of the North Eastern Province within their flight ranges. This is reflected in *COI* locus having a wide distribution in Kotile with representation in lineages II, III and IV. The reason why we have greater diversity within Kotile population may be due to its proximity and importance of the Tana River and surroundings as a convergence zone for human/livestock during times of drought. The flooding that causes the Tana River to burst its banks may help to move the mosquito population towards Kotile (on the river banks) and also upstream. Following the pattern of livestock mobility in North Eastern Province of Kenya, Tana River becomes a convergent point during dry spells and when it rains, thereafter animals move back from the riverbanks to other sites like Jalish, Bulagolol, Koranhidi, Wakabhare, Mare Diiso, Elhumow, etc. Nomadic animal movement, therefore, might play a greater role in dispersal of this mosquito possibly through transport of eggs.

The overall diversity of *Ae. mcintoshi* individuals, throughout the sampled area is high as it is evident from the relatively high estimates of the haplotype and nucleotide diversities. This may be indicative of a stable population with a large long-term effective population size or an admixed sample of individuals from historically separated populations [45]. The general lack of shared haplotypes between lineages I and II, suggests these two lineages (I and II) are genetically discrete. This is corroborated by their high F_{ST} values from the other two lineages and high mean evolutionary divergence estimates and hence their genetic distinctiveness. The divergence between these two lineages and the rest (>3%) is clearly at a level expected for interspecific

rather than intraspecific relationships in mosquitoes [46]. Therefore, lineages I and II probably represent separate species under the phylogenetic species concept. Intraspecific variation is constrained to 2–3% for mosquito species based on the DNA barcode region [47], values which overall are comparable to those found between lineage III and IV for both markers and other studies [48,49]. At least three species of what is currently described as *Ae. mcintoshi* are suggested from the *COI* barcoding region analyses as three well-supported monophyletic lineages displaying high levels of evolutionary divergence, were recovered (Figure 4; Tables 3 and 4). The consistently low estimates of evolutionary divergence between lineage III and IV for both markers (1.9% and 2.2% for *COI* and *ITS*, respectively) coupled with shared haplotypes among these lineages suggest a variant of this species or a cryptic species. Moreover, the possibility of a variant population of *Ae. mcintoshi* has previously been suggested [16]. The degree of sub-structuring with consequent haplotypes observed among lineages III and IV is most likely due to incomplete lineage sorting.

Aedes mcintoshi is known to be engaged in the transovarial maintenance and spread of RVFV [14,15]. Transovarial transmission of symbionts is known to influence polymorphism in mosquito populations as they confer some selective advantage on their hosts thereby favoring them by natural selection [50]. Selective pressures as a result of transovarial maintenance of the RVF virus in this mosquito could play a role in population sub-structuring or polymorphisms. Additionally, fine scale ecological partitioning of *Anopheles gambiae* populations have facilitated the expansion of malaria transmission spatially and temporally [51] and it remains unknown if a similar mechanism is at play in the RVFV-vector scenario.

Conclusion

Four distinct lineages have been identified within what is currently described as *Ae. mcintoshi*, which shows geographic restriction reflecting the magnitude of RVF in Kenya, like influenced by prevailing environmental factors. However, further insights into the processes underlying the observed diversity is required including competence studies among individuals from the different lineages but particularly among those sourced from areas of distributional overlap between lineages III and IV to ascertain if there would be variation in the transmission of the virus by the different lineages. Additionally, cross breeding experiments between individuals from the two lineages to determine whether they are reproductively isolated, may be helpful although the

challenge of rearing flood water *Aedes* presents a major hurdle; therefore, the taxonomic delineation of the two lineages will have to rely on thorough morphology and sequence divergence. If both lineages are implicated as vectors, the characteristics of their breeding sites, their behaviors and their migration history could be used to predict changes in RVF transmission patterns in North Eastern area, the rest of the country, and provide useful information for viral surveillance and possibly targeted vector control. Moreover, further taxonomic elucidation of the status of this species requires additional stringent markers such as single nucleotide polymorphism or microsatellite analysis in areas especially North Eastern Province of Kenya where lineages overlap so as to bypass any problems associated with pre-mating barriers between populations and this should be backed by extensive sampling to cover the entire range of this species.

Materials and Methods

Study sites, specimen collection, identification and processing

Adult female *Ae. mcintoshi* mosquitoes were sampled using CO_2-baited CDC light traps from 2009 to 2011 in 14 localities including endemic and non-endemic areas and foci of past RVF outbreaks in Kenya (Figure 4, Table 5). The sites were selected as part of an on-going project monitoring the inter-epidemic circulation of the disease in these communities. Specimens were morphologically identified using the taxonomic keys of Edwards [52] and Jupp [22], placed individually in 1.5ml eppendorf tubes and then stored in liquid nitrogen for transport to the laboratory. Once in the laboratory, the samples were transferred and stored at -80^{o}C until DNA extraction.

Ethics Statement

No specific permits were required for the described field studies. No specific permissions were required for these locations/activities.

DNA extraction and amplification

Genomic DNA was extracted from individual whole mosquitoes using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, GmbH-Hilden, Germany) as per manufacturer's instructions. The extracted DNA was stored at -20^{o}C until required for amplification. A number of samples per

site were amplified and sequenced for the mitochondrial cytochrome oxidase subunit 1 (*COI*) and ribosomal internal transcribed spacer (*ITS*) (Table 1). A 1500-bp fragment of the *COI* gene was amplified using primers LCO1490 (5'- GGTCAACAAATCATAAAGATATTGG-3') [53] and TL2-N-3014 (5'- TCCAATGCACTAATCTGCCATATTA-3') [30]. Genomic amplification reactions were performed in a final reaction volume of 20 µl containing 5X Phusion HF Reaction Buffer, 50 mM $MgCl_2$, 10mM of each dNTP, 0.5 unit of Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Thermo Scientific, New England Biolabs, Hitchin, United Kingdom) 0.5 µM primers each of the forward and reverse primers, 100% DMSO and approximately 1–10 ng of genomic template DNA. The 1100bp *ITS* fragment inclusive of *ITS*1 and part of *ITS*2, was amplified with primers CAS18S (5'- TACACACCGCCCGTCGCTACTA-3') [54] and ITS2-Porter (5'- ATGCTTAAATTTAGGGGGTAGTC-3') [55] under similar reaction conditions but without additional $MgCl_2$.

The thermal profile for *COI* amplification was; enzyme activation at 98°C for 15 min, followed by 39 cycles of 98°C for 10 sec, 55°C for 40 sec, 72°C for 1 min, and a final elongation step 72°C for 10 min. Thermal cycling conditions for *ITS* amplification were; enzyme activation at 98°C for 15 min, followed by 39 cycles of 98°C for 10 sec, 55°C for 40 sec, 72°C for 1 min 20 sec, and a final elongation for 10 min at 72°C. Amplicons were sized by 1.5 % agarose gel electrophoresis against a 1kb DNA ladder (O' GeneRuler, Fermentas).

DNA purification, sequencing and analysis

Individual PCR products were purified with an ExoSap PCR purification kit (USB Corporation, Cleveland, OH) according to the manufacturer's recommended protocol. Both strands of each purified PCR product were sequenced with each of the external PCR primers in separate reactions (Macrogen, South Korea and Inqaba, South Africa).

Forward and reverse sequences for the *COI* and *ITS* gene regions were visually inspected and aligned in MEGA version 5.0 [56]. Multiple sequence alignments of the resulting contiguous sequences for each gene were performed using ClustalW for the *COI* dataset [57] and MUSCLE for the *ITS* dataset [58] in MEGA v 5.0 using the default parameters of the program. Each dataset was trimmed to 1448bp and 1065bp, respectively, and thereafter imported into other programs for sequence analyses. The *COI* gene sequences were translated to ensure that no stop codons occurred and the mutational frequency at first, second and third base position was recorded to

further rule out the possibility of nuclear mitochondrial pseudogenes (numts) in the mitochondrial dataset.

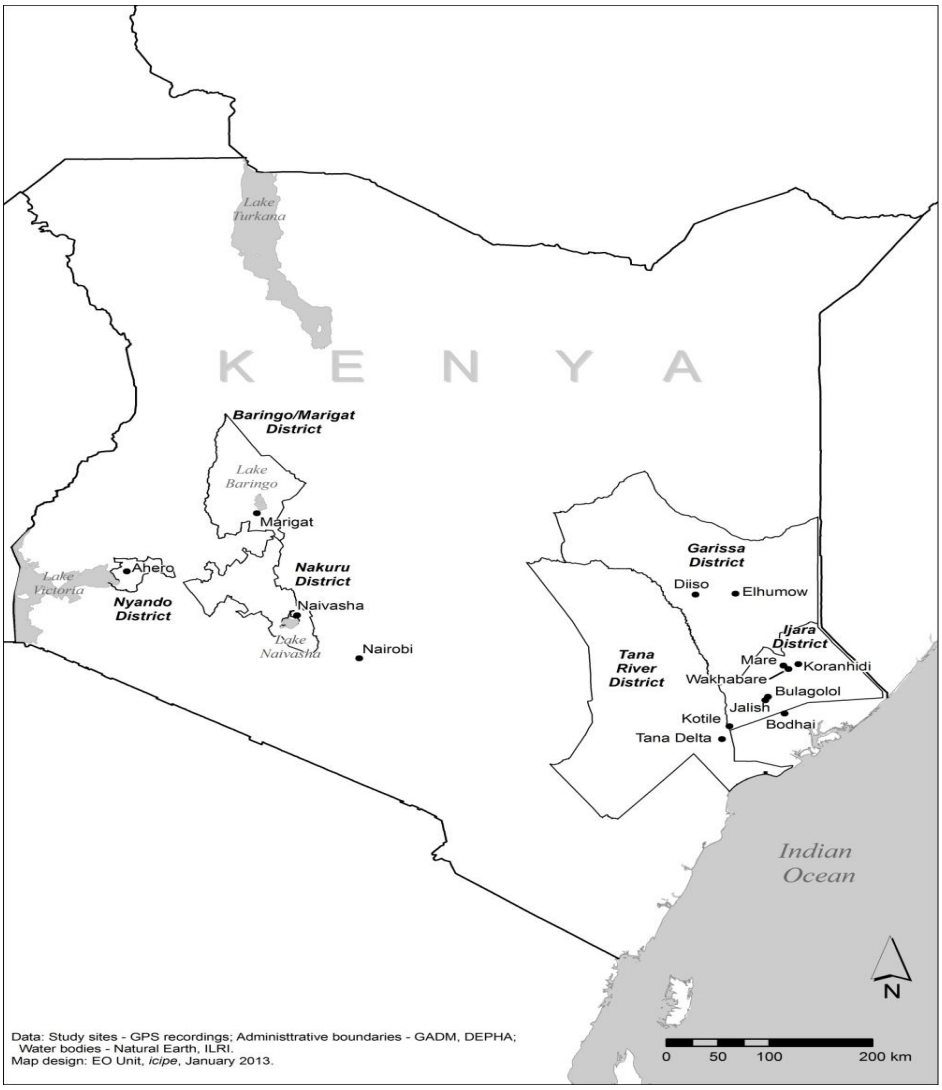

Figure 7. Map of Kenya showing location of study sites (in dots).

Table 7. Summary of the sampling sites and genetic data generated for each of the fourteen populations of *Aedes mcintoshi* used in this study.

Sites	Site abbreviation	Latitude	Longitude	Geographic region (Province of Kenya)	*COI*	*ITS*
Kotile	KO	S01.974	E040.197	NE	11	7
Tana Delta	TA	S02.124	E040.131	NE	10	5
Marigat	MG	N0.500	E036.059	RV	14	7
Ruiru	RU	S1.184	E036.956	Central	11	4
Naivasha	NV	S0.685	E036.412	RV	10	4
Ahero	AH	S00.174	E034.920	Western	10	3
Disso	DO	S00.445	E039.898	NE	12	6
Elhumow	EH	S00.434	E040.249	NE	12	8
Mare	MA	S01.269	E040.668	NE	12	6
Wakabhare	WA	S01.310	E040.712	NE	12	6
Jalish	JA	S01.671	E040.511	NE	14	7
Bulagolol	BU	S01.631	E040.535	NE	10	6
Bodhai	BO	S01.826	E040.679	NE	12	6
Koranhidi	KR	S01.253	E040.799	NE	12	4
Total					165	79

NE, North Eastern Province; RV, Rift Valley Province of Kenya.

Sequence analyses

A principal component analysis (PCA) was performed to cluster the sequence data into genetically and geographically homogeneous populations. A table of mean evolutionary divergence estimates over sequence pairs within sites for *Ae. mcintoshi* was constructed in MEGA v 5 (Table S1). This table was used to generate the principal component plots using GenAlEx 6.41 [59].

Phylogenetic analyses were initially performed for each of the two genes, to permit comparison of the single gene topologies, and subsequently extended to include a concatenated datasets for both gene fragments and a reduced *COI* data corresponsind to the barcoding region. The model of DNA sequence evolution that best fits each dataset was selected in MrModeltest version 2.3 [60] in cooperation with PAUP*4b10 [61] using the Akaike information criterion (AIC) [62].The general time reversible (GTR) model was selected for the *COI* locus and Hasegawa-Kishino-Yano (HKY) model for the *ITS* locus. Both loci were subject to a gamma distribution with a proportion of invariable sites. Distance trees were inferred using the neighbor-joining (NJ) algorithm [63] and the locus-appropriate model of sequence evolution in MEGA v 5.0. Nodal support was evaluated by bootstrap resampling with bootstrap values of less than 70 % being considered as weakly supported [64]. This was only applicable to NJ trees and not to Bayesian Inference (BI) for which we used a cut-off of 95 %. Homologous portions of partial sequences from *Aedes aegypti* (GenBank accessions AF380835 and GU980956 for *COI* and *ITS*, respectively) were included as outgroups for all phylogenetic analyses. For each analysis of Bayesian inference (BI) performed using MrBayes 3.1.2 [65] using the substitution model for each gene, four Markov chains were run, with each chain starting from random trees and run for 10 million generations, sampling each tree every 10,000 generations. A majority rule consensus tree was generated from the trees retained (after burn-in trees were discarded using likelihood plots), with posterior probabilities (PP) for each node indicated in percentage corresponding to the number of times the node was recovered. Relationships between the observed haplotypes were assessed by constructing median-joining networks. Phylip data files (PHY) were created with DnaSP and imported into Network v4.6.1.1 (Fluxus-Technology, www.fluxus-engineering.com) and networks were calculated with the median-joining algorithm using maximum parsimony post-processing [66].

Polymorphism, diversity and genetic structure

Initial estimates of DNA sequence polymorphism based on the full ingroup sequence data set were computed using DnaSP 5.0 [67], following which molecular indices of diversity based on the lineages identified from the phylogenetic analyses (NJ tree, Network and BI). Parameters included the number of variable sites, nucleotide diversity, haplotype number and diversity. Additionally, neutrality test statistics of Tajima's *D* [68] and Fu's *Fs* [69] were estimated to examine the demographic and selection forces affecting molecular evolution in *Ae. mcintoshi*,

and to detect signatures of past population expansions. The indices *D* and *Fs* were examined based on 1000 coalescent simulations with consideration of the recombination rate using DnaSP. Expectations of these statistics are nearly zero in a constant population size; significant negative values indicate a sudden population expansion while significant positive values indicate population subdivision or recent population bottlenecks.

Genetic structure was estimated in the program AMOVA in Arlequin 3.5 [70] to calculate pairwise F_{ST} values between each of the lineages of *Ae. mcintoshi* identified in the phylogenetic analyses with 10,000 permutations.

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Table S1. Pairwise nucleotide sequence divergence between *Aedes mcintoshi* from different sampling sites in Kenya.

Sites	Tana	Kotile	Ruiru	Marigat	Disso	Naivasha	Ahero	Mare	Jalish	Wakabhare	Elhumow	Bulagolol	Koranhidi	Bodhai
Tana		0.053	0.024	0.024	0.049	0.029	0.025	0.051	0.054	0.054	0.049	0.055	0.053	0.054
Kotile	0.045		0.051	0.051	0.015	0.053	0.050	0.016	0.015	0.012	0.015	0.011	0.012	0.008
Ruiru	0.050	0.063	−	0.010	0.050	0.008	0.004	0.051	0.051	0.050	0.050	0.051	0.051	0.051
Marigat	0.048	0.063	0.021	−	0.049	0.007	0.009	0.051	0.052	0.052	0.049	0.052	0.051	0.051
Disso	0.075	0.045	0.075	0.076	−	0.051	0.048	0.001	0.007	0.014	0.001	0.013	0.011	0.021
Naivasha	0.054	0.065	0.016	0.021	0.076	−	0.008	0.053	0.054	0.053	0.051	0.053	0.052	0.052
Ahero	0.057	0.067	0.022	0.023	0.076	0.020	−	0.049	0.050	0.049	0.048	0.050	0.050	0.050
Mare	0.081	0.044	0.077	0.077	0.016	0.077	0.077	−	0.008	0.015	0.002	0.015	0.012	0.023
Jalish	0.082	0.045	0.077	0.077	0.019	0.077	0.077	0.012	−	0.015	0.007	0.014	0.013	0.018
Wakabhare	0.080	0.044	0.077	0.077	0.017	0.077	0.076	0.010	0.012	−	0.014	0.012	0.013	0.010
Elhumow	0.081	0.044	0.076	0.077	0.018	0.076	0.077	0.011	0.013	0.011	−	0.014	0.011	0.021
Bulagolol	0.080	0.044	0.076	0.077	0.020	0.076	0.076	0.013	0.014	0.012	0.013	−	0.012	0.010
Koranhidi	0.081	0.044	0.077	0.077	0.020	0.077	0.077	0.013	0.014	0.012	0.013	0.014	−	0.013
Bodhai	0.079	0.044	0.075	0.076	0.021	0.075	0.076	0.015	0.016	0.015	0.015	0.016	0.015	−

COI gene sequences are given in the lower diagonal and *ITS* in the upper diagonal.

Chapter 6

Genetic diversity of *Aedes ochraceus*, an increasingly important primary vector of Rift Valley fever virus in Kenya

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This chapter has been formatted for submission to Parasites and Vectors.

Abstract

Background: The floodwater mosquito *Aedes ochraceus* has been incriminated as a key primary vector of Rift Valley fever (RVF) virus during an outbreak in Kenya. As part of the ecological dynamics in understanding the disease spread and transmission, we assess genetic diversity and fine-scale population structure in this species, by analyzing sequence variation in 1456 bp of cytochrome c oxidase subunit 1 (*COI*) and 1086 bp of internal transcribed spacer (*ITS*) regions of the mitochondrial and nuclear ribosomal DNA, respectively. Individuals were sampled from seven populations/sites within the RVF epidemic-prone area of northeastern Kenya.

Results: **Results**: For both markers, mean evolutionary divergence estimates among and within sites were low and the lack of sub-structuring from phylogenetic and network analyses suggestive of a single population. Despite the low nucleotide diversity, the high number of polymorphic sites and haplotype diversity within sampling localities was high indicating an admixture of individuals from different regions. Interestingly, we observed significant negative neutrality tests of Tajima's *D* and Fu's *Fs* for the *COI* locus only, which was supported by a unimodal curve for the mismatch distribution of frequencies of pairwise differences, indicative of a rapid population expansion for this species.

Conclusion: With the availability of suitable breeding sites in the vast arid to semi-arid area of northeastern Kenya, and clear preference for livestock which are susceptible hosts for the disease and abundant in this area, the population expansion as evident from our data suggests that this species will continue to increase in importance in RVF transmission in Kenya.

Key words: *Aedes ochraceus*; genetic diversity; cytochrome oxidase subunit I; internal transcribed spacer; Kenya.

Background

Rift Valley fever (RVF) poses not only a severe economic burden, but is of major public health and veterinary concern in sub-Saharan Africa (SSA). The disease is characterized by frequent and sporadic outbreaks in most parts of Africa especially in Kenya and the East African sub-region. Despite this, the pattern of maintenance and spread, especially between epidemics, remains poorly understood. Although several mosquito species of diverse genera have been implicated as vectors through virus isolation and experimental studies [1-3], there is strong

evidence that in Kenya, *Aedes mcintoshi* and *Ae. ochraceus*, members of the *Aedes* subgenera *Aedimorphus* and *Neomelaniconion*, respectively, play key roles in the transmission of the disease [4-6]. During the 2006/2007 RVF outbreak in predominantly the northeastern part of Kenya, these two species were identified as primary RVFV vectors, accounting for over 77% of positive pools of mosquitoes sampled in the field [6].

RVFV has historically been restricted to sub-Saharan eastern Africa and to the Rift Valley of Kenya and Tanzania, in particular [7]; however, spread outside Africa and records of the disease in the Arabian Peninsula [8] highlights the potential of the disease to spread to the rest of the world [9]. The introduction and spread into new areas has been associated with the migration of infected animals (which then serve as a source of virus-infected blood meals for susceptible, local mosquitoes) [10]. However, outbreaks of the disease without evidence of animal movement have also been noted, suggesting that outbreaks due to the movement and spread of infected mosquitoes is a likely possibility [11, 12].

Relatively little was known about the role of *Ae. ochraceus* as a vector of the disease in Kenya and East Africa until the 2006/2007 RVF outbreak in Kenya [6], although RVF-vectoring potential of this species based on numerous isolations of the virus in field-collected samples, is well-established for West Africa [2, 13]. The surprisingly high number of isolations of RVFV from this species during the 2006/2007 RVF outbreak signaled a new RVFV-vector association in East Africa, Kenya [6].

A complete understanding of the natural history of this virus is not possible without a better understanding of the key mosquito vectors [14]. Although attempts have been made to expand our knowledge about the vector species involved in transmitting the virus, relatively nothing is known about *Ae. ochraceus* apart from its taxonomy and role in disease epidemiology through viral isolation from field collected samples. The genetic background of mosquito species and even populations of the same species influences important traits such as vector competence which affects the potential for transmission, colonization and establishment of arboviruses [15-18] especially in the face of climate change.

The distribution of vector-borne diseases is influenced by climate change acting as an environmental driver [19, 20]. Whether the increasing importance of this species in Kenya's RVF epidemiology is as a result of the impacts of climate is unclear. Therefore to more fully understand the species' ecological dynamics for surveillance of the disease, it is important to shed light on its genetic diversity, population structure and demographic history by focusing on selected communities within the RVF epidemic-prone area of northeastern Kenya.

Methods

Study sites, mosquito sampling and processing

Adult female *Ae. ochraceus* mosquitoes were sampled using CO_2-baited CDC light traps from 2011 to 2012, across seven localities in the RVF virus epidemic-prone areas of northeastern Kenya, where the species predominantly occurs (Lutomiah et al, unpublished data). These include Kotile (S01.9742666, E040.196616), Mare (S01.269233, E040.668233), Wakabhare (S01.3095333, E040.71248333), Jalish (S01.670933, E040.51135), Bulagolol (S01.63125, E040.534583), Bodhai (S01.8264333, E040.67905) and Koranhidi (S01.252666, E040.7990333). These sites were selected as part of an on-going surveillance project to monitor RVF circulation. Specimens were morphologically identified using the keys of Edwards [21] and Jupp [22], placed individually in 1.5ml eppendorf tubes and then stored in liquid nitrogen for transport to the laboratory. Once in the laboratory, the samples were transferred to -80^{o}C storage until DNA extraction.

DNA extraction and amplification

Genomic DNA was extracted from individual, whole mosquitoes using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, GmbH-Hilden, Germany) as per manufacturer's instructions. The extracted DNA was stored at -20^{o}C until required for amplification. A number of samples per site were amplified and sequenced for the mitochondrial cytochrome oxidase subunit 1 (*COI*) and ribosomal internal transcribed spacer (*ITS*) (Table 1). A 1500-bp fragment of the *COI* gene was amplified using primers LCO1490 [23] and TL2-N-3014 [24]. For *COI*, reactions of 20 µl contained 5X Phusion HF Reaction Buffer, 50 mM $MgCl_2$, 10mM of each dNTP, 0.5 units of Phusion High-Fidelity DNA Polymerase (Finnzymes Oy,Thermo Scientific, New England Biolabs, Hitchin, United Kingdom), 0.5 µM of each the forward and reverse primers, 10%

DMSO and approximately 1–10 ng of genomic template DNA. Similar reaction mixes were prepared for amplification of the ~ 1100bp *ITS* fragment that includes *ITS*1 and part of *ITS*2, using primers CAS18S [25] and *ITS*2-Porter [26], but without additional $MgCl_2$.

The thermal cycling conditions for *COI* amplification were as follows; denaturation at 98°C for 15 min followed by 39 cycles of 98°C for 10 sec, 55°C for 40 sec, 72°C for 1 min, and a final elongation for 10 min at 72°C. The ITS thermal cycling profile comprised an initial denaturation at 98°C for 15 min followed by 39 cycles of 98°C for 10 sec, 55°C for 40 sec, 72°C for 1 min 20 sec, and a final elongation at 72°C for 10 min. All amplicons were resolved by1.5 % agarose gel electrophoresis against a 1kb DNA ladder (O' GeneRuler, Fermentas) after staining with Ethidium bromide (EtBr).

DNA purification, sequencing and analysis

Individual PCR products were purified using an ExoSap PCR purification kit (USB Corporation, Cleveland, OH) according to the manufacturer's recommended protocol. Both strands of each purified PCR product were sequenced (Inqaba Biotech, South Africa and Macrogen, South Korea) with each of the external PCR primers in separate reactions.

The forward and reverse *COI* and *ITS* sequences were aligned and edited using the Chromas package embedded in MEGA version 5.0 [27]. Multiple sequence alignments for each gene were performed using ClustalW (*COI*) [28] and MUSCLE (*ITS*) [29] using default parameters and trimmed to 1456bp and 1086bp for *COI* and *ITS*, respectively. As co-amplification of nuclear mitochondrial pseudogenes (numts) is possible and is known to overestimate lineage divergence in mitochondrial sequences [30], numts presence in the *COI* dataset was ruled out by translation of nucleotides to amino acids to assess stop codon presence and the frequency of 1^{st}, 2^{nd} and 3^{rd} base positions, which because of the degeneracy of the amino acid code occur at frequencies of $3^{rd}>1^{st}>2^{nd}$ for coding genes under functional constraints [31].

Phylogeny

Phylogenetic analyses were performed using the two genes separately to examine differences in the single gene topologies, prior to concatenating the datasets. The best model of DNA sequenced evolution was determined in MrModeltest version 2.3 [32] in cooperation with PAUP*4b10 [33] using the Akaike information criterion (AIC) [34]. For both loci, a gamma

distribution (G) with a proportion of invariable sites (I) was selected in combination with a general time reversible (GTR) model for the *COI* locus and the symmetrical model (SYM) for the *ITS* locus. Neighbor-joining trees [35] were initially inferred for each locus in MEGA, after which a concatenated (*COI* + *ITS*) dataset comprising of the taxa common to both datasets, was compiled. For each of the three datasets, homologous portions of partial sequences for 12 *Aedes mcintoshi* specimens from the same region on Kenya (Chapter 5) were included for outgroup purposes. Bayesian inferences (BI) were subsequently performed for each locus and the concatenated datset using MrBayes 3.1.2 [36]. Analyses were run for 5 million generations, sampling trees every 1,000 generations, with the initial 20% of trees sampled being discarded as burn in after inspecting trace plots.

Genetic structure and diversity

Estimates of average evolutionary divergence over sequence pairs both between and within sampling sites, taking into consideration the number of base differences per site from averaging over all sequence pairs, were calculated in MEGA. Indices of DNA polymorphism including number of haplotypes, haplotype diversity, number of polymorphic sites and nucleotide diversity were calculated using DnaSP 5.0 [37]. We used a statistical parsimony network to estimate genealogical relationships among *COI* and *ITS* haplotypes using TCS 1.21 [38] based on 95% confidence of connections among haplotypes [39] and constructed a median-joining network using Network v 4.6.1.1 [40].

Neutrality test statistics Tajima's *D* [41] and Fu's F_S [42] performed using DnaSP, were used to examine the demographic and selection forces affecting molecular evolution in *Ae. ochraceus*, and also to detect signatures of past population expansions. The indices *D* and *Fs* were examined based on 1000 coalescent simulations. Expectations of these statistics are nearly zero in a constant population size, whereas significant negative values indicate a sudden population expansion while significant positive values indicate population subdivision or recent population bottlenecks. The mismatch distribution model (MDM) for sudden expansion was also performed in DnaSP with the R2 and raggedness statistic (rg) based on the mismatch distribution also calculated [43].

Results

A total of 60 haplotypes were recovered from the 67 *COI* gene sequences corresponding to a haplotype diversity (Hd) of 0.9964 and a nucleotide diversity of 0.00619. There were a total of 107 variable sites; 47 being parsimony informative and 60 singleton variations. Of these, mutations occurred at 14 1^{st} base positions, two 2^{nd} base positions, with the remaining 91 occurring at the 3^{rd} base position. Due to sequencing difficulties, the full *ITS* dataset was limited to 32 sequences, yielding 32 haplotypes and 24 polymorphic sites (without gaps considered) comprising 8 singleton variable sites and 16 parsimony informative sites. The overall haplotype and nucleotide diversity values were 0.998 and 0.00537, respectively, for the *ITS* locus. Despite the high haplotype diversity estimates, which are indicative of considerable diversity within this species over the range sampled, the mean number of nucleotide differences (k) within each of the sampling sites ranged from 7.4 to 11.76 for *COI* and from 4.0 to 16.5 for *ITS* (Table 1). The individual gene trees and the phylogenetic tree inferred using the 32 specimens for which both *COI* and *ITS* data were available (Figure 1), consistently recovered a single monophyletic lineage, lacking intra-lineage sub-structure. The same pattern was observed for the NJ tree inferred using the DNA barcode region (Figure 2).

Of the six shared *COI* haplotypes, most were shared within sites; with only one haplotype shared among sites (Wakabhare and Koranhidi). The localities containing shared haplotypes were Kotile, and Jalish, Wakabhare and Koranhidi (Figures 2a and 2b). For *ITS*, all individuals sequenced had unique haplotypes, irrespective of the locality/site. Haplotype diversity was high among the localities, irrespective of sa mple size, for both markers.

The mean divergence estimates among and within sites were low for *Ae. ochraceus* (Tables 1 and 2). The mean genetic divergence between samples from each site varied from 0.05% to 0.07 % with a similar pattern reflected for *ITS*, which had values ranging from 0.05 to 0.09 % (Table 2).

Table 7 Indices of diversity per site and overall tests of neutrality of *Aedes ochraceus* for mitochondrial and nuclear genes

Locus	Parameter	Mare (MA)	Kotile (KO)	Jalish (JA)	Wakabhare (WA)	Koranhidi (KR)	Bodhai (BO)	Bulagolol (BU)
COI	n	7	10	11	12	12	8	7
	h	7	8	10	11	10	8	7
	hd	1	0.9556	0.98186	0.9849	0.9697	1	1
	pi	0.0058	0.0051	0.00662	0.00808	0.0061	0.0051	0.0055
	s	22	24	37	48	37	22	23
	k	8.4762	7.4222	9.6364	11.7576	8.8636	7.3929	8
	within site distances	0.0058	0.0051	0.0066	0.0081	0.0061	0.0051	0.0055
	Tajima' D				-2.057*			
	Fu's Fs				-64.455*			
ITS	n	3	6	5	7	4	3	4
	h	3	6	5	7	4	3	4
	hd	1	1	1	1	1	1	1
	pi	0.0026	0.004	0.0053	0.0061	0.0108	0.0057	0.0042
	s	6	12	24	25	30	9	19
	k	4	5.2	12.2	12.381	16.5	6	11
	within site distances	0.0025	0.0033	0.0069	0.0063	0.0116	0.0057	0.0051
	Tajima' D				-0.2074			
	Fu's Fs				-34.053			

* P< 0.05; n, number of samples sequenced per site; h, number of haplotypes; hd, haplotype diversity; pi, nucleotide diversity; s, number of polymorphic sites; k, mean number of differences.

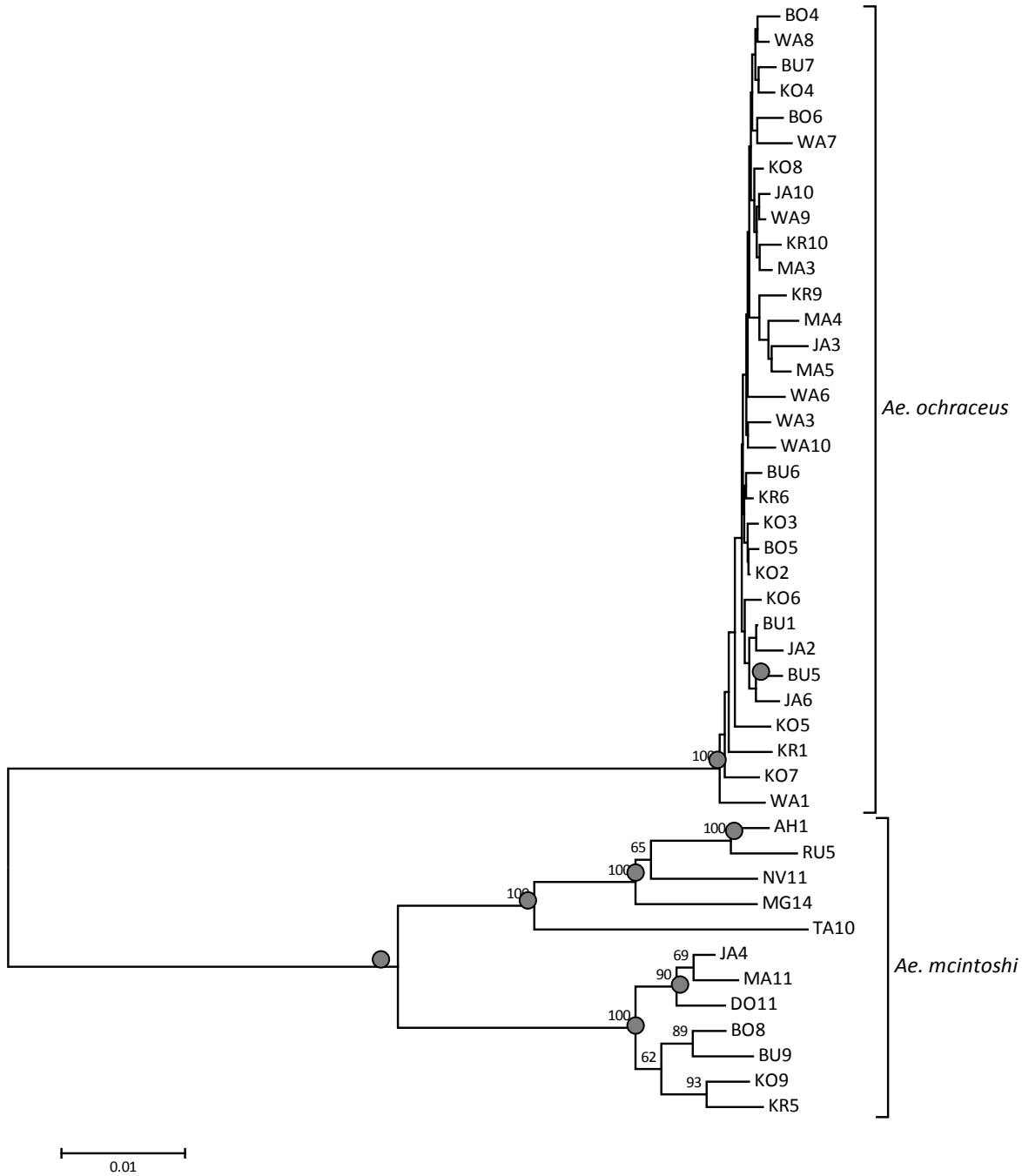


Figure 1 Neighbor-joining (NJ) tree inferred using concatenated *COI* and *ITS* sequence dataset for *Aedes ochraceus* in northeastern area of Kenya. *Aedes mcintoshi* was included as outgroups and regions containing gaps were excluded. Bootstrap support values >60 based on 10,000 replicates from the NJ analysis are indicated, with posterior probabilities >95 being denoted by a shaded circle on the relevant notes. Taxon codes follow those provided in Table 1.

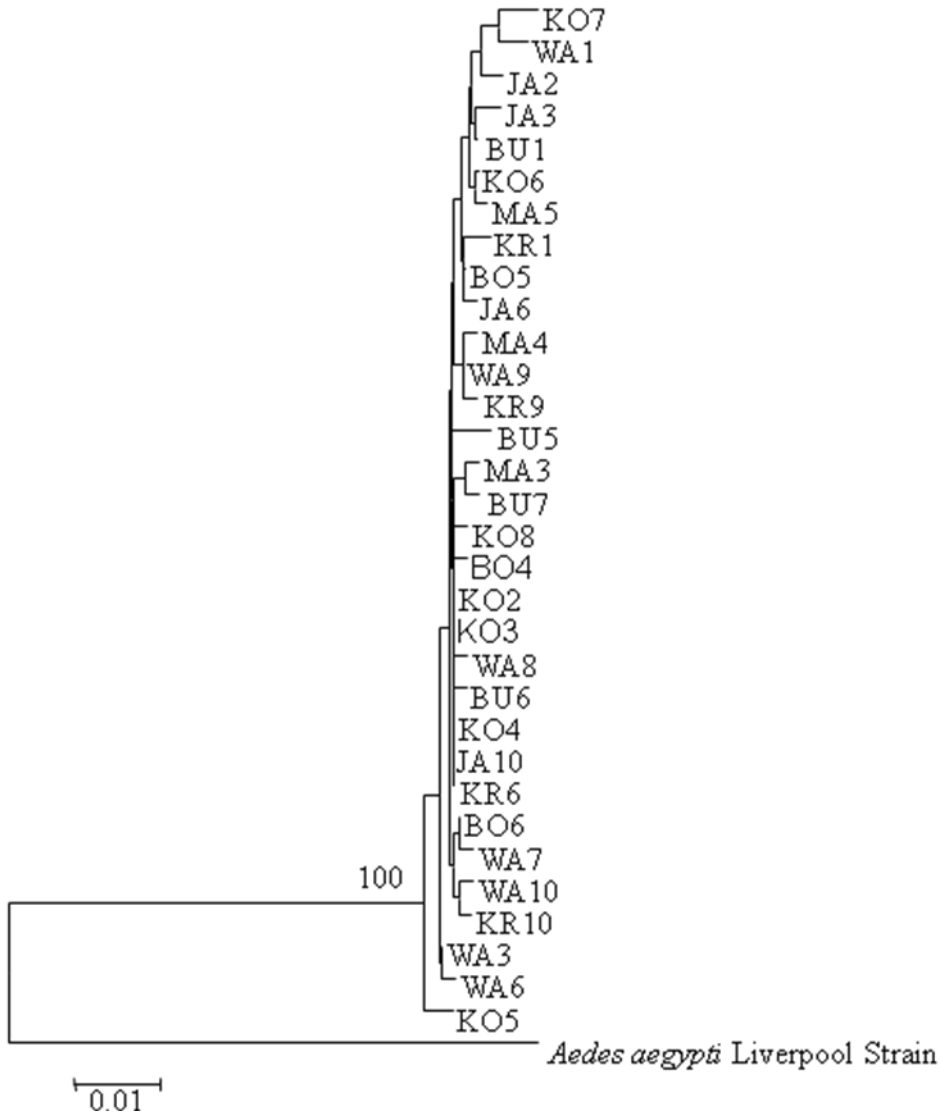


Figure 2. Neighbor-joining (NJ) tree inferred using DNA barcode region for *Aedes ochraceus* in northeastern area of Kenya.

The phylogenetic trees for the individual datasets and for the concatenated data set (Figure 1) yielded a star-like phylogeny with shallow divergences. The lack of sub-structuring contrasts markedly with results obtained for *Ae. mcintoshi* from Kenya (Chapter 5, Figures 2 and 3) and is consistent with a recent and rapid expansion. These features were also reflected in the TCS parsimony and network analyses (Figures 3 and 4). The TCS analysis could join haplotypes separated by a maximum of 16 and 14 mutational steps respectively for *COI* and *ITS* locus, based on the 95% confidence criterion (Figures 4a and b). Genealogical relationships among

haplotypes inferred using TCS indicated that ancestral haplotypes were from Kotile for both markers used (KO9 for *COI* and KO7 for *ITS*); and that the other haplotypes originated from this site (Figures 4a and b).

The neutrality tests for historical population expansion indicate that the mtDNA diversity of *Ae. ochraceus* is the result of a single rapid expansion. Both Fu's *Fs* and Tajima's *D* were significantly negative (*Fs* = -64.455, *D* = -2.057, P< 0.05). Further, the mismatch distribution also supported expansion of *Ae. ochraceus* (Figure 5) with an essentially unimodal distribution of frequency of pairwise differences with raggedness index (r) of 0.0035 and an R2 value of 0.0.0352 both consistent with population expansion or growth for this species (Figure 5). In contrast, for the *ITS* locus, both Tajima's *D* (*D* = -0.2074, P> 0.1) and Fu's *Fs* (*Fs* = -34.053, P >0.1) were negative but non-significant.

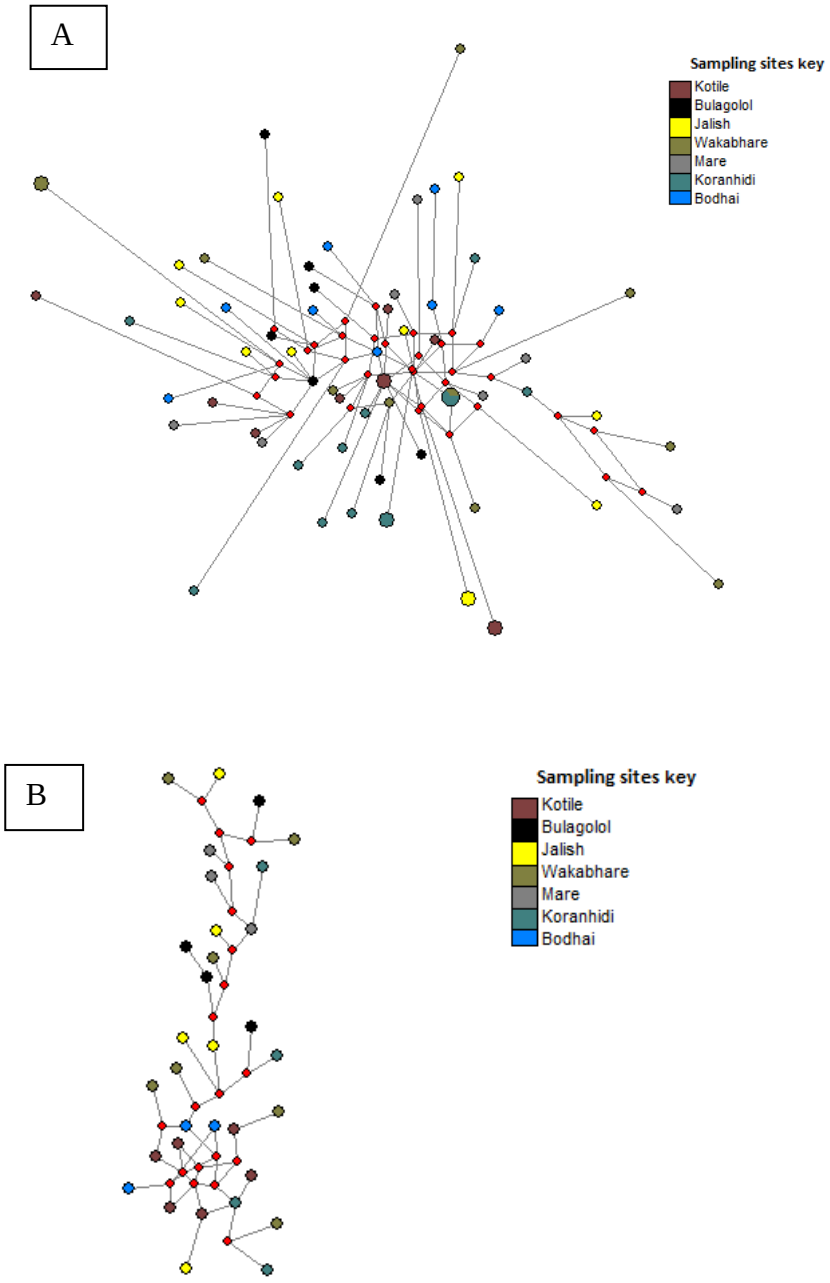
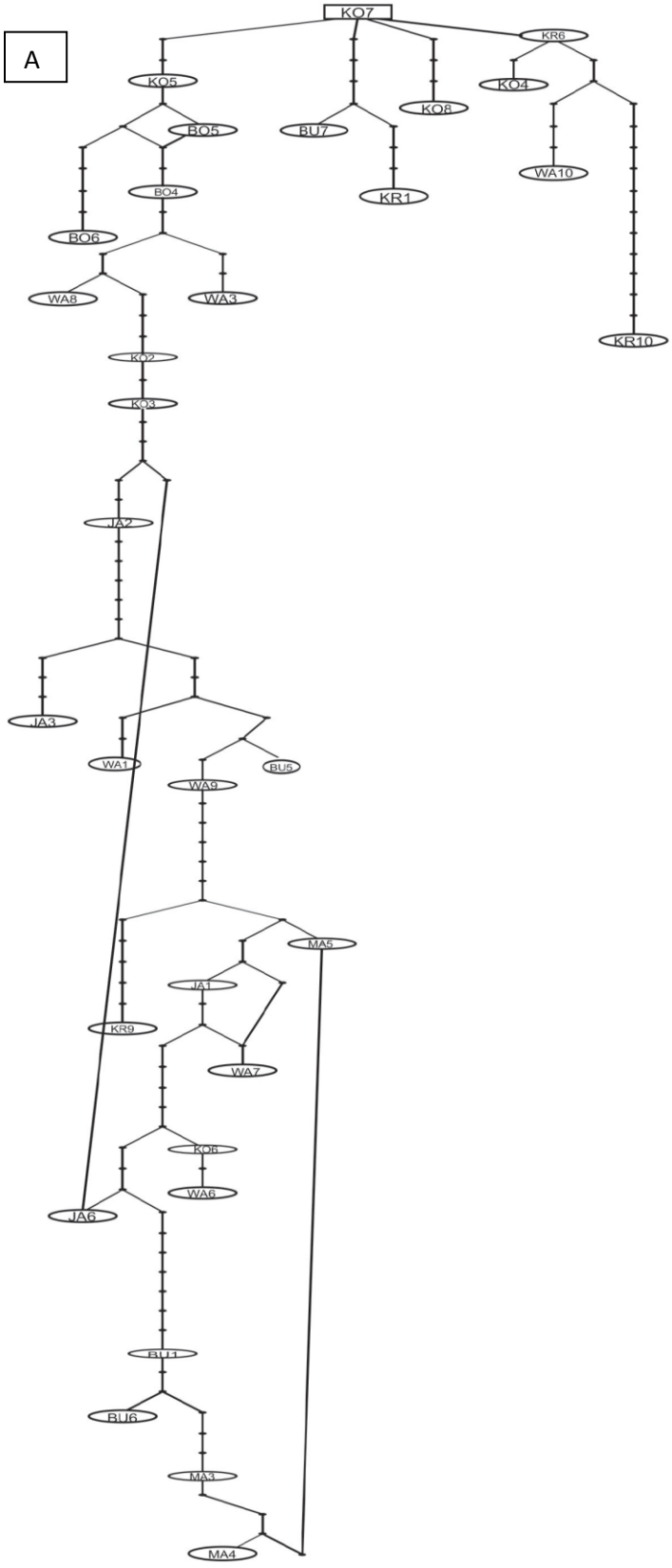


Figure 3 Median-joining network showing mutational differences among A). 60 cytochrome oxidase subunit I (*COI*) B). 32 Internal transcribed spacer (*ITS*) haplotypes (bp) representing 67 and 32 *Aedes ochraceus* specimens from Kenya. Each circle represents a haplotype and the color depicts the origin in terms of sampling site, and the frequency of each haplotype. The smallest circles denote unique haplotypes with labels corresponding to the singe clade identified and each small very red square representing the mutational steps. The area of circles is proportional to the frequency of the haplotypes.

Table 2 Estimates of average evolutionary divergence over sequence pairs within sites for *Aedes ochraceus* for both *COI* and *ITS* gene loci.

	Mare	Kotile	Jalish	Wakabhare	Koranhidi	Bodhai	Bulagolol
Mare	−	0.007	0.005	0.006	0.008	0.007	0.006
Kotile	0.006	−	0.006	0.006	0.008	0.005	0.005
Jalish	0.006	0.006	−	0.006	0.008	0.007	0.006
Wakabhare	0.007	0.007	0.007	−	0.008	0.006	0.006
Koranhidi	0.006	0.006	0.006	0.007	−	0.009	0.008
Bodhai	0.005	0.005	0.006	0.007	0.006	−	0.006
Bulagolol	0.006	0.005	0.006	0.007	0.006	0.005	−

Lower diagonal, *COI*; upper diagonal, *ITS*


A
KO7
KR6
KO5
KO4
KO8
BO5
BU7
WA10
KR1
BO4
BO6
WA8
WA3
KR10
KO2
KO3
JA2
JA3
WA1
BU5
WA9
MA5
JA1
KR9
WA7
KO6
WA6
JA6
BU1
BU6
MA3
MA4

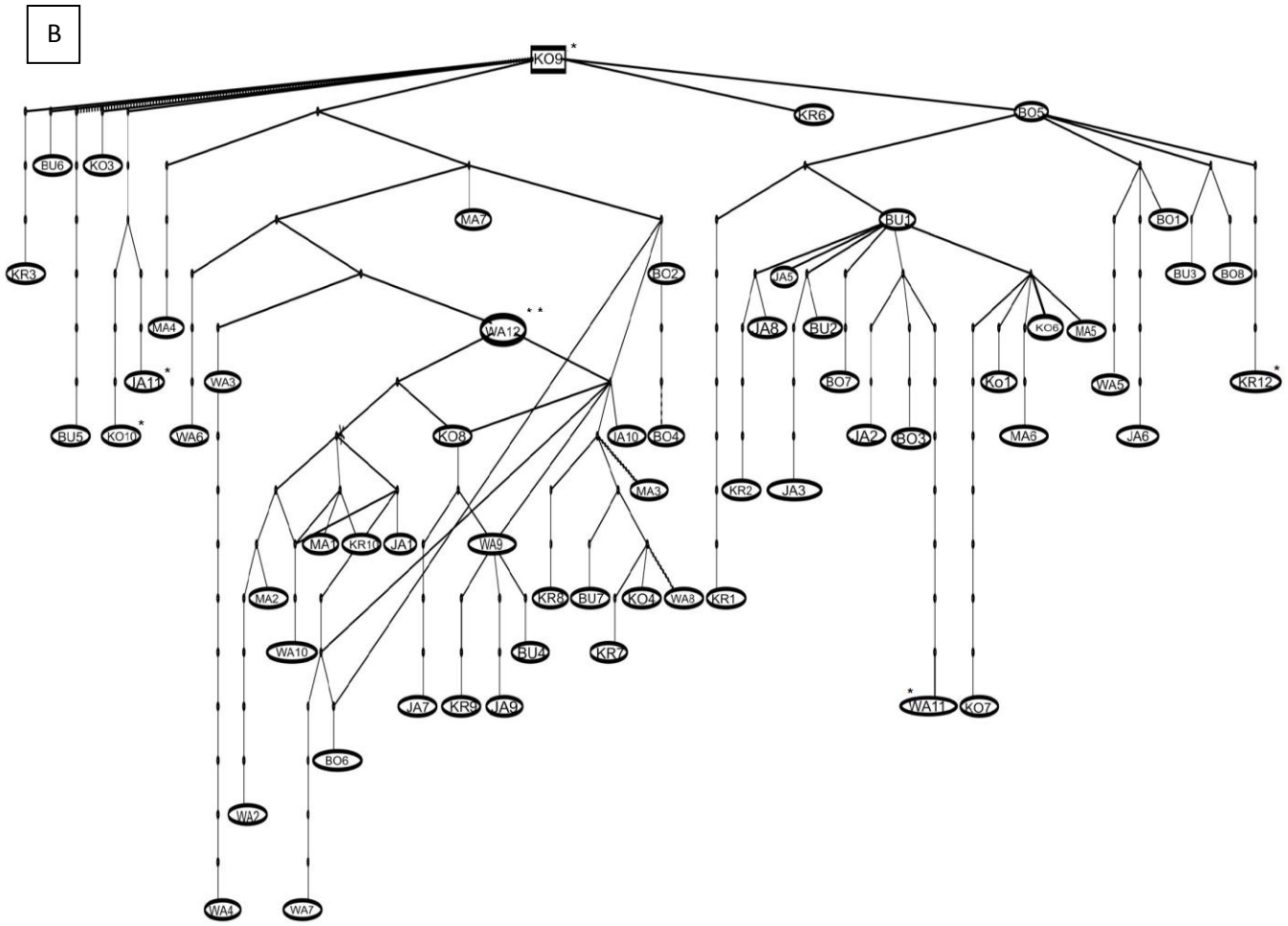


Figure 4. Statistical parsimony haplotype network of *Aedes ochraceus* based on (A) *ITS* locus (B) *COI* locus.

Labels in the circles correspond to the haplotype origin; Black dots on the interconnecting branches represent the number of mutational steps. * shared haplotype from same site; ** shared haplotypes from two different sites (WA and KR). Taxon codes follow those provided in Table 1.

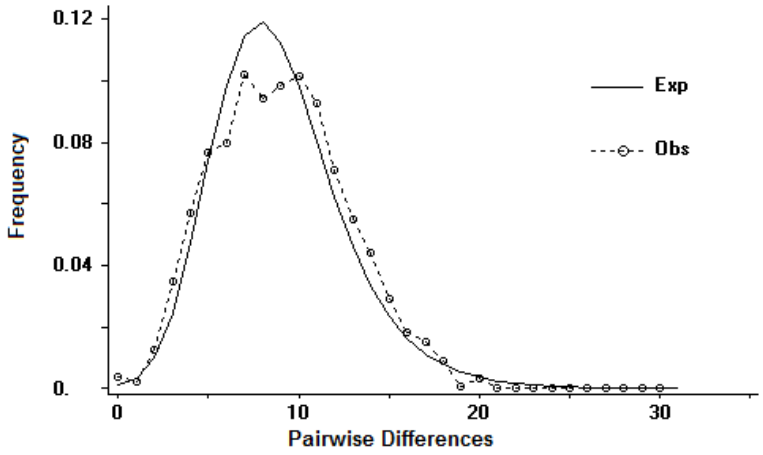


Figure 5 Mismatch distribution showing the frequency of pairwise differences in cytochrome oxidase subunit I sequence for all sampled *Aedes ochraceus*. Observed distributions are represented by the black line, and the expected distribution under the sudden expansion model is represented by the dotted line.

Discussion

We have explored the level of genetic diversity in *Ae. ochraceus* from RVF epidemic-prone areas of northeastern Kenya, for the first time, using two DNA markers. Based on mitochondrial and nuclear molecular data we have shown that *Ae. ochraceus*, is composed of a single large clade, suggesting a uniform homogeneous population as evidenced from a low intra and inter site average evolutionary divergence and from the recovery of a single lineage within limited intra-clade sub-structure.

The genetic diversity of *Ae. ochraceus* individuals in the sampled areas of northeastern Kenya is high as evident from the relatively high estimates of the haplotype, but nucleotide diversity was low indicative of an admixed and widely dispersed sample of individuals belonging to a single population / lineage. The overall low frequency of shared haplotypes and ancestral haplotype allocation to Kotile for both gene markers is suggestive of rapid population growth from an ancestral population at Kotile (Figures 3a and b). This is supported by both neutrality tests as well as mismatch distributions for the *COI* data set, which had a unimodal distribution consistent with a rapid population expansion [44].

The differences in demographic patterns observed for both data sets, may relate to differences in the mutational rates for both markers. Nuclear mutation rates are generally slower than mitochondrial ones [24, 45], thus the lack of expansion detected in the nuclear sequences in this species in the present study suggests either the differentiation is restricted to the mitochondrial genome, or it is recent and not yet visible in the nuclear genome. However, as the same genome targets were characterized for *Ae. mcintoshi* (Chapter 5), which co-occurs at many of the same localities as *Ae. ochraceus* and yielded different results, it is probable that the difference is due to a recent expansion. The differences in population parameters of these two species are suggestive of *Ae. ochraceus* being a relatively recent introduction to Kenya and requires further investigation. As a medically important insect group, mosquitoes are dependent on specific climatic conditions for their survival and spread, therefore understanding the factors that influence their distribution is critical in the face of a range of emerging arboviruses. While significant insights have been gained from our analyses, comparable studies of *Ae. ochraceus* from different countries especially from West Africa, where it has been known as a vector for long time, could shed light on origin, spread, genetic variability and patterns of diversification.

Genealogical relationships among haplotypes inferred using TCS indicated that ancestral haplotypes were from Kotile for both markers used (KO9 for *COI* and KO7 for *ITS*); and the other haplotypes originated from this site. This is not surprising as Kotile is in close proximity to the River Tana; as such provides conditions for more frequent breeding due to the riverine effect and possibilities of movement along the Tana River and off shore when the river bursts its banks as well as during pastoral migration in search of pastures and new markets. The shared ancestral origin as depicted by both markers, suggest this site could therefore provide a refugial population from which active dispersion of females would have originated into different populations within their flight ranges for spread and dispersal to other areas. Although studies on the flight range for this species is currently unknown, such dispersal could be facilitated by wind [10, 46] or through sequences of shorter range flights culminating in apparent long distance [47] or animal movements from one location to another [10]. The tendency for recent, extensive dispersal, however, is also reflected in the low genetic differentiation seen between locations.

As a prerequisite for an optimal epidemic arboviral vector, a mosquito species needs to be susceptible to infection, spatially and temporally abundant, long-lived and willing to blood feed on amplification hosts including dead-end ones [48-50]. In the context of RVF, *Ae. ochraceus* fulfills most of these requirements, in particular, susceptibility is tied to a number of isolations from field-collected samples and there is a clear preference for amplifying hosts of RVF virus [6, 51-53].

Conclusion

Northeastern Kenya is a typical pastoral and arid area with livestock trade constituting over 90% of the inhabitants' source of income and livelihood [54]. *Aedes ochraceus* which our data suggest is a relatively recent introduction to this country, is increasingly becoming an important vector of RVF especially in Northeastern Kenya, the epicenter of the disease [6]. With the availability of suitable breeding sites in vast drier savannahs of northeastern Kenya [11], and clear preference for livestock [53] which are susceptible hosts for the disease, the population expansion of this species as evident from our data suggest *Ae. ochraceus* will continue to increase its importance in RVF transmission especially in this epidemiologically important region of Kenya.

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General Conclusions and Recommendations

Monitoring mosquito populations and mosquito-borne virus activity are the cornerstones of surveillance programs (Leal *et al*., 2008; Gu *et al*., 2008). Most monitoring systems target adult female mosquitoes as only they seek hosts for blood and are thus responsible for disease transmission (Qiu *et al*., 2007). The standard monitoring tool for mosquitoes which involves the use of CO2-baited CDC light traps, catches a wide range of insects making sorting of mosquitoes from captured insects for viral testing laborious and time consuming. Additionally, the method is inadequate and less sensitive for use during the low intensity inter-epidemic period (IEP) of enzootic virus transmission where viral activity may remain undetected among mosquito species (EFSA, 2005; Labeaud *et al*., 2007, Labeaud *et al*., 2011a); thus the necessity to develop more sensitive and effective monitoring tools so as to increase trap captures of mosquito vectors and maximize detection of virus activity.

The purpose of this study was to develop an odor-baited trapping system for improved surveillance of Rift Valley fever virus (RVFV) vectors through understanding the cues used by adult female mosquitoes in host location. In addition to this we aimed to evaluate coloured light preferences for important RVF vectors. The project also sought to characterize the population genetics of key RVFV vectors (*Aedes mcintoshi* and *Ae. ochraceus*) using cytochrome oxidase subunit I (*COI*) and a long stretch of the internal transcribed spacer (*ITS*) to gain deeper insight into the role of the vectors' genetics and spread of RVF, for surveillance and control purposes. Samples were collected from several localities in Kenya encompassing RVF endemic, epidemic and disease-free areas. Consequently a number of research questions listed in the 'General Introduction' each of which is associated with the six research chapters in this study were formulated to guide the course of the present investigation. In order to address the research questions formulated in this study, the derived data were subjected to a range of analyses including field-tested behavioral bioassays, chemical, physiological and molecular.

In an effort to improve sampling of mosquito vectors using artificial light, Chapter 2 investigated if light emitting diodes (LEDs) could be effective substitutes for incandescent lamps commonly

used in CDC light traps for mosquito surveillance, and if so, determine the best color for attracting important RVFV vectors. The overall data revealed better performance of the control incandescent light relative to the other colored LEDS (blue, green, red, violet, combined b/g/r) although the difference in terms of mosquito captures was only significantly different from red and violet. However, there was evidence of seasonal preference of *Ae. mcintoshi* and *Ae. ochraceus* at Ijara with a bias towards BGR and blue traps, respectively, in one trapping period but with a declining pattern during another period at same site. The trapping design and color used in the experiment suggest that none of the LEDs out-competed the standard incandescent light. The data however provided preliminary evidence that a preference might exist for some of the RVF mosquito species based on observed differential attraction to the different light colors requiring future studies to compare reflected versus transmitted light and the incorporation of colored light of varying intensities.

Initial field trapping assays using sheep the most susceptible RVFV host (Findlay *et al*., 1936; Swanepoel & Coetzer, 2004; EFSA, 2005), showed that, the addition of fur (representing skin volatile) from sheep to the standard CO_2-baited light trap improved captures of key RVFV vectors and this is the subject of Chapter 3. The data provided for future investigations to identify attractive components present in these natural odors, so that they can be incorporated into existing traps to serve as a population density-monitoring tool for improved arbovirus disease surveillance during IEP.

Furthermore, understanding interspecific host preferences can reveal new semiochemicals that could be exploited to maximize development of better attractants. Therefore, in addition to sheep, the attractiveness of different RVF virus hosts (cow, donkey, goat, human) were assessed further (as guided by molecular blood meal analysis - Omondi *et al*., 2011; Omondo *et al*., unpublished data) to RVFV vectors in field experiments. This formed the basis for Chapter 4 whereby in the comparative field trapping experiments, an analogous pattern was observed whereby an increase in mosquito captures were recorded with the addition of skin odors from cow, donkey, goat, sheep and humans to CO_2 traps compared to control traps having CO_2 alone. An interesting finding was also that a higher proportion of engorged mosquitoes (bloodfed + gravid) were recorded in CO_2 traps containing skin odors from these animal hosts relative to control CO_2 trap alone. Electrophysiological studies to find out which compounds the RVFV

mosquitoes responded to, revealed a similarity in response profile to the aldehyde components, heptanal, octanal, nonanal and decanal, common to all the hosts evaluated. We further exploited these components as attractants by field-testing in traps baited with lures using the compounds individually or in different blends. Blends were representative of the mean ratio of occurrence of the aldehydes in each of the animals and a blend of the four aldehydes based on the doses of individual components that elicited optimal attraction in the preliminary field assays. In field trials, synergism was observed to each of these compounds combined with CO_2 in increasing captures of these mosquito vectors in a dose-dependent manner. The blend formulated from an optimal attractive dose of each of the compounds synergized with CO_2 significantly increased trap captures than control traps baited with CO_2 alone. The four-component blend attracted multiple mosquito vectors of the disease under field conditions suggesting that a trapping system based on this formulation offers opportunity for its use as a tool for RVF disease surveillance.

In chapter 5, both mitochondrial and nuclear markers were used to characterize the genetic structure of *Ae. mcintoshi* populations, a key vector of RVFV, across virus-endemic, -epidemic and free areas of Kenya. The results revealed the presence of geographically distinct lineages that coincide with magnitude of RVF in Kenya. Both gene loci recovered four well-supported lineages with notable levels of genetic diversity being detected between lineages across the study area. This was evident from combined phylogenetic analyses, and median-joining networks and analysis of molecular variance (AMOVA). Broadly for both markers, lineage I was restricted to RVF endemic areas and lineage III and IV restricted to the epidemic-prone areas of northeastern Kenya. However within northeastern Kenya, the epicenter of RVF epidemics in Kenya, these two lineages occur in sympatry in most of the localities sampled but overall low genetic distances between the clades suggest a variant or cryptic species in this region. Furthermore, disproportionate abundance of these lineages in these localities and their presence/absence may drive differential transmission and outbreak pattern of the disease in different communities of northeastern Kenya. The identification of four distinct lineages within what is currently described as *Ae. mcintoshi*, paves the way for further insights into the processes underlying the observed diversity including competence studies among individuals from the different clades but particularly among those sourced from areas of distributional overlap between lineages III and IV. The taxonomic elucidation of the status of this species requires additional stringent markers such as single nucleotide polymorphism or microsatellite analysis in areas especially

northeastern Kenya where lineages overlap, so as to bypass any problems associated with pre-mating barriers between populations and this should be backed by extensive sampling to cover the entire range of this species. Equally important will be to perform vector competence studies to ascertain if there would be variation in the transmission of the virus by the different lineages especially sourced from areas of distributional overlap between lineages III and IV, but inclusive of the other lineages as well. Additionally, cross breeding experiments between individuals from the two lineages to determine whether they are reproductively isolated, may be helpful although the challenge of rearing flood *Aedes* presents a major hurdle; therefore, the taxonomic delineation of the two lineages will have to rely on thorough morphology and sequence divergence. If both lineages are implicated as vectors, the characteristics of their breeding sites, their behaviors and their migration history could be used to predict changes in RVFV transmission patterns in the northeastern region, and other endemic regions in the rest of the country, and to provide useful information for viral surveillance and possibly targeted vector control.

In Chapter 6, similar markers as detailed in chapter 5 were applied in the study of the population structure and diversity of *Aedes ochraceus*, another primary vector of RVFV in Kenya. As revealed by the data, not only were the mean evolutionary divergence estimates among and within sites low, Neighbor-joining trees showed a single large lineage with very low bootstrap support which was also corroborated by network and TCS parsimony analyses, suggestive of a homogenous population. Despite the low nucleotide diversity, the number of polymorphic sites and haplotype diversity within sites was high indicating an admixture of individuals from different origins. Interestingly, significant negative neutrality tests of Tajima's *D* and Fu's *Fs* were evident for the *COI* locus only, which was supported by a unimodal curve for the mismatch distribution of frequencies of pairwise differences, indicative of a rapid population expansion for this species. With the availability of suitable breeding sites in the vast drier savannahs of northeastern Kenya, and clear preference for livestock which are susceptible hosts for the disease and abundant in this area, the population expansion as evident from our data suggest this species will continue to increase in importance in RVFV transmission in Kenya. While significant information have been gained from our analyses, comparable studies of *Ae. ochraceus* from different countries especially from West Africa where it has been known as a vector for long

time is likely to provide additional insights into genetic variability and patterns of diversification of this species.

Overall, this study represents the most detailed investigation of the chemical basis for mosquito attraction (leading to the development of an improved trapping tool) which in combination with the genetics of key vectors of RVFV assists in explaining transmission and spread of the disease in Kenya. The improved understanding of the vectoring capability and the means by which sampling methods developed can maximise detection probability is seminal to effective monitoring of arboviral disease processes. As a medically important insect group, mosquitoes are dependent on specific climatic conditions for their survival and spread, therefore understanding the factors that influence their distributions is crucial in the face of a range of emerging arboviruses.

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Appendix

Table 1. Primers used for PCR amplification of the different gene fragments.

Target	Primer	Sequence (5'-3')	Annealing temperature (^{O}C)
COI	LCO1490F[a]	GGTCAACAAATCATAAAGATATTGG	55
	TL2-N-3014R[b]	TCCAATGCACTAATCTGCCATATTA	55
ITS	CAS18SF[c]	TACACACCGCCCGTCGCTACTA	55
	ITSPorterR[d]	ATGCTTAAATTTAGGGGGTAGTC	55

[a]Folmer et al, 1994; [b]Simon et al, 1994; [c]Ji et al, 2003; [d]Porter and Collins, 1991.

Table 2. Sample location, code and haplotype number of *Aedes mcintoshi* for the two gene loci used in this study.

Sampling site	Sample codes	*COI*	*COI* haplotype number	*ITS*	*ITS* haplotype number
Kotile	KO1	x	2	−	
Kotile	KO2	x	5	−	
Kotile	KO3	x	10	−	
Kotile	KO4	x	11	−	
Kotile	KO5	x	14	−	
Kotile	KO6	x	58	−	
Kotile	KO7	x	84	x	32
Kotile	KO8	x	90	x	6
Kotile	KO9	x	108	x	2
Kotile	KO10	x	118	−	
Kotile	KO11	x	121	x	1
Kotile	KO12	x		−	
Kotile	KO13	−		x	1
Kotile	KO14	−		x	10
Kotile	KO15	−		x	29
Tana	TA1	x	1	x	43
Tana	TA2	x	3	x	42
Tana	TA3	x	4	−	
Tana	TA4	x	7	x	40
Tana	TA5	x	9	−	

Tana	TA6	x	12	−	
Tana	TA7	x	15	−	
Tana	TA8	x	16	−	
Tana	TA9	x	18	−	
Tana	TA10	x	19	x	41
Tana	TA11	−		x	42
Bulagolol	BU1	x	62	x	49
Bulagolol	BU2	x	74	−	
Bulagolol	BU3	x	80	−	
Bulagolol	BU4	x	99	−	
Bulagolol	BU5	x	105	x	9
Bulagolol	BU6	x	105	−	
Bulagolol	BU7	x	111	−	
Bulagolol	BU8	x	113	−	
Bulagolol	BU9	x	119	x	12
Bulagolol	BU10	x	120	x	16
Bulagolol	BU11	−		x	15
Bulagolol	BU12	−		x	54
Jalish	JA1	x	51	−	
Jalish	JA2	x	56	−	
Jalish	JA3	x	61	−	
Jalish	JA4	x	70	x	19
Jalish	JA5	x	75	x	24
Jalish	JA6	x	91	x	17
Jalish	JA7	x	94	x	20
Jalish	JA8	x	97	−	
Jalish	JA9	x	100	x	24
Jalish	JA10	x	112	−	
Jalish	JA11	x	129	−	
Jalish	JA12	x	130	−	
Jalish	JA13	x	130	x	11
Jalish	JA14	x	131	−	
Jalish	JA15	−		x	19
Bodhai	BO1	x	67	−	
Bodhai	BO2	x	82	−	
Bodhai	BO3	x	83	−	
Bodhai	BO4	x	96	−	
Bodhai	BO5	x	101	−	5
Bodhai	BO6	x	101	x	2
Bodhai	BO7	x	123	−	
Bodhai	BO8	x	124	x	3

Bodhai	BO9	x	125	−	
Bodhai	BO10	x	126	−	
Bodhai	BO11	x	128	x	5
Bodhai	BO12	x	132	x	4
Bodhai	BO13	−		x	2
Bodhai	BO14	−		x	4
Wakabhare	WA1	x	54	−	
Wakabhare	WA2	x	55	x	8
Wakabhare	WA3	x	57	x	30
Wakabhare	WA4	x	68	x	14
Wakabhare	WA5	x	78	−	
Wakabhare	WA6	x	79	x	26
Wakabhare	WA7	x	88	−	
Wakabhare	WA8	x	90	−	
Wakabhare	WA9	x	92	x	18
Wakabhare	WA10	x	106	−	
Wakabhare	WA11	x	106	−	
Wakabhare	WA12	x	115	x	7
Mare	MA1	x	49	−	
Mare	MA2	x		−	
Mare	MA3	x	66	x	21
Mare	MA4	x	69	−	
Mare	MA5	x	69	x	22
Mare	MA6	x	72	−	
Mare	MA7	x	73	−	
Mare	MA8	x	81	−	
Mare	MA9	x	87	x	31
Mare	MA10	x	95	x	28
Mare	MA11	x	135	x	20
Mare	MA12	x	136	−	
Mare	MA13	−		x	20
Koranhidi	KR1	x	65	−	
Koranhidi	KR2	x	76	x	22
Koranhidi	KR3	x	76	−	
Koranhidi	KR4	x	93	x	53
Koranhidi	KR5	x	93	x	1
Koranhidi	KR6	x	98	−	
Koranhidi	KR7	x	98	−	
Koranhidi	KR8	x	104	−	
Koranhidi	KR9	x	109	−	
Koranhidi	KR10	x	118	−	
Koranhidi	KR11	x	122	−	

Koranhidi	KR12	x	127	−	
Koranhidi	KR13	−		x	13
Elhumow	EH1	x	59	x	19
Elhumow	EH2	x	63	x	20
Elhumow	EH3	x	64	−	
Elhumow	EH4	x	77	−	
Elhumow	EH5	x	85	x	25
Elhumow	EH6	x	86	x	23
Elhumow	EH7	x	89	−	
Elhumow	EH8	x	103	x	51
Elhumow	EH9	x	107	x	19
Elhumow	EH10	x	110	−	
Elhumow	EH11	x	117	−	
Elhumow	EH12	x	134	−	
Elhumow	EH13	−		x	19
Elhumow	EH14	−		x	19
Disso	DO1	x	13	−	
Disso	DO2	x	48	−	
Disso	DO3	x	48	−	
Disso	DO4	x	48	x	22
Disso	DO5	x	50	−	
Disso	DO6	x	53	x	55
Disso	DO7	x	60	x	27
Disso	DO8	x	71	x	52
Disso	DO9	x	102	−	
Disso	DO10	x	114	−	
Disso	DO11	x	116	x	50
Disso	DO12	x	133	−	
Disso	DO13	−		x	51
Marigat	MG1	x	8	−	
Marigat	MG2	x	17	x	38
Marigat	MG3	x	20	−	
Marigat	MG4	x	21	−	
Marigat	MG5	x	22	−	
Marigat	MG6	x	22	x	36
Marigat	MG7	x	26	−	
Marigat	MG8	x	27	−	
Marigat	MG9	x	33	−	
Marigat	MG10	x	33	x	44
Marigat	MG11	x	36	−	
Marigat	MG12	x	40	−	

Marigat	MG13	x	43	−	
Marigat	MG14	x	43	x	33
Marigat	MG15	−		x	36
Marigat	MG16	−		x	37
Marigat	MG17	−		x	45
Naivasha	NV1	x	22	x	47
Naivasha	NV2	x	28	−	
Naivasha	NV3	x	29	−	
Naivasha	NV4	x	30	−	
Naivasha	NV5	x	31	−	
Naivasha	NV6	x	31	x	36
Naivasha	NV7	x	32	−	
Naivasha	NV8	x	37	−	
Naivasha	NV9	x	38	−	
Naivasha	NV10	x	41	−	
Naivasha	NV11	x	42	x	36
Naivasha	NV12	x	42	−	
Naivasha	NV13	x	42	−	
Naivasha	NV14	−		x	39
Ruiru	RU1	x	6	x	48
Ruiru	RU2	x	25	x	47
Ruiru	RU3	x	25	−	
Ruiru	RU4	x	27	x	46
Ruiru	RU5	x	27	x	46
Ruiru	RU6	x	27	−	
Ruiru	RU7	x	27	−	
Ruiru	RU8	x	27	−	
Ruiru	RU9	x	27	−	
Ruiru	RU10	x	36	−	
Ruiru	RU11	x	36	−	
Ahero	AH1	x	23	x	46
Ahero	AH2	x	24	−	
Ahero	AH3	x	34	−	
Ahero	AH4	x	34	−	
Ahero	AH5	x	35	−	
Ahero	AH6	x	39	−	
Ahero	AH7	x	44	x	34
Ahero	AH8	x	45	−	
Ahero	AH9	x	46	−	
Ahero	AH10	x	47	x	35

x, samples sequenced for each marker; -, samples without any sequence

Table 3. Sample location, code and haplotype number of *Aedes ochraceus* for the two gene loci used in this study.

Sampling site	Sample name	*COI*	*COI* haplotype number	*ITS*	*ITS* haplotype number
Kotile	KO1	x	17	−	
Kotile	KO2	x	3	x	7
Kotile	KO3	x	18	x	8
Kotile	KO4	x	19	x	9
Kotile	KO5	x	4	x	11
Kotile	KO6	x	20	x	10
Kotile	KO7	x	21	x	2
Kotile	KO8	x	22	x	3
Kotile	KO9	x	3	−	
Kotile	KO10	x	4	−	
Bulagolol	BU1	x	46	x	26
Bulagolol	BU2	x	47	−	
Bulagolol	BU3	x	48	−	
Bulagolol	BU4	x	49	−	
Bulagolol	BU5	x	50	x	27
Bulagolol	BU6	x	51	x	28
Bulagolol	BU7	x	52	x	29
Jalish	JA1	x	28	−	
Jalish	JA2	x	29	x	15
Jalish	JA3	x	30	x	16
Jalish	JA4	x	5	−	
Jalish	JA5	x	31	−	
Jalish	JA6	x	32	x	17
Jalish	JA7	x	33	−	
Jalish	JA8	x	34	−	
Jalish	JA9	x	35	−	
Jalish	JA10	x	36	x	18
Jalish	JA11	x	5	−	
Wakabhare	WA1	x	6	x	19
Wakabhare	WA2	x	37	−	
Wakabhare	WA3	x	38	x	20
Wakabhare	WA4	x	39	−	
Wakabhare	WA5	x	40	−	
Wakabhare	WA6	x	41	x	21
Wakabhare	WA7	x	42	x	22
Wakabhare	WA8	x	43	x	23

Wakabhare	WA9	x	44	x	34
Wakabhare	WA10	x	45	x	25
Wakabhare	WA11	x	6	−	
Wakabhare	WA12	x	7	−	
Mare	MA1	x	23	−	
Mare	MA2	x	24	−	
Mare	MA3	x	25	x	12
Mare	MA4	x	26	x	13
Mare	MA5	x	27	x	14
Mare	MA6	x	1	−	
Mare	MA7	x	2	−	
Koranhidi	KR1	x	53	x	30
Koranhidi	KR2	x	54	−	
Koranhidi	KR3	x	55	−	
Koranhidi	KR4	x	8	−	
Koranhidi	KR5	x	5	−	
Koranhidi	KR6	x	56	x	32
Koranhidi	KR7	x	57	−	
Koranhidi	KR8	x	58	−	
Koranhidi	KR9	x	59	x	31
Koranhidi	KR10	x	60	x	1
Koranhidi	KR11	x	7	−	
Koranhidi	KR12	x	8	−	
Bodhai	BO1	x	11	−	
Bodhai	BO2	x	12	−	
Bodhai	BO3	x	13	−	
Bodhai	BO4	x	14	x	4
Bodhai	BO5	x	15	x	5
Bodhai	BO6	x	16	x	6
Bodhai	BO7	x	9	−	
Bodhai	BO8	x	10	−	

x, samples sequenced for each marker; -, samples without any sequence