ECOLOGY AND POPULATION GENETICS OF THE MALARIA VECTOR *Anopheles arabiensis* PATTON IN THE REPUBLIC OF SUDAN

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by

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LIST OF ABBREVIATIONS

s.l	sensu lato
s.s	sensu stricto
WHO	World Health Orgnization
GIA	Gezira Irrigated Area
DDT	**D**ichloro**d**iphenyl**t**richloroethane
dATP	deoxyadenosine triphosphate
ddw	distilled de-ionized water
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
PCR	Polymerase chain reaction
rDNA	ribosomal DNA
mtDNA	mitochondrial DNA
IGS	Intergenic spacers
ITS	Internal transcribed spacer
ND5	NADH dehydrogenase subunit 5
FMOH	Federal Ministry of Health Sudan

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EKOLOGI DAN GENETIK POPULASI VEKTOR MALARIA

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ABSTRAK

Objektif utama kajian ini adalah untuk mengemaskini taburan geografi *Anopheles gambiae* spesies kompleks di Sudan dan menyiasat struktur genetik populasi *An. arabiensis*, vektor malaria utama di Sudan. Sebanyak 4932 nyamuk termasuk 3047 larva dan 1885 dewasa (jantan dan betina) telah dikutip dari Abu Algoni dan Algerif Barat di Sudan pada bulan Jun 2010 hingga Mei 2011. Daripada jumlah nyamuk yang dikutip, 88.9% nyamuk adalah dari Algoni dan 11.1% dari perkampungan Algerif Barat. Dua spesies, *An. rufipes dan An. arabiensis* telah ditemui, spesies pertama hanya ditemui di Abu Algoni, manakala spesies kedua ditemui di kedua-dua perkampungan, dan mewakili lebih daripada 99% daripada jumlah tangkapan. Nyamuk didapati membiak di banyak jenis habitat termasuk terusan, air kolam sementara, jejak tapak haiwan, air dari paip pecah, dan bekas air. Dalam kedua-dua kampung kelimpahan bermusim *An.arabiensis* adalah yang paling tinggi semasa musim hujan; ini boleh dikaitkan dengan terdapatnya habitat pembiakan yang berlebihan terbentuk akibat curahan hujan. Majoriti adalah parus menunjukkan kadar kemandirian yang tinggi, maka menunjukkan keupayaan vektor yang tinggi vektor dalam mentransmisikan malaria. Sampel untuk ND5, gen ITS dan kajian mikrosatelit telah dikutip dari kawasan-kawasan yang berlainan di Sudan pada bulan Jun 2010 hingga Mei 2011. Tapak koleksi termasuk Mygoma, Alhaj Yousif dan Algerif Barat (Negeri Khartoum), Alkrmota dan Alhalanga Shemal (Negeri Kassala) dan Abu Algoni (Negeri Sennar). Gen separa mtDNA dan ND5 telah digunakan untuk mengkaji variasi jujukan dalam spesies di Sudan. Kepelbagaian

haplotip adalah tinggi (0.61- 0.90) dengan kepelbagaian nukleotida (0.003-0.004) yang rendah. Aliran gen yang tinggi dan tiada pengasingan secara jarak diperhatikan antara populasi. Dengan gen ITS, terdapat kepelbagaian haplotip pada tahap rendah dalam kebanyakan populasi dan kepelbagaian nukleotida rendah dalam semua populasi. Satu set tujuh lokus mikrosatelit telah digunakan untuk menyiasat dengan lebih lanjut struktur populasi *An.arabiensis* di Sudan. Sampel populasi *An. arabiensis* telah dikutip dari tiga lokasi di Negeri Khartoum dan satu lokasi di Negeri Sennar yang mewakili ekologi biotop ekologi yang berbeza yang dipisahkan oleh Sungai Nil dan cabang nya. Populasi dari Negeri Kassala juga telah dimasukkan dalam analisis untuk menyiasat sebarang pengasingan secara jarak dan kemungkinan hadirnya keturunan populasi *An. arabiensis* yang berbeza di Sudan. Analisis genetik mendedahkan perbezaan populasi yang tinggi dan aliran gen yang berterusan di kalangan populasi yang dikaji, tanpa sebarang tanda-tanda pengasingan. Kadar penghijrahan yang tinggi dan kekurangan variasi genetik antara populasi di Sudan telah dikaitkan dengan pergerakan manusia dan haiwan ternakan yang berterusan antara lokasi yang dikaji, yang mungkin membantu dalam penyebaran *An. arabiensis*.

ECOLOGY AND POPULATION GENETICS OF THE MALARIA VECTOR *Anopheles arabiensis* PATTON IN REPUBLIC OF SUDAN

ABSTRACT

The main objectives of this study were to update the geographical distribution of *Anopheles gambiae* complex and to investigate the genetic structure of *Anopheles arabiensis*, the main malaria vector in Sudan. A total of 4,932 mosquitoes including 3047 larvae and 1885 adults (males and females) were collected from Abu Algoni and Algerif West in Sudan during June 2010 to May 2011. Of the total mosquitoes collected, 88.9% were from Algoni and 11.1% from Algerif West. Two species, *An. rufipes* and *An. arabiensis* were encountered, the former was only found in Abu Algoni, but the latter was found in both villages, and represented more than 99% of the total collection. Mosquitoes were found breeding in many types of habitats including canals, temporary pools of water, animal hooves, water from broken pipes, and water storage containers. In both villages the seasonal abundance of *An.arabiensis* was highest during the rainy season; this could be associated with the availability of more breeding sites created by rainfall. The majority were parous, indicating high survival rates and thus high vectorial capacity in transmitting malaria**.**

The samples for ND5, ITS genes and microsatellite study were collected from different regions of the Sudan during June 2010 to May 2011. The collection sites include Mygoma, Alhaj yousif and Algerif West (Khartoum State), Alkrmota and Alhalanga Shemal (Kassala State) and Abu Algoni (Sennar State). The mtDNA ND5 partial and Nuclear DNA ITS genes were utilized to examine the intraspecies sequence variation in Sudan. Haplotype diversity was high (0.61- 0.90) with low nucleotide diversity (0.003-0.004). High gene flow and no isolation by distance were

observed between populations using ND5 gene. With ITS gene there were low levels of haplotype diversity in most population and low nucleotide diversity in all populations.

A set of seven microsatellite loci was utilized to further investigate the population structure of *An. arabiensis* populations in Sudan. Samples of *An. arabiensis* populations were collected from three localities in Khartoum State and one locality in Sennar State which represented different ecological biotopes separated by the River Nile and its tributaries. Populations from Kassala State were also included in the analysis to investigate any isolation by distance and the possibility of the presence of different lineages of *An. arabiensis* populations in Sudan. Genetic analysis revealed high population differentiation and continuous gene flow among the studied populations, however without any signs of isolation. The high migration rate and lack of interpopulation genetic variation among the Sudanese population was attributed to the continuous human and domestic animals movement among the studied localities that might help in the distribution of *An. arabiensis*.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Malaria is a parasitic disease transmitted through arthropod vectors which are members of the genus *Anopheles.* In 2011 there were about 216 million cases reported globally with an approximate 655,000 reported deaths in 2010 (WHO, 2011). Malaria remains the main obstacle to social and economic development in countries plagued by the disease (Sachs and Malaney, 2002). *Anopheles* is responsible for the transmission of the disease. Although, there are 500 species of the genus *Anopheles*, only 70 are transmitters of human malaria (Hay *et al.*, 2010). Malaria is also a most important reason of death in children under five years of age (WHO, 2008). Pregnant women are also in the danger group as malaria causes severe problems including abortion, low birth weight and maternal anaemia (Newman *et al.*, 2003; Adam *et al.*, 2005; Rogerson *et al.*, 2007). The disease is also a burden on the economy because of the decrease in productivity. In countries with severe transmission, malaria has been responsible for a loss in economic growth estimated at an average of 1.3% annually (Killeen *et al.*, 2004). In countries with high malaria transmission, the control of the disease acting a main role in reducing the level of poverty (WHO, 2005). The estimated number of global malarial deaths has however, fallen from about 985.000 in 2000 to about 655.000 in 2010 (WHO 2010; WHO 2011). This reduction is due to the implementation of effective malaria prevention and control measures for the past 10 years. These include the extensive use of

impregnated bed nets, precise diagnostics and the wider availability of malarial drugs.

In Sudan the use of insecticides as indoor residual spray is the most important approach for controlling malaria vectors. However, *An. arabiensis* has been reported to shown high levels of DDT, malathion, Fenitrothion, Bendiocarb, Propoxur, deltamethrin, lambdacyhalothrin and permethrin resistance in Khartoum, eastern Sudan, El Rahad, Gezira and Central Sudan (El Gaddal *et al.*, 1985; Himeidan *et al.,* 2004; Abdalla *et al.,* 2008; *Seidahmed et al.,* 2012; Yagoop *et al*., 2013). Besides residual spraying, the utilize of insecticide-treated bed nets (ITNs) have also been introduced (WHO, 2005). In Sudan, malaria is one of the major public health problems, constituting about 40% of all infectious diseases. It is the primary reason for morbidity and mortality in the country. Symptomatic malaria constitutes for approximately 17.5% of out-patients visits and 11% of hospital admissions (Annual Health Statistical Report, 2009). The entire population of Sudan is at risk of malaria, although to different degrees. The incidence of malaria ranged from 0.7 to 20.9% between children aged 2 to 10 years, between 0.4 to 15.5% among children more than 10 years old and between 3.7 to 10.3% among pregnant women, (FMOH, 2006). Based on climate models, 75% of the population of Sudan is estimated at danger of endemic malaria whereas the other 25% of the population is at danger of epidemic malaria (FMOH, 2006). In Eastern Sudan, malaria remains the leading cause of morbidity with incidence rate of 14 cases per 10.000 population in Kassala State and 12.5 per 10.000 populations in Gedaref State (WHO, 2011). The intensity of the disease results from the high vectorial capacity of *An. gambiae sensu stricto, An arabiensis* and *An. funestus* in addition to social, economic, behaviour and

cultural reasons (Gillies and Coetzee, 1987; Gomes and Salazar, 1990; Agyepong, 1995; *Espino et al.*, 1997; Sachs and Malaney, 2002).

1.2 Malaria Vector

Malaria is a mosquito-borne disease. The disease is transmitted by the anopheline mosquitoes. Although, there are approximately 500 species of the genus *Anopheles*, only 70 are vectors of malaria under natural conditions, with approximately 40 being regarded as of real importance in malaria transmission (Service and Townson, 2002). The *An. gambiae* complex which is one of the most important group of vectors comprises seven species (White, 1974; Hunt *et al.*, 1998). All except for *An. quadriannulatus*, are responsible for the transmission of malaria parasites in humans. Two members of the complex, *An. gambiae* s. s. Giles and *An. arabiensis* Patton are considered as the major vectors transmitting malaria in Africa. Although they are mainly found in sympatry, the two species differ deeply in their behaviour and physiology. In addition *An. arabiensis* is zoophilic and exophilic (Gillies, 1955), and it has ability to tolerate to the upper temperatures and lesser humidities than *An. gambiae* s. s. (Kirby and Lindsay, 2004) whereas the latter is more anthropophilic and endophilic (Gillies, 1955). As a result *An. arabiensis* is widely distributed in arid areas (Coetzee *et al.*, 2000) and more likely to change its behaviour in response to interventions such as residual insecticides than *An. gambiae* s. s. (Geissbühler *et al*., 2007). *Anopheles arabiensis* is therefore likely to replace *An. gambiae* s. s. (Lindblade *et al.*, 2006) in some areas and to be present as the single vector of malaria in other places.

The behavioral and ecological characteristics stem from the process of evolution over a certain period of time and are never static. Human natural

environment continuously influenced vector species resulting in adaptation and giving rise to genetic variants. Within species, it has been found that subpopulations of identifiable genotypes demonstrated adaptation, for example, to particular climatic conditions (Appawu *et al*., 1994), to certain parts of the human environment (Coluzzi *et al.*, 1979) host preference (Petrarca *et al*., 1992), resting habits (Mnzava, 1995) and infection rates (Coosemans *et al.*, 1989). All these factors have significant epidemiological outcomes. Therefore, to determine the role played by a species in disease broadcast might necessitate to test at the intraspecific rank to get a much clearer grasp of the outbreak of the malaria. At this juncture, the dynamic of these alteration and the association of these genes throughout the *Anopheles* population are also important. In Sudan, cytogenetic experiments confirmed that *An. arabiensis* is the principal species found in the country (El Gaddal *et al.*, 1985; Petrarca *et al*., 2000; Mustafa, 2002). Ecological variation has been recommended to be one of the characters that can break off the movement of genes by making scenery or genetic barriers like fast chromosomal inversions that can eventually effect in reproductive isolation and speciation (Schneider, 2000). Therefore by understanding the association among populations and their surrounding, it can give the knowledge regarding the movement of genes from individual or population to another. This knowledge is essential not only in considerate species development, furthermore in planning, for their control. The COI gene had been utilised to investigate six populations of *An. arabiensis* extending from Khartoum to Dongla in Northern Sudan (Azrag, 2007). The study detected little or no evidence of genetic variation among the six populations along the River Nile.

The choice of markers for this study was based on several considerations. The study on the origins of mosquito and its association to human history have been investigated through a number of markers of varying mutational rates but changed adequately slowly were utilised to capture chronological gene flow. The utilization of mtDNA combined with the highly evolving internal transcribed spacer – ITS (rDNA) could address this problem in elucidating historical gene flow. This could satisfactorily complement data obtained on contemporary gene flow through frequency-based such as microsatellite markers (Sunnucks, 2000). Marshall *et al*., 2008 observed that complementary ITS-rDNA and mtDNA data had permitted novel insights into the origin and genetic structure of *An. gambiae* s.s. populations on the islands of Sau Tome´ and Princpe.

The ecology and population genetics of *Anopheles arabiensis* would be the major focus of this study. Using NADH dehydrogenase subunit 5 (ND5), nuclear internal transcribed spacer (ITS) and microsatellite molecular markers. Mosquitoes will be sampled from six geographical areas in Sudan. Ecological study of *An. arabiensis*, breeding habitat, distribution and seasonal abundance are important factors in understanding the vector's role in malaria transmission and therefore crucial in formulating and developing control programmes. In addition, ecological studies will be conducted in two of the sampling sites.

1.3 Objectives

1. To conduct an ecological study of *An. arabiensis* in two selected locations in Sudan and to produce continuous maps of their spatial distribution.

2. To estimate the parity rate and gonotrophic cycle in relation to the transmission of malaria in the two sites.

3. To conduct a population genetics and phylogenetics study of *An.arabiensis* inferred from mitochondrial ND5 and nuclear ITS genes in six populations within Sudan.

4. To conduct a population genetics and phylogenetics study of *An. arabiensis* in six populations within Sudan using microsatellite markers.

CHAPTER 2

LITERATURE REVIEW

2.1 General Classification and Morphological Characteristics of the Mosquitoes: family Culicidae

Mosquitoes have a worldwide distribution, absent only in the Antarctica. They feed on a variety of hosts from birds to mammals (Chaves *et al.*, 2010). They are insects belonging to the order Diptera and family Culicidae. This family is divided into three subfamilies: Toxorhynchitinae, Culicinae and Anophelinae. The subfamily Toxorhynchitinae comprises of only one genus, *Toxorhynchites*, which contains about 76 species and occurs mainly in the tropical and subtropical regions of the world (Service, 1996). They are large mosquitoes which do not suck blood and therefore are of no medical importance. However, the larvae are predacious and feed on other mosquito larvae, making it a good biological control agent. The subfamily (Culicinae) contains more than 2930 species of which the main genera of medical importance are *Aedes*, *Culex*, *Mansonia*, *Haematogogus*, *Sabethes* and *Psorophora* (Service, 1996). They are important as vectors of filariasis and arboviruses such as dengue and yellow fever. The subfamily Anophelinae contains 500 species of the (Hay *et al.*, 2010) belonging to three genera of which *Anopheles* is the only one of medical importance being the sole vector of malaria.

Anophelines can be differentiated from the culicines at all stages of their life cycle. Anopheline eggs are laid singly on the water surface and each possesses a pair of floats, while culicine eggs are either laid singly, in rafts or in masses at the water's edge, and without floats (Kettle, 1995). Larvae of anopheline do not possess siphons, lie parallel to the water surface and have abdominal palmate hairs and tergal plates

whereas culicine larvae have short or long siphons, generally rest at an angle to the water surface and lack palmate hairs and tergal plates (Service, 1996). The respiratory trumpets in the anopheline pupae resembles a cone, short and broad distally, with abdominal spines on segments 2-7 or 3-7 whereas in most culicines the trumpets are more cylindrical and without abdominal spines on segments 2-7 (Kettle 1995). Adult *Anopheles* usually rests at an angle to the surface, but adult culicines rest parallel to the surface (Service, 1996).

2.2 Distribution of Anopheline mosquitoes

Mosquitoes of the genus *Anopheles* are the only species that have ability to spread the human malaria worldwide. These include familiar vector species such as *An. gambiae*, *An. arabiensis* and *An. funestus* in sub -Saharan Africa; *An. dirus*, *An. maculatus*, *An. balabacensis*, *An. sinensis* and *An. minimus* in Asia; *An. punctulatus* and *An. farauti* in Southwest Pacific, and *An. darlingi* and *An. albimanus* in South America (Sinka *et al.*, 2010). The most important vector with Anopheline mosquitoes was *An. gambiae* complex the main malaria vector in Sub- Saharan Africa which it was our consideration in this study.

2.3 *Anopheles gambiae* complex

The *An. gambiae* complex consists of seven sibling species (Hunt *et al.*, 1998). Sibling species is defined as any two or more related species that are morphologically almost indistinguishable other than are incapable of producing fertile hybrid (Mayr, 1942). Taxonomic resolution of species complex can only be resolved through genetics, biochemical, behavioural, or ecological approaches. Their divergence is thought to have occurred very recently. This complex includes the majority of important vectors of malaria in Sub-Saharan Africa. *Anopheles gambiae*

s.s and *An. arabiensis* are two members of the complex, breeding in freshwater and the most important vectors of malaria in Africa. *Anopheles arabiensis* is zoophilic and exophilic (Gillies, 1955), and are able to tolerate higher temperatures and lower humidities than *An. gambiae s.s.* (Kirby and Lindsay, 2004) which is anthropophilic and endophilic (Gillies, 1955). Three other members of the complex; *An. merus, An. melas* are the saltwater – breeders and important vectors in the coastal regions while *An. bwambae* is limited to area near to the Buranga hot springs in Uganda (White, 1985). Two other members, *An. quadriannulatus* subspecies the zoophilic subspecies A, which is highly distributed in southern Africa, and subspecies B are of no medical importance, but anthropophilic behaviour of *An. quadriannulatus* subspecies B was lately reported in Ethiopia (Pates *et al.*, 2006). The species have different preferences in breeding location distribution, host, resting-site and vectorial position. Previous to 1962, *An. gambiae* was assumed to represent as sole, biologically changeable species. On the other hand, in an early literature (Gillies and De Meillon, 1968), variations in the larval breeding sites and adult female resting behaviour and feeding choices have been reported numerous times. This gave rise to scepticisms regarding the adoption of indoor residual spraying of insecticides as a routine control strategy against malaria. Number one is the irritancy property of DDT which resulted in adult vectors avoiding sprayed surfaces. This change in behaviour was responsible for the reduced effectivenesss of the malaria management plans in West Africa. Subsequently, great records of *An. gambiae* larvae might still be collected in regions where it had been successfully controlled, and outdoor – resting females were reported to have fed on cattle. At that time, this was understood to be a selection for behavioural resistance within a single species. The causes for these obvious alter in behaviour were not identified, and it was dread that the outdoor mosquitoes could

make a come back again to their earlier human – biting, indoor – resting ways (Hunt and Coetzee, 1995).

There is still considerable controversy on the taxonomic standing of the West African *An. gambiae* species. However, more evidence is pointing towards it being a distinct biological species (Favia *et al.*, 1997). Cytogenetic data has suggested that *An. arabiensis* is monomorphic for the 2La chromosomal arrangement while *An. gambiae s. s*. is a complex of five chromosomal forms, referred to as Bamako, Bissau, Forest, Mopti and Savanna (Toure *et al.*, 1998; della Torre *et al.*, 2002).

Species identification is usually done using morphological keys, but sometimes the morphological characters fail to distinguish members of a species complex especially when they are found symmpatrically or they overlap in the edges of their distribution. To solve this problem a variety of methods have been developed to differentiate closely related species e.g. cross mating, polytene chromosome banding patterns, species-specific allozymes and DNA markers.

2.4 *Anopheles arabiensis*

Anopheles arabiensis is a member of the *An. gambiae* complex transmitting malaria in Sub-Saharan Africa. It is a zoophilic, exophagic and exophilic species (White, 1972), recognized to have an extensive choice of feeding and resting patterns, and based on geographical position (White, 1974; Gillies and Coetzee, 1987; Sharp and Lesueur, 1991). This preference flexibility permits *An. arabiensis* to acclimatize rapidly to counteract indoor IRS control, where specific genotypes are present (Coluzzi *et al.*, 1979). Thus, they have become conditioned to avoid certain types of insecticide (White, 1974; Ameneshewa and Service, 1996).

2.4.1 Larval Habitat

Anopheles arabiensis larvae breeding site are comparable to those of *An. gambiae.* In general they include little, momentary, sunny, with clear fresh water and shallow ponds (Abdullah and Merdan *et al.*, 1995; Gimnig *et al.*, 2001; Edillo *et al.*, 2002; Himeidan *et al.*, 2008), even though it has the ability to use a higher diversity of sites than *An. gambiae*. This includes slow flowing, and streams that do not receive direct sunlight (Abdullah and Merdan *et al.*, 1995; Shililu *et al.*, 2003; 2007; Kamau *et al.*, 2007) and an assortment of big and small innate and artificial breeding sites. It has been established in turbid waters (Charlwood and Edoh, 1996; Ye-Ebiyo *et al.*, 2000) and, on occasion, in brackish habitats (Bøgh *et al.*, 2003).

On the other hand, the favoured breeding places of *An. arabiensis* are ditches, drains, burrow pits, ponds, irrigated fields and edges of streams, water collected in natural depressions as well as unconventional sites like salt water in the Red Sea coast (Anon,1980) and along the banks of the River Nile with aquatic vegetations (Dukeen, 1981). In particular, *An. gambiae* and *An. arabiensis* mostly breed in habitats that are formed through human activity such as hoof prints and irrigated rice fields which are well lit. Both species have high preference for human feeding but cattle may be an alternative choice when present. *Anopheles gambiae senso strict* is more intimately connected with humans. In general, they are indoor feeders and complete egg development inside the human dwellings (Coluzzi *et al*., 1979). *Anopheles gambiae* has been commonly found in pools near human dwellings, whereas the density of *An. arabiensis* is seen higher in pools near cattle shelters (Minakawa *et al*., 2002). In experimental box in rice irrigation schemes in Tanzania, *An. arabiensis* was found to prefer outdoor resting (Kweka *et al*., 2009). The larvae occupy habitat formed by rains such as sunlit pools, puddles, borrow pits, car tracks

and hoof prints and/or more stable sources such as rice fields and the edges of seasonal swamps (Gillies and Coetzee, 1987; Koenraadt *et al*., 2003). Recently *An. arabiensis* was found breeding in the highlands of Kenya, but there was no confirmation for the occurrence of *An. gambiae* larvae (Chen *et al*., 2006). Furthermor, the larvae of *An. arabiensis* were found to be more successful in competition with *An. gambiae* larvae when they breed in the same pools under similar conditions (Paaijmans *et al.,* 2009).

2.4.2 *Anopheles arabiensis* adult feeding and resting behaviour

Anopheles arabiensis feeding occur at night at varying frequencies. Biting times peak in the early hours of the evening (19:00) or early morning (03:00) (Githeko *et al.*, 1996; Lemasson *et al.*, 1997; Shililu *et al.*, 2004; Tirados *et al.*, 2006; Oyewole and Awolola, 2006). It is obviously clear that behavioural change occurs in *An. arabiensis* as reported by numerous studies on the anthropophilic or zoophilic preference of the mosquito. Bøgh *et al.* (2001) reported that there is a large disparity in the feeding behaviour of the mosquito depending on the availability of hosts. Generally, as stated by Tirados *et al.* (2006) and Oyewole *et al.* (2007), the biting patterns are inclined to be exophagic, although such preference is frequently stated in contrast with extremely endophagic species such as *An. gambiae.* For instance, Fontenille *et al.* (1997) stated that *An. arabiensis* is more exophagic than *An. gambiae* and *An. funestus*. This species nevertheless, demonstrates a tendency to be exophilic or partial exophilic in spite of where it has taken up a blood meal and the source of its blood meal (Fontenille *et al.*, 1990; Mosha *et al.*, 1992; Mnzava *et al.*, 1995; Fontenille *et al.*, 1997; Shililu *et al.*, 2004; Tirados *et al.*, 2006; Mahande *et al.*, 2007).

2.4.3 Nomenclature

The classification of *Anopheles arabiensis* is as follows (Gillies and De Mielon, 1968)

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Diptera

Suborder: Nematocera

Family: Culicidae

Subfamily: Culicinae

Tribe: Anophelini

Genus: *Anopheles*

Species: *arabiensis*

2.5 Cytogenetic Identification

Cytogenetic identification is enabled by the occurrence of polytene chromosomes also called giant chromosomes. They are present in the cells of specific tissues in dipterans for example in the salivary glands of larvae stage 4 and ovarian nurse cells. The latter is considered to produce better chromosomal preparations. Through the use of various stains such as Orcein stain, light and dark banding patterns are generated that are critical milestones in chromosomal karyotyping. Dark banding is considered to represent inactive chromatin areas while light banding is generally found at regions with higher transcriptional activity.

These cytogenetic studies have permitted the elucidation of taxonomic status and population differentiation among mosquito taxa based on specific banding

patterns as well as chromosomal structural variations. Early cytogenetic studies in *An. gambiae* have described population specific paracentric inversions (Coluzzi and Sabatini 1967, 1968, 1979). More recently Pombi *et al*. (2008) defined 82 infrequent and seven shared inversions detected in natural populations. Inversions are not randomly dispersed between chromosomes, but occur most regularly on chromosome 2 (2R). In *An. gambiae* complex studies on the investigations of correlations of specific physical or behavioural characters with inversions have been limited. In consensus with an expectation founded on climatic relations, inversion 2La was found to confer superior larval heat forbearance and adult resistance to aridity (Gray *et al.*, 2009; Rocca *et al.*, 2009). Furthermore, this inversion polymorphism is nonrandomly distributed geographically, micro spatially, and seasonally with respect to ecological and climatic variables, particularly dryness (Coluzzi *et al.*, 1979; Toure *et al.*, 1998; Costantini *et al.*, 2009; Lee *et al.*, 2009; Simard *et al.*, 2009). To date, 130 paracentric inversions in *Anopheles gambiae* group of complex have been identified (Coluzzi *et al.*, 1979). Of these, 10 inversions have been recognized as important in sibling species differentiation. On the other hand, fixed inversion disparity between species and populations are the exclusion in the *An. gambiae* complex. The majority of inversions remain polymorphic in natural populations. Although most species in the complex carry at least one polymorphic inversion, *An. gambiae* and *An. arabiensis* have the majority of the identified inversion polymorphisms, suggesting their enormous geographic and ecological sharing across Africa (Coluzzi *et al.*, 1979, 2002). However, while these cytogenetics studies have been very useful in the early taxonomic studies of species complex. Modern approaches such as molecular markers are now widely utilized for accurate classification.

2.6 Molecular markers in mosquito studies

Proper classification of the insect vector is one of the main factors in the study of arboviral diseases (Cook *et al.,* 2005). Additionally, accurate identification of species has medical importance, particularly in developing vector control strategies. In the past, the taxonomy of mosquito has been largely based on morphological features, cytogenetics and allozyme markers. In recent times, the DNA molecular approach has greatly improved the reliability of species identification (Kumar *et al.,* 2007). Genomic DNA has been used as a molecular tool for differentiating species which are morphologically indistinguishable. Generally, well preserved tissues (frozen, in alcohol or even dried samples) could be easily extracted for DNA material in various tissue types. These include those at the juvenile and adult stages in both male and female specimens (Marrelli *et al.,* 2006). These methods could be applied not only to sibling species but also to members of complex groups that are difficult to be distinguished morphologically (Garros *et al.,* 2005; Goswami *et al.,* 2005). Vectorial and behavioural variations found among these species groups or complexes constitute the main reason that requires perfect and accurate identification (Garros *et al.,* 2004).

Molecular markers such as mitochondrial Cytochrome oxidase C subunit I and II (COI & COII), Cytochrome oxidase B, ND5 gene, 16S rRNA gene, internal transcribed spacers (ITS1 & ITS2) of ribosomal DNA genes, third domain (D3) of 28S rDNA gene and microsatellite markers are useful in species classification, phylogenetic analyses and other related studies (Fritz *et al.*, 1994; Marrelli *et al.*, 1999; Hackett *et al.*, 2000). In recent times molecular markers have been used for a diversity of genomic-based classification, phylogenetic, population and evolutionary

investigations in animal species (Hillis, 1996; Wilkerson *et al.,* 2005; Khan *et al*., 2008).

2.6.1 Ribosomal DNA (rDNA) Gene

Ribosomal DNA of the nuclear genome is effective taxonomic markers in anophelines. The rDNA transcriptional unit is tandemly replicated and interspersed by non-transcribed spacers. Variation between species is higher than within species at the nucleotide sequences of these spacer areas. Thus, this genomic segment is very useful for taxonomic differentiation of species complex through its length or sequence polymorphisms (Fritz *et al.,* 1994; Marrelli *et al.,* 1999; Hackett *et al.,* 2000).

Ribosomal DNA genes (rDNA) are found in all organisms and have a basic shared function. They have been extensively utilised to investigate the phylogeny from species to kingdom level. They occur in multiple copies per genome. *Anopheles arabiensis* has approximately 700 sets of rRNA genes. These rRNA sequences and inter-genic spacer regions are efficient markers for phylogenetic reconstruction including in *An. arabiensis* populations. The rDNA transcriptional unit is tandemly replicated (>100 copies per genome) and interspersed by a non-transcribed intergenic spacer (IGS). Every transcribed unit has two internally transcribed spacer designated the ITS1, which partitions the ssrDNA (18S) from the 5.8S rDNA subunits while the ITS2, separates the 5.8S from the large subunit rDNA (28S). These units are transcribed to produce the rRNA component of the ribosomal structure (Figure 2.1).

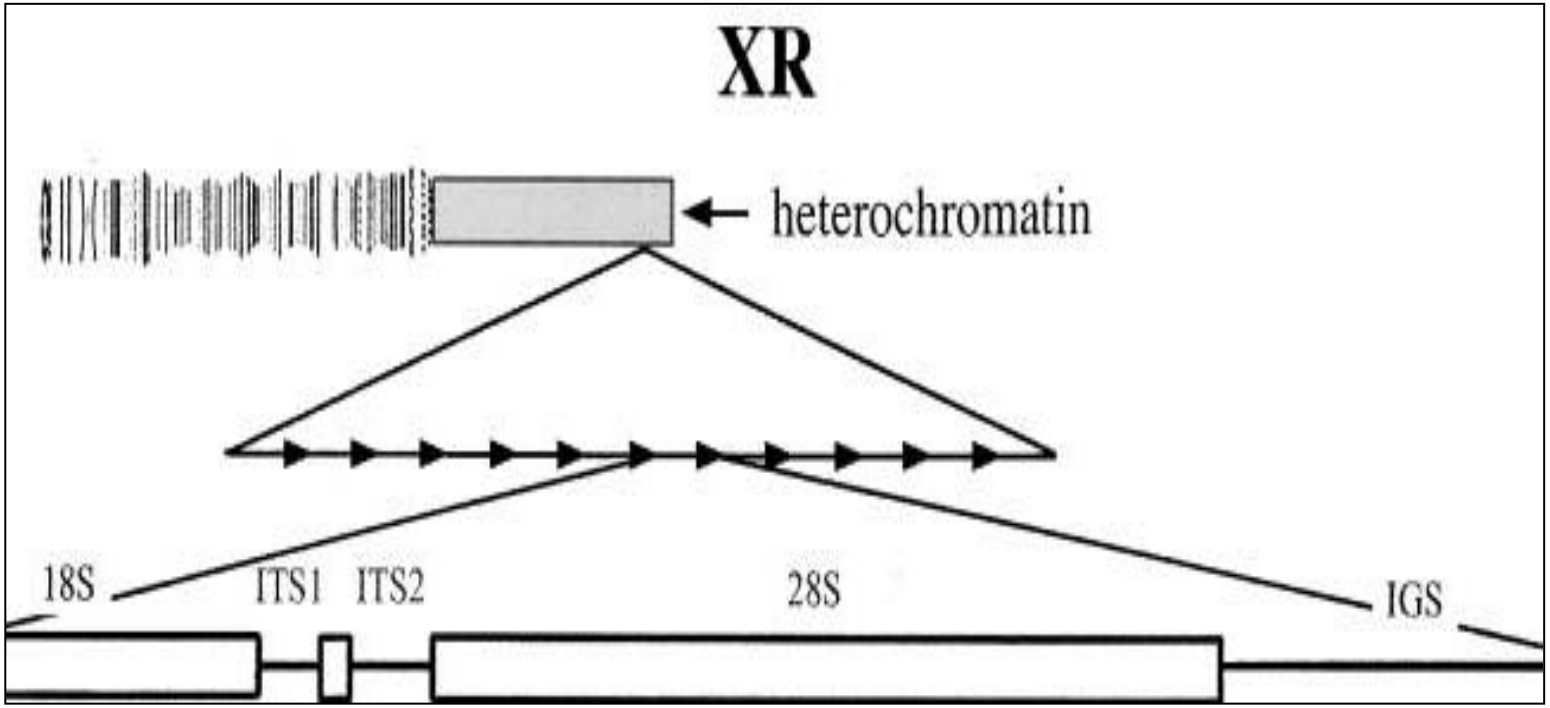


Figure 2.1: Location and organization of the rDNA in *An. gambiae*. The rDNA locus consists of one very long array of repeat units (500–700 repeats per genome) located in the heterochomatic region of the X chromosome. Each repeat is 9 kb long and consists of the genes for the 18S, 5.8S, and 28S rDNA (open rectangles). These genes are separated by spacers (solid lines), the internal transcribed spacers (ITS1 and ITS2), and the intergenic spacer (IGS) (modified from Gentile *et al.*, 2002).

2.6.1.1 Intergenic spacer gene IGS

Intergenic spacer (IGS) has been found to be more variable than the other ribosomal gene subunits in an organism. PCR primer flanking the variable regions are designed based on the combination of the variable spacer and conserved genes. Species and population genetics are easily studied based on the high variability of this gene family. The IGS contains species-specific sequence which permits species delineation in *Anopheles* complexes (Scott *et al.,* 1993; Paskewitzet *et al.,* 1993). Through RFLP analysis of the IGS region, Collins *et al.* (1987) successfully differentiated each member of the *An. gambiae* complex. The *An. gambiae* complex is an excellent model for highlighting the intricacies of speciation. Studies by Lehmann *et al.* (2003) and della Torre *et al.* (2005) have revealed complex patterns of genetic structuring within *An. gambiae* sensu stricto. While Coluzzi *et al.* (1979, 1985), Bryan *et al*. (1982) and Coluzzi (1982) suggested two chromosomal forms based on different patterns of paracentric inversions in chromosome 2, later studies established small concordance between chromosome forms and molecular markers (Besansky *et al.,* 1994; Mukabayire *et al.,* 1996, 2001; Gentile *et al*., 2001). Favia *et al.* (1997) and della Torre *et al.* (2001) observed two distinct molecular forms, termed M and S. These were defined based on fixed differences in the intergenic spacer gene region (IGS) of the ribosomal DNA (rDNA). Interestingly, heterozygotes for these genotypes are rarely detected even in sympatric populations (Tripet *et al.,* 2001; Wondji *et al*., 2002; Diabate *et al.,* 2003; della Torre *et al*., 2005; Barnes *et al*., 2005). The taxonomic status of these forms is still unresolved. Several studies (Weill *et al*., 2000; Gentile *et al*., 2001, 2002, 2004; Diabate *et al*., 2003; Barnes *et al*., 2005; della Torre *et al*., 2005) have shown the genetic integrity of each form based on other nuclear markers where near complete linkage disequilibrium,

significant genetic distance, or large frequency differences were observed. In contrast, other studies have shown little differentiation between the two molecular forms (Gentile *et al.,* 2001; Lehmann *et al.,* 2003) based on other loci.

2.6.1.2 Internal transcribed spacers ITS

The internal transcribed spacer (ITS) has high variability between species. It is an area of rapid mutation but surrounded by highly conserved regions, has unique sequence for each species and has therefore proven to be highly effective in species delineation (Krzywinski and Besansky, 2003). Intra species variation is very high for ITS1 and therefore it is very useful in resolving taxonomic issues at lower level such as population and strain differences (Beebe *et al.*, 2000; Fairley *et al.*, 2005). However, ITS2 variation is highly conserved and is therefore more useful in resolving among than within species variability. Therefore, ITS2 is a good marker for taxonomic investigation.

The ITS2 of the ribosomal DNA genes is useful for distinguishing among specimens belonging to a species complex. For example, the *An. maculipennis* complex (Porter & Collins 1991), *An. quadrimaculatus* complex (Cornel *et al.*, 1996) and *An. culicifacies* complex (Goswami *et al.*, 2005) have been resolved using this marker. The ITS2 provides an effective molecular marker for sibling species resolution (Marrelli *et al.*, 2006; Kronefeld *et al.*, 2012).

In anophelines, the ITS2 fragment has high variability and seems to be longer than in other mosquito genera (Severini *et al.*, 1996; Miller *et al.*, 1996). As in other mosquito groups, this marker is useful for taxonomic resolution in the genus *Anopheles.* Sequence slippages of simple sequence motifs in this gene results in insertion and deletion. The decreased selection pressure in this gene leads to high

occurrence of replication slippage of simple sequence, attributing to the high evolution of the rDNA units (Wesson *et al.*, 1992; Tang *et al.*, 1996). However, in other complexes, composed of recently-diverged members, such as the *An. gambiae*, the ITS2 interspecies differences are minimal, from 0.4 to 1.6% (Paskewitz *et al.*, 1993). Thus, the use of this marker alone for taxonomic purposes may not be effective.

2.6.2 Mitochondrial DNA

The mitochondrial genome in animals is a single, compact, double stranded closed circular DNA molecule (16 kb long). Selection pressure and evolution in mitochondrial DNA often occurs independent of the nuclear DNA (Moritze *et al.*, 1987). Mitochondrial DNA is a highly conserved genome in *Anopheles gambiae s. l.* Like in most eukaryotes, it is tightly packed with 37 genes coding for proteins (ATPase 6 and 8, cytochrome oxidase subunits COI, COII, cytochrome B and NADH dehydrogenase, twenty two genes coding for 22 transfer RNAs or tRNAs, two genes coding for rRNAs genes-12S and 16S). In addition, there is also a non coding region known as the control region or D-loop (A+T rich region) that is roughly 0.9 kb long and appears to control mtDNA replication and RNA transcription (Beard *et al.,* 1993). The mtDNA genome in *An. gambiae* complex has a high adenine and thymine content of approximately 77.6 %, According to Beard *et al.,* (1993), the composition of nucleotides for the sense strand (excluding the A+T D-loop rich region and intergenic spacer) is 43% T, 34% A, 12.4% G and 10.7%C.

Mitochondrial DNA has numerous characteristic advantages for studying population genetics, molecular evolution and phylogenetic relationships of insects including *Anopheles.* These include a comparatively uncomplicated genetic structure,

mutation rate that is up to ten folds higher than nuclear DNA replacement (Avise *et al.*, 1987). Thus, mtDNA sequences have been considered to be useful markers for studying population structuring.

Several studies have provided support for the theory that *Anopheles gambiae* s.s had undergone a series of population expansion (Donnelly *et al.*, 2001; Lehmann *et al.*, 2003). It was postulated that the distributional range of this anthropophilic species paralleled human population colonisation in Sub-Saharan Africa throughout the agricultural uprising in past years (Coluzzi, 1982), although this region has also been subjected to great intermittent famine (Reader, 1997).

2.6.3 Microsatellite markers or simple sequence repeats (SSRs)

Microsatellite markers also known as short tandem repeats (STRs) (Edward *et al.*, 1991) are short stretches of DNA that occur throughout the genome of many eukaryotic organisms, including insects (Weber and May, 1989). Typically tandemly arranged with a motif of 1 to 6bp repeated up to 100 times nucleotides, they can contain mono, di, tri or tetra tandem repeats (Beckman and Weber, 1992).

Microsatellite are widely used as genetic markers because they are codominant, widely distributed in the genome, highly polymorphic, and easy to score (Muturi *et al.*, 2010). Microsatellite loci have been used to demonstrate the gene flow and the population structure within and between members of *An. gambiae* complex (Kamau *et al.*, 1999; Kamau *et al.*, 2007; Moreno *et al.*, 2007). Most of thes studies have been conducted on *An. gambiae* and a limited number on *An. arabiensis*. Population genetic structure data allows the management and control of mosquitoes with regards to health related issues. These include the development and dispersal of insecticide resistance and outbreak of *Anopheles* transmitted disease. Furthermore,

innovative approaches such as transgenic strategies could be designed when such information is available (Tripet *et al.*, 2001; Bennett *et al.*, 2002; Fanello *et al.*, 2003)**.** Although, molecular markers applied in population genetics are assumed to be neutral, in several instances they are closely linked to loci that are under selection. In such cases, they can be effectively utilised for elucidating selection mechanisms. In this study, the molecular markers are used for studying the population genetic structure and gene flow of *An. arabiensis* populations in Sudan.

CHAPTER 3

BIONOMICS OF *Anopheles arabiensis* IN TWO STATES IN SUDAN

3.1 Introduction

In Sudan malaria remain as one of the most important public health problems plaguing its population. The disease accounts for about 40% incidence of all infectious diseases (Ministry of Health, 1994). It is the most important cause of mortality and morbidity especially among children and pregnant women. Between 1970 and 1975, more than a million malaria cases were recorded in the country. It was then considered as a major health problem and the most widespread of all diseases throughout the country (Malaria Administration, 1983). In 1987, the number of malaria cases in hospitals was over a million with a mortality rate of 0.9%. In 1988, heavy rains and floods caused an epidemic during which more than 10 million cases were reported in the country (Malaria Administration, 1990).

Four *Plasmodium* species have been reported in the country but the predominant species is *Plasmodium falciparum* which accounts for about 90 % of the reported malaria cases (Omer, 1968; Taha and Broadhead, 1986), followed by *P. vivax* and *P. malariae*. Previous unpublished reports, recorded *P. ovale* in rare cases in Sennar and Western Sudan. In addition *P. vivax* and *P. ovale* have been reported in Khartoum (Elsayed, 1998).

3.1.1 Malaria Vectors in Sudan

In Sudan, 31 *Anopheles* species have been recognized but only a few of them are malaria vectors (Nugud *et al.*, 1997). Figure 3.1 shows the distribution pattern of malaria vectors in Sudan. *Anopheles gambiae* and *An. funestus*, are

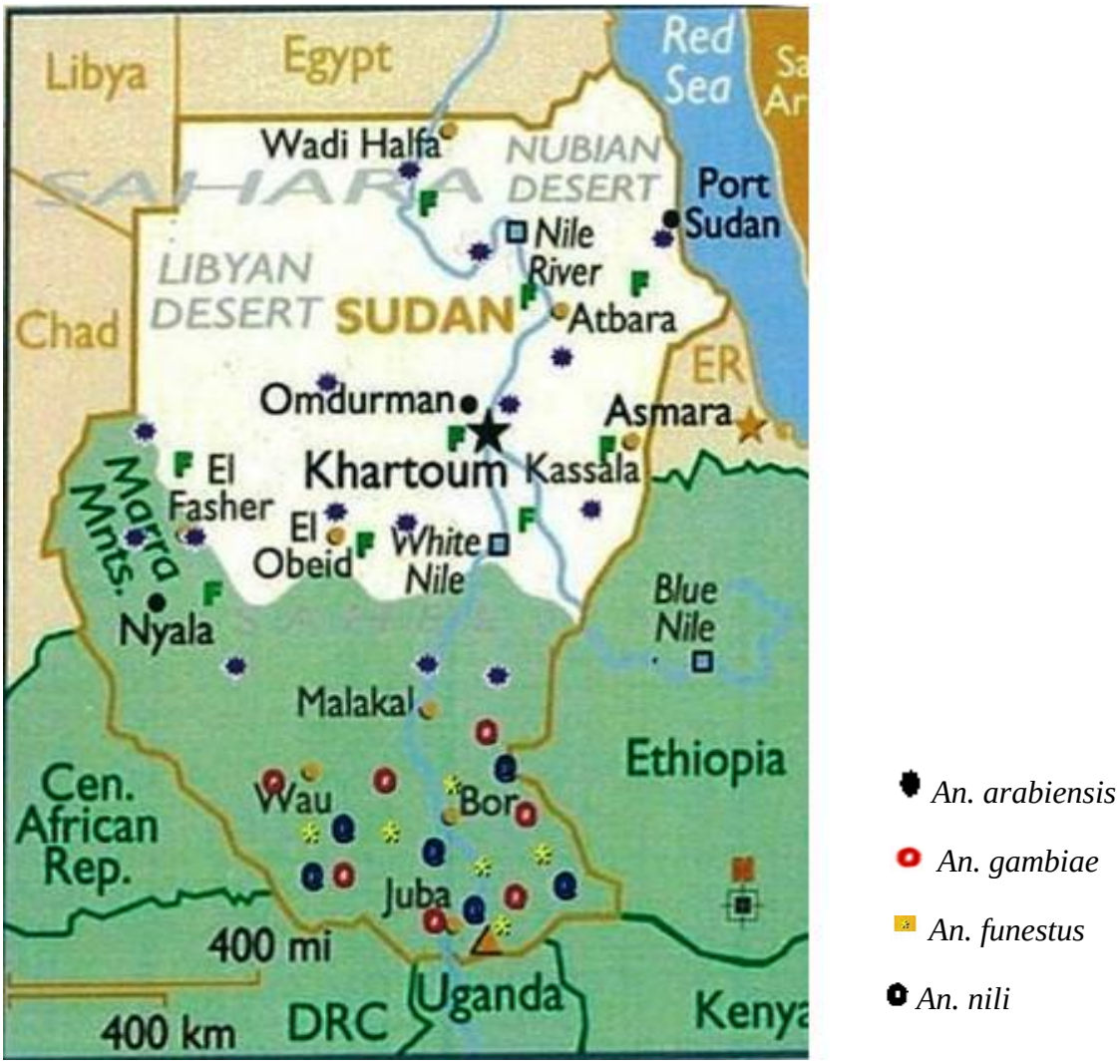


Figure 3.1: Distribution of malaria vectors in Sudan.
(Source: National Strategic Plan for RBM 2007-2012
(Federal Ministry of Health, Sudan, 2006).

essential vectors of malaria in the Southern parts of Sudan and their vectorial capacity maybe similar to that of *An. arabiensis* (El Sayed, 1998). *Anopheles pharoensis* is a known malaria vector in Egypt. It is widespread but seems to have little or no role in malaria transmission in Sudan due to its exophilic and zoophilic behaviour (Akood, 1980; Nasir 2010). While *An. arabiensis* is considered to be the main malaria vector in Sudan (Mustafa, 2002; Hamza, 2003; Himeidan *et al.*, 2004; Abdalla *et al.*, 2008; Tasneem *et al*., 2010; *Seidahmed et al.*, 2012; Yagoop *et al*., 2013). Other anopheline mosquitoes present in Sudan such as *An. nili* and *An. rufipes* are of no medical importance due to their predominantly zoophilic tendencies and their extremely low densities even during the rainy season (Nugud *et al.*, 1997). In the poor Savanna area of central Sudan, it has been established that *Anopheles* mosquitoes disappeared during the dry months of the year and reappeared during or soon after the first rainfall (Omer and Cloudsley- Thompson, 1970). Experimentally *An. arabiensis* shows a remarkable tolerance for water shortage and low humidity (Colluzzi, 1965; White, 1972). For the duration of the dry season, the vector was found breeding in habitats along the western bank of the White Nile between Khartoum and Jabel Aulia (Omer, 1968; Omer and Cloudsley - Thompson 1970). During the periods of aestivation often lasting several months, *An. arabiensis* may take several blood meals, and during this period the gonotrophic maturation is arrested or proceeds very slowly. Elsayed (1998) revealed that the density of this vector reaches its maximum value during the rainy season especially in irrigated areas. However, mesoendemicity of malaria has been observed in much of this region and in many areas of Khartoum state which are not adjacent to the Nile (Anon, 1980). Several of these areas are surrounded by small agricultural schemes that provide favorable breeding sites for *Anopheles* species.

3.1.2 Malaria control

Many efforts have been carried out to control malaria**.** There has been a renewed emphasis on preventive measures, both at the community and individual levels. Early detection of the disease and appropriate treatment can save lifes. Necessary precautions are known to prevent the incidence of the disease and to avert or control epidemics and other critical cases. Ecological study of the insect vector and the behaviour of the people in endemic regions are necessary to formulate an effective control against the disease (WHO, 1999). All the technologies required to control, malaria are already in existence. However, it only needs to be adapted to the situation regional and be applied through the local and national malaria control programmes (WHO,1999).

The current stratagies of malaria control include methods that deal with :-

1. Parasite control by the use of chemotherapy.
2. Vctor control by the application of chemicals, biological and mechanical methods.
3. Reducing the chance of exposure to the infected mosquito by appling simple control measures such as mosquito nets, oils and repellants (Bradley *et al.*, 1991; Alonso *et al.*, 1991), in addition to screening of houses and constructing them away from breeding places. However the Global Malaria Control Strategy which was adopted in 1992 calls for the creation of new epidemiological tools to support and reinvent the current tools that are used in the management of malaria (WHO, 1993).

In Sudan, the first organized efforts to control mosquitoes were concentrated only in the Gezira Irrigated Area (GIA) due to its economic importance. These efforts that started in 1925, were simply directed towards water management of the irrigation system (WHO, 1993). In 1935, the use of Paris green larvicide was substituted by oil and with the discovery of residual insecticide, DDT with oil was

sprayed in 1948 (WHO, 1956). On the other hand, in 1952, gammaxane was used instead of DDT and continued until 1965 when vector resistance was detected (El Gaddal, 1968). DDT also became ineffective in 1970 due to vector resistance (Haridi, 1972). Great efforts have been carried out by the WHO and many other organizations to control the malaria transmission all over the world, especially in Africa (Alilio *et al.*, 2004). Research in the development of a vaccine is high on the priority to control malaria in the African continent which bears 90% of the disease burden (Breman *et al.*, 2004). The current available control methods include chemical control, the use of Curtis and Townson, 1998 insecticide treated bednets beside biological control, environmental management and mosquito targeted genetic control (Collins and Besansky, 1994; Curtis *et al.*, 1996; WHO, 1997;; Collins *et al.*, 2000; Curtis *et al.*, 2003; Benedict and Robinson, 2003). Unless new tools and strategies are developed to augment those currently used for malaria control, malaria will persist to be a considerable barrier to the social and economic progress of endemic countries and will be a risk to the millions of people who travel to those countries (Snow *et al.*, 2005).

3.1.3 Morphological identification

Morphological identification is still extensively used to study the systematics and taxonomy of mosquitoes and the external classification of the genus *Anopheles* is based primarily on morphological characters (Harbach, 1994, 2004). In addition, external feature differences between populations have sometimes provided clues or evidence for the existence of sibling or cryptic species. This is true of the egg morphology in *An. maculipennis* (Fantini, 1994) and of melanism in *An. melas*. It has been mentioned that the morphological studies of cryptic species have not been thorough sufficient. As such, more detailed clarification on the different

developmental stages of their life cycle may provide useful identification characteristics (White, 1974). Schmidt *et al.* (2003) have recently been able to develop taxonomic keys for members of the *An. punctulatus* complex in the Solomon Islands that are based on conventional morphology characters of the adults and larvae. In the field, morphological characteristics are still important in identifying unrelated species and in sorting out the field samples for other identification techniques.

3.1.4 Effect of climatic factors on the population dynamics of mosquitoes

Environmental changes affect the stability of the ecological system within which the vectors and and their *plasmodium* breeding, expand, as well as the disease transmission (Patz *et al.*, 2000). Little increases in temperature have an effect on *Anopheles* and the parasites they transmit (Bruce-Chwatt, 1987). Malaria transmission is also temperature dependent because of its effect on the development of the mosquito eggs and larvae as well as the development rate of the parasites. Temperature also influences the life span of the adult mosquito (Depinay *et al*., 2004). Mosquito eggs, larval stages and parasites tend to develop more rapidly at higher temperature, but the immature stages of the mosquito will die beyond a temperature of 40°C (Jepson *et al*., 1947). At the same time, the adult longevity of anopheline mosquito decreased with increased in temperatures (Martens, 1998). Therefore, in areas where the mean temperature is either adequately low or adequately high, *Plasmodium* maturity period within the mosquito surpasses the average lifetime of the vector, obviously preventing the occurrence of endemic malaria in these regions (Craig *et al.*, 1999). The borders of endemic regions are defined by temperature limitations which are further confined by the availability of water for the formation of breeding habitats and microclimates (Thomson *et al.*,

2004). Zones outside of these malaria prone areas are free of sustained endemic transmission, but may suffer periodic outbreaks when the weather changes to become conducive for malaria transmission (Kiszewski and Teklehaimenot, 2004). Shifts in weather conditions may include variations in temperature which influence developmental rates, or changes in rainfall pattern creating more mosquito breeding habitats.

The period of gonotrophic cycle is the time from when mosquitos take up a blood meal, through digestion of the blood and oviposition or egg laying (Santos *et al.*, 2002). A decrease in the duration of the gonotrphic cycle would result in an increase in the occurence of human biting from an average of once every 5 days to once every 3 days (Afrane *et al.*, 2006). This would mean that the vector anophelines would feed more regularly on humans, pick up the parasites and become infective before transmitting the disease to a new host. A change in microclimatic would also caused the parasites within the mosquitoes to undergo a reduced sporogonic cycle beginning from average of 14 days to 12.6 days (Afrane *et al.*, 2008), thus reducing the infection time from mosquitoes to humans.

This study was carried to determine the distribution and abundance of mosquitoes in two sites in Sudan in association with the climatic factors, and to estimate the parity rate and gonotrophic cycle in relation to the transmission of malaria in the designated sites.

3.2 Material and Methods

3.2.1 Geography and Climate

The Republic of Sudan is located in North Africa and the Middle East. It is surrounded in the north by Egypt, northeast by the Red Sea with a shoreline of about

853km. To the east, the Republic of Sudan is bordered by Eritrea and Ethiopia, and in the south by South Sudan (Figure 3.1). To the southwest of the republic is located the Central African Republic, to the west Chad and to the northwest Libya. With an area of 1,886,068 square kilometres (728,215 sq mi), the Republic of Sudan is the biggest country in the African continent. It is dominated by the River Nile and its tributaries. The topography consists mainly of flat plains with several mountain ranges; the Jebel Marra is the highest range in the west; in the south, the highest mountain is Mount Kinyeti Imatong, close to the border with Uganda and the Red Sea Hills are located in the east. The River Nile is formed by the Blue and White Niles, which meet in Khartoum and runs northwards all the way through Egypt into the Mediterranean Sea. The Blue Nile meanders through Sudan for almost 500 miles and is connected to the rivers Dinder and Rahad between Sennar and Khartoum. The White Nile has no important tributaries within Sudan. In the south the rainfall is high with the presence of swamps and rainforests whereas in the north is located the very dry Nubian Desert. The rainy season in the north Sudan lasts for about three months (July to September), while in the south up to six months (June to November). In the dry regions, sand storms, called haboob, are common occurence.

3.2.2 Study sites

Two study areas were selected for the current bionomics study (Figure 3.2).

(a) Algerif West Village

This study site is a farming area which lies on the Western bank of the Blue River Nile in Khartoum state, located between latitude15º 35`394N and longitudes 32º 35 160 E. Algerif West mainly grows citrus and breeds farm animals namely

cattle and chickens. The land is flat with fertile clay soil and irrigated by the Blue Nile.

(b) Abu Algoni Village

This study site is a village in Sennar State located between longitudes 33 38 E and latitude 13 31 N. The soil is mainly alluvial, and naturally very fertile. The main economic activity is agriculture, especially the cultivation of sorghum, cotton and sugar cane. A number of fruit farms (including bananas and mangos) are located on the banks of the Blue Nile.

3.2.3 Entomological Survey:

3.2.3.1 Mosquito Sampling Techniques

Anopheline populations in Algerif West and Abu Algoni villages were sampled over a period of 12 months beginning in June 2010 to May 2011. Larval surveys and spray captures of resting adults inside houses were conducted once a month throughout the period following the standard WHO (1975) procedures. Three mosquito collectors assisted in the field work throughout the study.

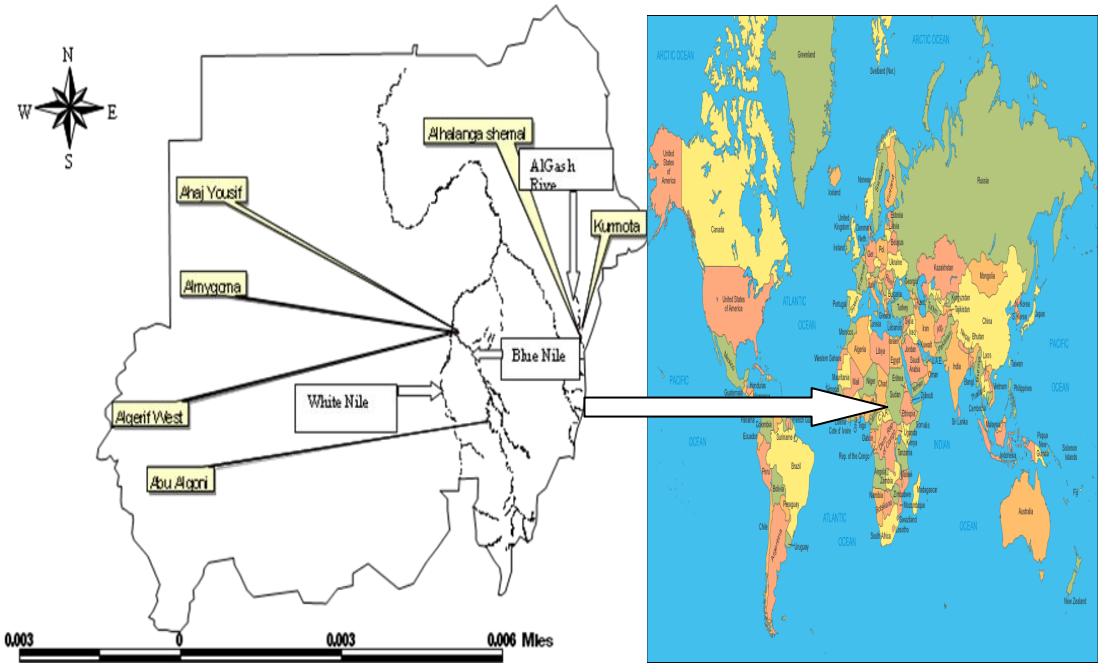


Figure 3.2: Map showing the sampling sites in the Republic of Sudan

3.2.3.2 Larval Collection: Dipping Collection Method

Aquatic stages of *An. arabiensis* were regularly collected from a fixed productive site near the main waterworks of the village in the form of water pools created by the running water, water drained to the vegetations through canal and animal hooves. Standard dipping collection using a ladle measuring 8 cm in diameter and 3 cm deep with a metal handle of 50 cm length was conducted. Ten random dips were taken from the fixed breeding sites. The ladle was lowered gently at an angle of about 45º until one side was just below the water surface. By means of a pipette, mosquito larvae and pupae were transferred into large plastic containers; natural predators (e.g. dragonflies, water beetles, tadpoles, etc.), were removed to avoid predation of the mosquitoes. The samples were then transferred to the laboratory for identification.

3.2.3.3 Adult Collection

Two methods were used for the collection of adult *Anopheles* mosquitoes resting inside houses, the indoor pyrethrum spray catch and indoor resting catch using an aspirator.

(a) Indoor Pyrethrum Spray Catch

Indoor pyrethrum spray catch or knock down collection (WHO, 1975) was carried out early in the morning, usually between 0600 -1000 hr. For this collection 10 houses were selected randomly as fixed capture stations, taking into consideration that each house is occupied by a number of people and is near to one or more mosquito breeding sites. People were first requested to leave the house and then the whole floor surface as well as beds and any other areas were completely covered from wall to wall with white cloth sheets. All windows, door, eves and other

openings through which mosquitoes could otherwise escape were firmly closed. The house was filled with the mist of 0.2% solution of pyrethroid diluted in kerosene, discharged using a hand atomizer (spray pump) of minimum capacity. After spraying, the house was kept closed for 10 minutes and then opened to collect mosquitoes found on the sheets; dead mosquitoes were carefully collected and placed gently into a plastic cup lined with moist cotton wool and covered with a damp filter paper. Samples were carefully transported to the laboratory for examination and identification.

(b) Indoor Resting Catch

Indoor resting adult females of *An. arabiensis* from unsprayed houses were collected using an aspirator. The aspirator consists of a glass tube 24 cm in length and 1 cm internal diameter. A fine mosquito net was fixed on one end of the tube. This end of the tube was inserted into a piece of rubber tube measuring 60cm long. The mosquitoes collected were emptied into a plastic cup covered with mosquito net with a central hole plugged with a wet piece of cotton wool. Samples were transported to the insectary, well protected to minimize any damage for examination and identification.

3.2.3.4 Laboratory Techniques

In the laboratory, records were made of adults and mosquito larvae collected. All adults and anopheline larvae collected were identified and their numbers counted. The abdominal appearance was classified according to Sella's stages, the ovaries were dissected following the procedure described by WHO (1975) to determine the ovarian developmental stages (Christopher's stage) and parity rate.

3.2.4 Identification of Mosquitoes

All adults and larval anopheline mosquitoes collected by the different techniques were identified to species level using the morphological key prepared by Gillies and De-Meillon (1968).

3.2.4.1 Age –Grading

Females of *An. arabiensis* collected by the pyrethrum spray method were age-graded by three methods, Sella's stage (Sella, 1920), Christopher's stage (Christopher, 1911) and parity rate.

(a) Sella's Stages

All females *An. arabiensis* collected resting indoors were classified as one of four Sella's stages:

Unfed**:** Newly emerged females or older ones that have not fed again after laying eggs. The abdomen appears collapsed and the stomachs were empty.

Blood fed**:** Abdomen largely filled with fresh blood or dark red clot; ovaries occupy one third or less of the abdomen.

Half- gravid: Blood dark red; ovaries occupying two and a half to three segments ventrally and six dorsally.

Gravid**:** Traces of a little blood or no clot remaining; ovaries occupying two thirds or more of the abdomen.

The ratio of fed/gravid *An. arabiensis* was determined at the two study location and was used to estimate the length of the gonotrophic cycle. Half-gravid and gravid females were grouped together. The duration of the gonotrophic cycle was determined based on the assumption that mortality rate is constant throughout

the ovarian cycle. For this, the ratio of fed/gravid is about 1:1 for a 2-day gonotrophic cycle and 1:2 for a 3-day cycle, the reason that for an additional is because of the half-gravid individuals as stated by Kulkarni *et al.* (2006).

(b) Christopher's Stages

The ovaries of female *An. arabiensis* collected resting indoor were dissected for the identification of the ovarian developmental stages and were classified as one of the following five stages:

Stage I**:** This stage was identified by rounded egg follicles and absence of yolk granules; nucleus of oocyte was indistinguishable from that of the nurse cell.

Stage II**:** The egg follicle is oval, yolk granules present and occupying up to half the follicle, but not obscuring the nucleus of the ovum. This stage has been further subdivided into three stages:

Early stage II**:** The yolk only visible under the high power magnification (X40).

Mid stage II**:** The yolk easily visible under low power magnification (X10).

Late stage II**:** The yolk easily visible under a low dissecting microscope (total magnification not more than X10).

Stage III**:** Egg follicle oval, yolk occupying more than half the follicle and the nucleus of the ovum is obscured.

Stage IV**:** Egg follicle elongated, sausage shaped, yolk completely filling the follicle.

Stage V**:** Ovaries containing ripe ova; floats fully formed.

(c) Parity Rate Determination

Unfed and freshly fed females collected by indoor resting spray catch with developing ovaries not beyond Christopher's stage II were dissected and examined monthly for parity rate and estimation of mosquito longevity. The method followed was that of Detinova (1962). The technique is a simple age- grading method based upon studying the presence or absence of coiled tracheolar skeins in fresh ovaries. Nulliparous young mosquitoes in Christopher's stage I and II were identified by the presence of the coiled tracheolar skeins in the ovaries which are absent in parous females. The ovaries were extracted in physiological saline (0.65%), detached, covered with cover slip and examined immediately under a compound microscope at a magnification of X40. The parity rate was then determined as a proportion of parous among all dissected females.

3.2.5 Rearing of *Anopheles arabiensis* Under Laboratory Conditions

An experiment was carried out in July 2010 to May 2011 at the insectary of the Malaria Research Unit in Sennar to identify the larvae and pupae collected during this study. Individual batches of eggs were obtained by isolating fed females in cages measuring 30 cm X 30 cm X 30 cm and provided with a wet dish for oviposition. The wet dish comprised of a thick layer of damp cotton wool in a Petri dish and covered with a piece of filter paper. After oviposition the eggs were transferred to the rearing bowls on the same day of oviposition. The larvae were provided food in the form of dry bread powder 24 hours after hatching. The rearing bowls were filled with tap water to a known depth. Records were made of the temperature, relative humidity and the duration of each aquatic stage.

3.2.6 Estimation of the Duration of Sporogonic Cycle

The duration of the sporogonic cycle correspond to the period required for the development of the infectious sporozoites within the anopheline mosquitoes. Once the sporozoites travel and enter the salivary glands, the vector *Anopheles* becomes fully infectious and hence transmission can occur. For *P. falciparum* sporozoites to develop, require 111 degree/days of >16 °C, resulting in the following formula by Detinova (1962):

$n = 111/ (T – 16)$

Where 111ºC are the sum of heat in degree days necessary for the sporogonic cycle of *P. falciparum*, n = the duration of time in days for sporozoite development, and T is the average temperature prevalent in the study area during the current study, estimated as an average of the daily mean month's temperatures. The asymptotic rise at 16 °C obviously points out that malaria incubation at this temperature and lower is not possible, and that by 16 °C, the incubation period far surpasses the normal life span of a mosquito.

3.2.7 Molecular Identification

Molecular identification was carried out using the rDNA-PCR technique to confirm the taxonomy of the species among the *Anopheles groups of* complex as morphological characteristics may not be sufficient for precise identification (Scott *et al.*, 1993). Molecular classification of *An. gambiae* species complex in this study was conducted based on the ribosomal DNA intergenic spacers (IGS) (Scott *et al.* 1993).

3.2.8 DNA Extraction

DNA was isolated from individual *Anopheles* using the DNeasy blood and Tissue E kit, (QIAGEN, Valencia, CA). Each mosquito was homogenized with the aid of a microtube pestle in a 1.5 ul microcentrifuge tube. Avolume of 180ul Buffer ATL and 20 ul proteinase K were added and thoroughly mixed by vortexing. This was followed by incubation at 65 ºC with occasional vortexing to disperse the sample until the mosquitoes were completely lysed with a further vortexing for 15 seconds. A volume of 200 ul Al buffer was added to the speceimen and thoroughly mixed by vortexing. After that 200 ul ethyl alchohol 96-100% was added and vortexed. The mixture was pipetted into a Dneasy mini spin column placed in a 2ml collection tube and centrifuged at 8000 rpm for 1 min. The flow-through and the collection tube were discarded. The Dneasy mini spin column was then placed into a new 2 ml collection tube. A volume of 500 ul AW1 buffer was added and centrifuged at 8000 rpm for 1 min. The flow-through and collection tube was again discarded and the Dneasy mini spin column placed into a new 2ml collection tube. A volume of 500 ul AW2 buffer was added and centrifuged at 12000 rpm for 5 min to dry the Dneasy membrane. The flow-through and collection tube were discarded. The Dneasy mini spin column was placed into a new 1.5 ml microcentrifuge tube. A volume 200 ul AE buffer was added and centrifuged at 8000rpm for 1 min to elute the DNA. The DNA extracted from a single mosquito was amplified in a MasterCycler® Gradient (Eppendorf Scientific), 25 ul PCR reaction containing 11.8ul sterile H_2O, 2.5 µl *Taq* DNA polymerase, 10X PCR Buffer with MgCl2, 2.5 µl dNTP (2 mM mix G,A,T,C), 1.0 µl MgCl2 (25 mM), 1.0 µl Universal (Forward 25 pmol/µl) [GTGTGCCCCTTCCTCGATGT], [AAGTGTCCTTCTCCATCCTA], 1.0 µl *An. arabiensis* (Reverse 25 pmol/µl) [CTGGTTTGGTCGGCACGTTT], 1.0 µl *An.*

gambiae (Reverse 25 pmol/µl) [TGACCAACCCACTCCCTTGA], 1.0 ul *An. melas* (Reverse, 25 pmol/ul) [CAGACCAAGATGGTTAGTAT], 2.0 µl *An. quadriannulatus* (R, 25 pmol/µl) 0.2 µl *Taq* DNA polymerase (5 U/µl), and 1 µl template DNA. The PCR profile was at first step of 10 min at 94ºC to make the DNA polymerase active followed by 30 cycles, for each cycle 30 sec denaturation at 94ºC, 30 sec annealing at 50ºC and 30 sec extension at 72ºC. The final extension was at 72ºC for 7 min. The PCR products were mixed with 2 ul of loading dye and electrophoresed through a 2% agarose gel followed by staining with ethidium bromide for 15 minutes and washed with (ddH2O). Then the gel was picture among UV light and the DNA band photograph was taken by a digital camera connected to a UV transilluminator (SynGene's InGenius L Gel Documentation and Analysis System).

3.2.9 Statistical Analysis

The data collected during the study was analyzed using the computer program SPSS (Statistical Package for Social Science) for windows version 18. T-Test was done to compare the difference between the two means of females and larvae collected from the two collection sites. Correlation test was used to find out the relation between the larvae, adults and the climatic factors. The test was considered statistically significant when the P.value was less than 0.05.

3.3 Results

3.3.1 DNA Identifications

All samples identified by rDNA as in Plate 3.1 show the characteristic 315 pb banding pattern indicating the amplification of *An. arabiensis* in the collection sites. This means that *An. arabiensis* is the main malaria vector in

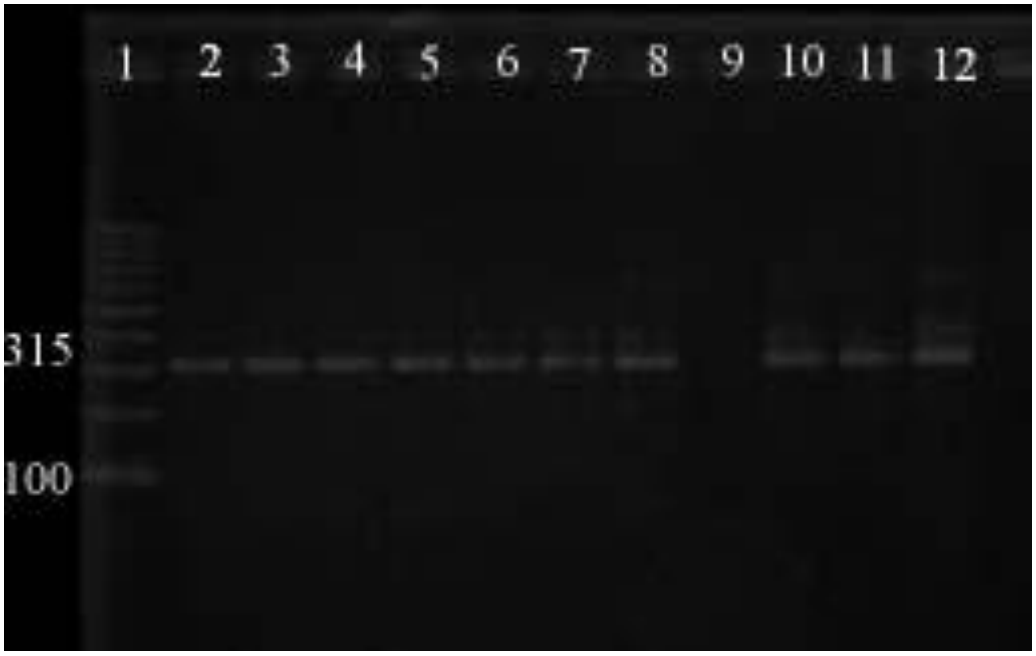


Plate 3.1: Ethidium bromide stained 2% agarose gel electrophoregram of PCR products obtained from the amplification of *Anopheles arabiensis* DNA for species identification. of *Anopheles gambiae* complex. Lane 1 100bp markers Lane 9 negative control; 2, 8 and 10-12 wild- caught *An. arabiensis* 315bp.

Sudan in the six study sites. This also explained that the only species of the *An. gambiae* complex found was *An. arabiensis*.

3.3.2 Anopheline Mosquito Fauna of the Study Area

The mosquitoes collected were *Anopheles* and *Culex* species but the only species taken into consideration was the *Anopheles* species. A total of 3041 larvae were collected using the dipping method and a total of 1897 adult females and males anopheline mosquitoes were collected by the indoor pyrethrum spray and indoor resting catches. The *Anopheles* species found in Algerif West was only *An. arabiensis*, but in Abu Algoni *An. arabiensis* and *An. rufipes* (12) were collected by both methods during the period from June 2010 – May 2011.

3.3.3 Breeding of *Anopheles arabiensis* in the Study Area

3.3.3.1 Types of Larval Habitats

Immature stages of *An. arabiensis* occurred in various types of water habitats. These habitats can be categorized as follows:

(a) Rain Pools

During the rainy season, different aquatic stages of *An. arabiensis* were abundant in rain pools. Rain pools are permanent or semi – permanent standing fresh water collections that are formed after heavy rainfall (Plate 3.2).

(b) Irrigation Water

Agricultural practices in the horticultural farms provided the species with excellent opportunities to breed during all months of the year. Between each two successive irrigation operations a number of puddles were formed. Breeding frequently occurred in these puddles. Types of puddles include:

Plate 3.2: Larval breeding habitat: Rain pools

1. Puddles formed from stagnant water which was left in the beds of parts of the irrigation canals that are lined with earth or bricks and the concrete troughs of the irrigation canals.

2. Puddles formed from seepage or overflow of water from irrigation canals due to incomplete or bad construction.

3. Puddles formed near the irrigation canals as a result of misuse of water sources by the inhabitants or by animals.

(c) Neglected Wells

Deserted or neglected wells, which are scattered throughout the horticulture farms, were found to harbor aquatic stages of *An. arabiensis*.

(d) Water From Broken Water Pipes

Water from broken water pipes was found to serve as positive breeding sites for *An. arabiensis*.

(e) Pools Close to Waterworks

Aquatic stages of *An. arabiensis* were also found in pools close to water tanks Plate 3.3. These pools were formed by water collecting on the ground due to continuous flow of water resulting from the improper use of taps and closing devices.

Plate 3.3: Larva breeding habitat: Near water tank as shown by arrow.

(f) Man- Made Ditches

Aquatic stages of *An. arabiensis* were found in many places where human activities produced water bodies suitable for mosquito breeding e.g:

1. Man – made ditches for the collection of tap water.

2. Man – made ditches used by the inhabitants for building purposes. Rainwater also collects in these ditches during the rainy season.

3. Puddles left on the ground by animal's hooves (Plates 3.4) and vehicle wheel tracks.

Plate 3.4: Larva breeding habitat: Animals hooves

3.3.4 Seasonal Density of Larvae in Abu Algoni Village, Sennar State

A total of 2007 larvae were collected from Abu Algoni village during the period from June 2010- May 2011. The abundance of *An. arabiensis* recorded from breeding sites in Abu Algoni village at different months of the year is shown in Table 3. 1. Breeding of *An. arabiensis* was recorded throughout the year except in February and June. The rain started in June but very little or no larvae were collected during this month. The peak in larval density occurred in August (680), followed by September (560) and began to decline in October (234) at the end of the wet rainy season. In the cool dry season, larval density declined in November (48), December (3), and increased again in January (81); no larvae were collected towards the end of the cool dry season. In the hot dry season, larvae started to increase and peaked in April (100). Eventually the density declined in May (55) towards the end of the hot dry season reaching the lowest level zero in June. The monthly variations in the numbers of larval mosquitoes in Abu Algoni are shown in Figure 3.3 A. The average density of larvae throughout the study period was 16.73 per dip per month (Table 3.1). A significant difference was observed between mean numbers of female ($P< 0.01$) and larvae ($P< 0.05$) of *An. arabiensis* ($P< 0.01$).

Table 3.1: Density of larvae of *Anopheles arabiensis* collected by dipping method at Abu Algoni village, Sennar State from June 2010 – May 2011.

Month	Total larvae	Average No. of larvae per one dip
June 2010	0	0
July	160	16.0
August	680	68.0
September	560	56.0
October	234	23.4
November	48	4.8
December	3	0.3
January2011	81	8.1
February	0	0.0
March	86	8.6
April	100	10.0
May	55	5.5
Total	2007	16.73 average per month

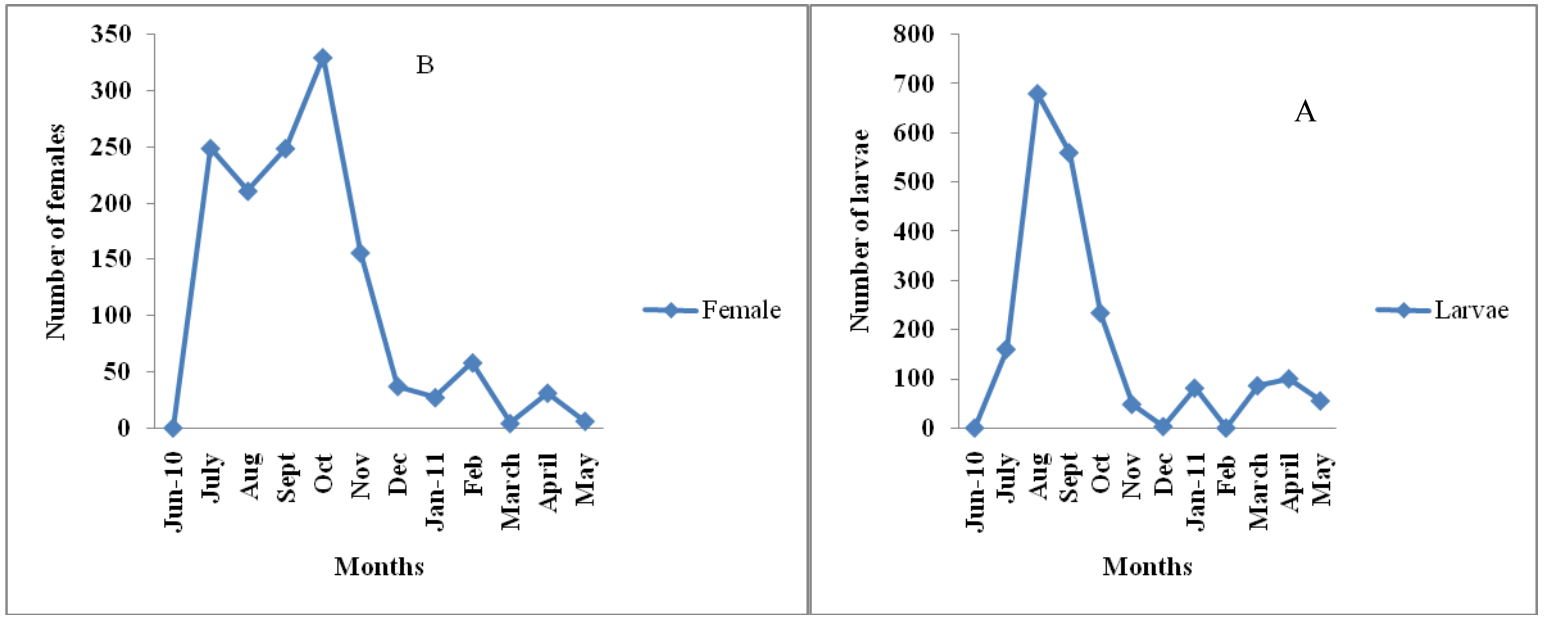


Figure 3.3: Monthly variations of numbers of A= larval and B= adult mosquitoes in Abu Algoni Village Sennar State during study period from June 2010 to May 2011.

3.3.4.1 Effect of Climatic Factors on the Larval Populations in Abu Algoni Village, Sennar State

A correlation between the prevalence of the monthly total number of the larvae and adults of *An. arabiensis* and climatic factors was made in Abu Algoni village during the study period. Breeding sites were encountered in the study area throughout the study period, so one assumes that the climatic conditions represented the main factors that determined the seasonal abundance of mosquitoes. Larval density was positively correlated with rainfall ($r = 0.81$, $P = 0.001$) and humidity ($r = 0.79$, $P = 0.002$), respectively (Figure 3.4).

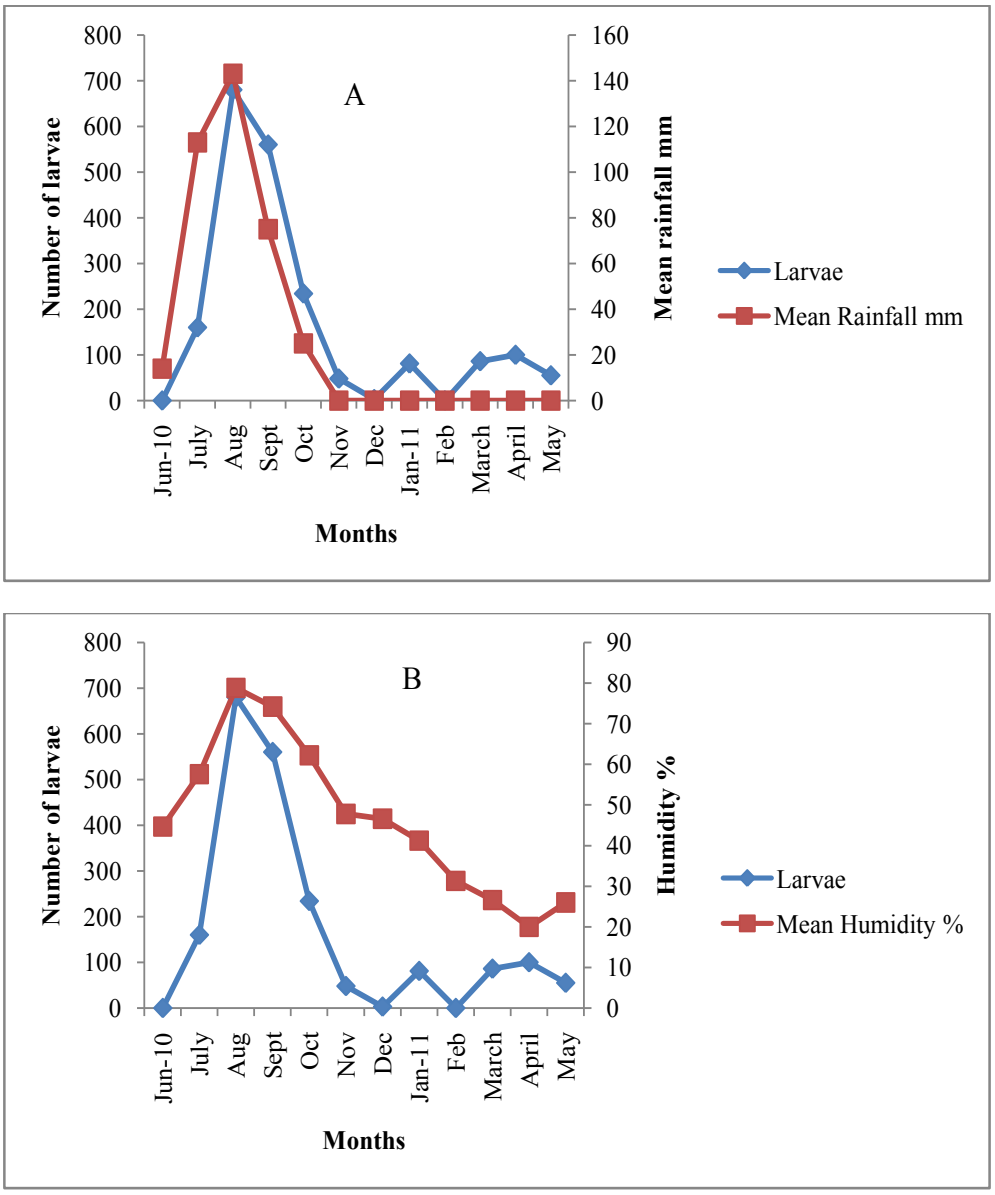


Figure 3.4: Seasonal abundance of the larvae of *Anopheles arabiensis* and their relationships with A= rainfall and B= humidity in Abu Algoni during the period from June 2010 to May 2011.

3.3.5 Adult Population Density of *An. arabiensis* in Abu Algoni Village, Sennar State

3.3.5.1 Seasonal Abundance of Indoor Resting Density

The numbers of adult females exhibited marked seasonal fluctuations consistent with fluctuations of the larval density (Figure 3.3B). The number of adults collected from June 2010 to may 2011 is shown in Table 3.2. The major peak of indoor resting density was in October 2010 (330) and progressively declined from November 2010 (156) at the start of the cool dry season. An increase in density however was recorded for February and April of 2011.

3.3.5.2 Sella's Stages

Table 3.2 illustrates the Sella's stages of resting female *An. arabiensis* collected from Abu Algoni. The occurrence of the different Sella,s stages was reported during the wet rainy (July - October), cool dry (November – February) and hot dry seasons (period) were mostly absent of half gravid and gravid females. The ratio of (fed/gravid) females which peaked in May during the hot dry season was 1: 2.0, followed by the wet rainy season in August with a ratio of 1: 1.8 and lowest ratio was during the cool dry season in December with a ratio of 1:0.03. This ratio fluctuated monthly during the study period.

Table 3.2: Frequency of Sella's feeding stages of the total pyrethrum spray monthly catches of female *Anopheles arabiensis* in Abu Algoni village, Sennar State during the period June 2010 – May 2011.

Months	T. No. of females	Unfed	Fed	Half gravid	Gravid	Fed/Half +Gravid
June2010	00	00	00	00	00	00
July	249	31	09	177	32	0.04
August	211	05	132	43	31	1.8
September	249	21	55	27	146	0.3
October	330	09	42	127	152	0.2
November	156	14	19	47	76	0.2
December	37	01	01	11	24	0.03
January2011	27	04	06	09	08	0.4
February	58	05	11	27	15	0.3
March	04	01	03	00	00	-
April	31	00	12	00	19	0.6
May	06	00	04	02	00	2.0
Total	1358	91	298	470	503	5.87
%	-	6.70	21.94	34.61	37.04	-

3.3.5.3 Christopher's Stages

The proportion of Christopher's stages of biting female *An. arabiensis* during the period of the study is illustrated in Table 3.3 and Figure 3.5. Six stages were recognized, these are stages I, II early and late, III, IV and V. A high proportion of early stages II and IV (34.15%) were found during the hot dry season, stage V (33.33%) during the cool dry season and stage III (26.37%) during the wet rainy season. The lowest proportion of late stages II and III (2.44%) were found during the hot dry season.

Table 3.3: Frequency and proportion of Christopher's stages of the total pyrethrum spray monthly catches of females *Anopheles arabiensis* in Abu Algoni village, Sennar State during the period June 2010 – May 2011.

Season	StageI	Early StageII	Late StageII	Stage III	Stage IV	Stage V	Total
Rainy July-Oct	62 5.97%	147 14.15%	195 18.77%	274 26.37%	178 17.13%	183 17.61%	1039
Cool Nov-Feb	30 11.76%	34 13.33%	45 17.65%	35 13.73%	26 10.20%	85 33.33%	255
Hot March-June	06 14.63%	14 34.15%	01 2.44%	01 2.44%	05 12.20%	14 34.15%	41

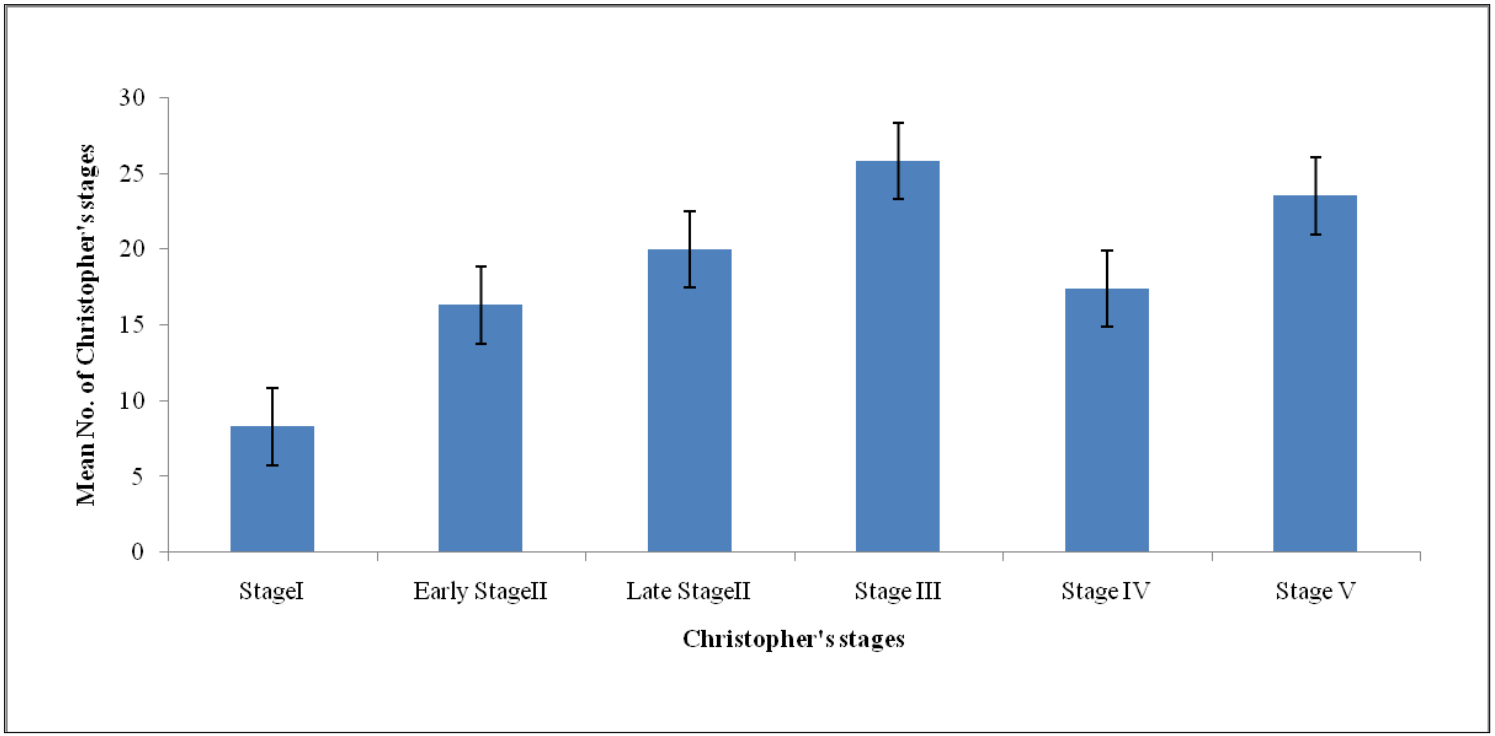


Figure 3.5: The mean with standard error of Christopher's stages of *An. arabiensis* in Abu Algoni village, Sennar State during the period of the study from June 2010 to May 2011.

3.3.5.4 Parity Rate and Probability of Survival

The majority of *An. arabiensis* dissected between June 2010 and May 2011 were parous. The highest parity rate was recorded in January 2011 (100%) and the lowest was in February (25%) as shown in Table 3.4 and Figure 3.6. The parous rate was negatively correlated with temperature (r = -0.39, P = 0. 19) and positively correlated with rainfall (r = 0. 36, P = 0.24), and humidity (r = 0.33, P = 0. 29) respectively. However, the correlations were not significant. Generally, the parity rate of *An. arabiensis* decreased when temperature increased (in the hot dry season, parity is 41.66%), while the rate increased with increased rainfall (in the wet rainy season, parity is 76.5%) and humidity (in the cool dry parity is 75.43%). There was significant difference between parous rate and seasons which effect the transmission of malaria.

Table 3.4: Monthly parous rates of females *Anopheles arabiensis* collected by pyrethrum spray methods in Abu Algoni village, Sennar State during period June 2010 – May 2011.

Month	No. mosquitoes dissected	No. parous	No. nulliparous	Parity rate %
June	00	00	00	00
July	40	36	04	90
August	137	132	05	96
September	76	54	22	71.05
October	51	25	26	49.02
November	33	29	04	87.87
December	09	08	01	88.88
January2011	10	10	00	100
February	16	04	12	25
March	03	01	02	33.33
April	12	10	02	83.33
May	06	03	03	50

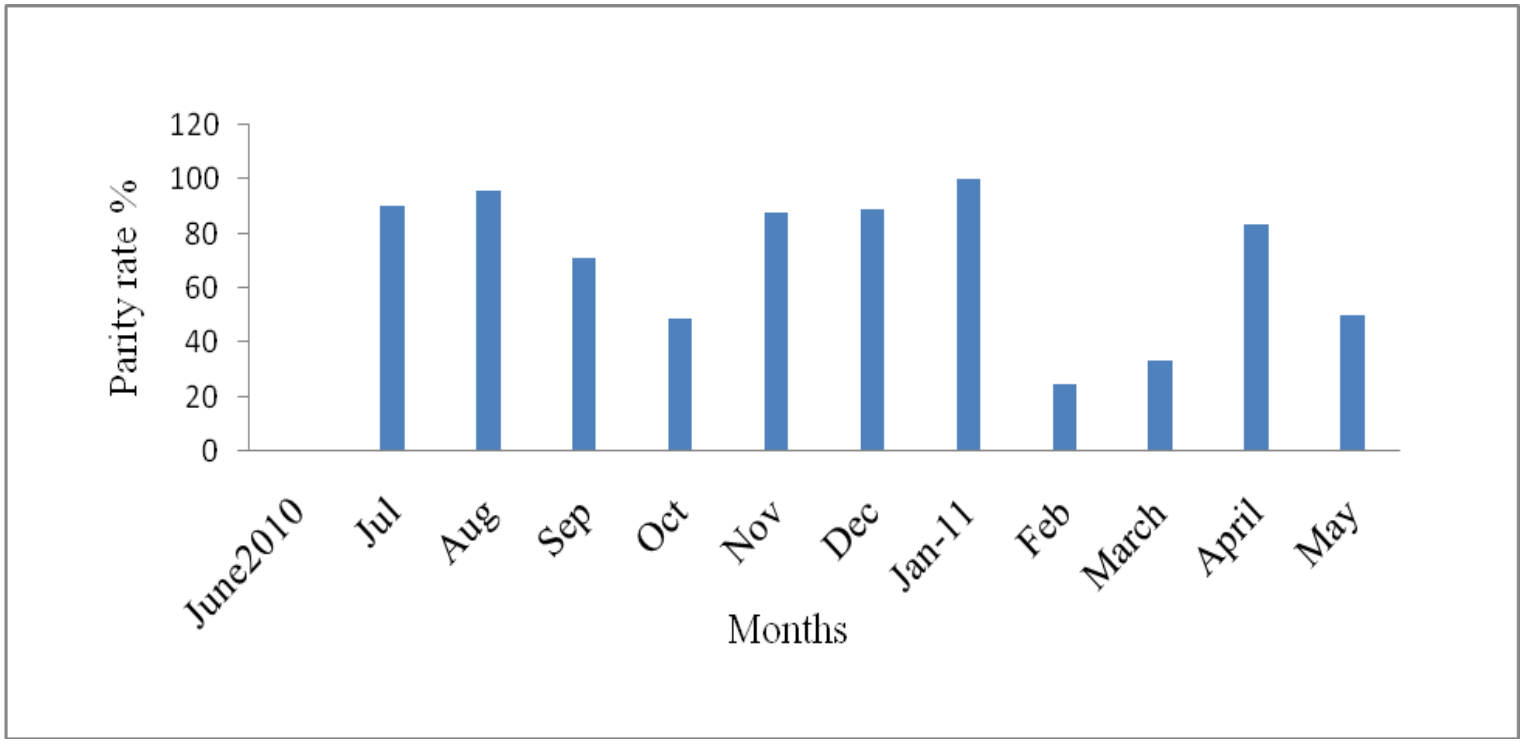


Figure 3.6: Parity rates of *An. arabiensis* in Abu Algoni village, Sennar State during the period from June 2010 to May 2011

3.3.5.5 Sporogonic Cycle

Table 3.5 shows the seasonal variation in sporogonic cycle in relations to the influence of temperature in Abu Algoni. The variation ranged from 5.90 – 15.20 days. The highest value in sporogonic cycle during January was 15.20 days while the lowest one during May was 5.90 days. This means that when the temperature was low and weather was cool the cycle takes a longer time (15.2 days); in contrast, when the temperature is high the cycle takes only a few days (5.9 days). Depending on the temperature, parasites will take a shorter time to become infective inside the mosquitoes and then will be transmitted more rapidly to humans.

Table 3.5: Monthly duration of sporogonic cycle of *Plasmodium falciparum* in *Anopheles arabiensis* calculated from the monthly average mean temperature of Abu Algoni village, Sennar State during the period between June 2010 and May 2011.

Month	Mean temperature (°C)	Sporogonic cycle (days)
June 2010	32.2	6.85
July	29.4	8.28
August	27	10.09
September	27.7	9.48
October	29.4	8.28
November	27.7	9.48
December	23.9	14.05
January2011	23.3	15.20
February	26.2	10.88
March	27.7	9.48
April	32.8	6.60
May	34.8	5.90

3.3.5.6 Effect of Climatic Factors on the Adult Populations of *An. arabiensis*

Adult females *An. arabiensis* in Abu Algoni showed insignificant negative correlation with temperature (r = -0.09, P= 0.78), and high positive significant correlation with rainfall (r = 0.66, P = 0.02), and humidity (r = 0.80, P=0.002) as shown in Figure 3.7.

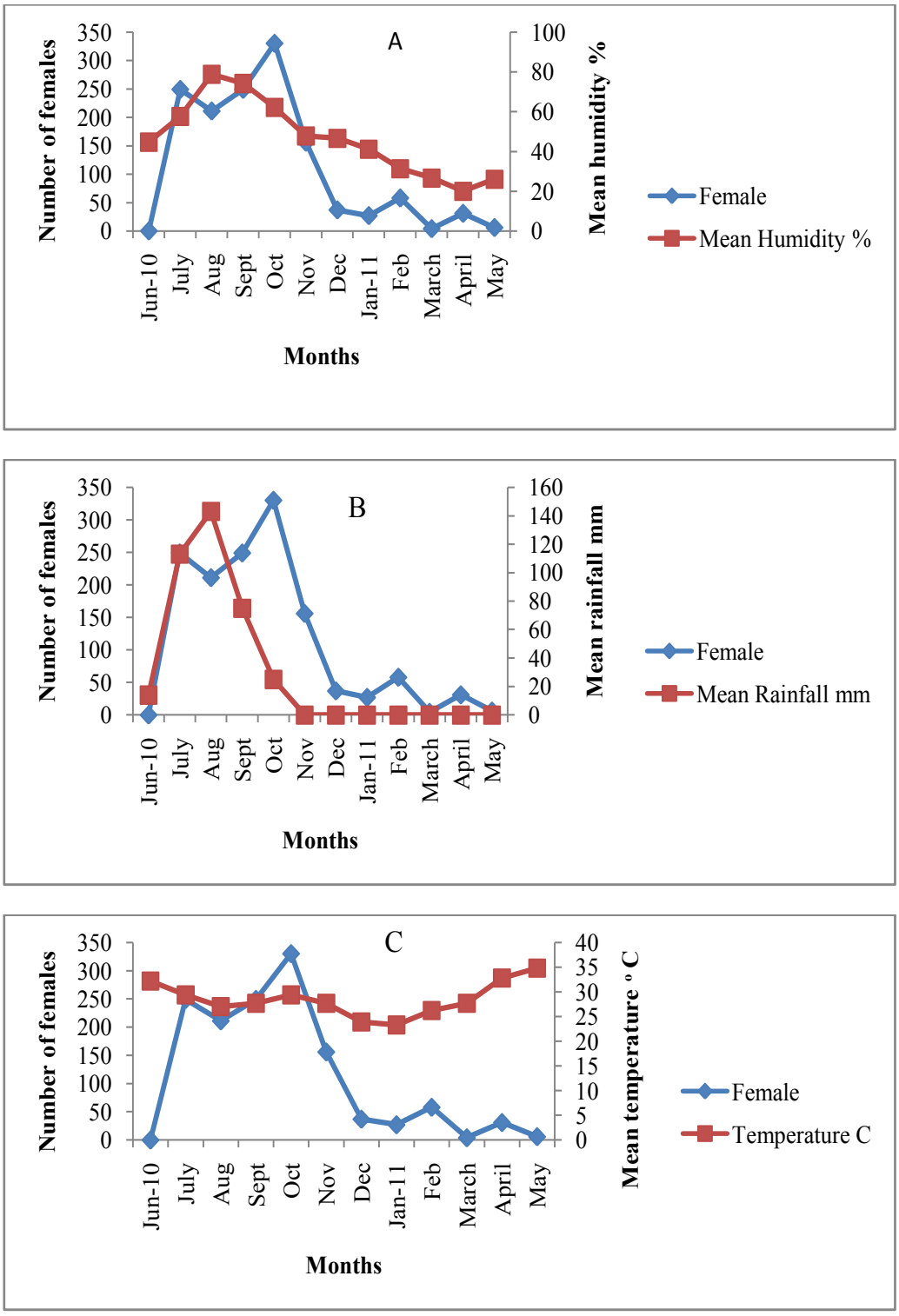


Figure 3.7: The relationship between number of female, A= mean humidity, B= mean rainfall and C= mean temperature in Abu Algoni during the period from June2010 to May 2011.

3.3.6 Seasonality of Larval Density in Algerif West Village, Khartoum State

In Algerif West, a total of 1034 larvae were collected between June 2010 - May 2011. High density was collected during the wet rainy season (955), low numbers were recorded in the cool dry season, and no larvae were found in the hot dry season. The peaks in larval density were recorded in September (523) followed by October (234). Low peaks were recorded in November (48), December (23) and January (08), and larvae were absent in February till June. The average density of larvae throughout the study period was (8.62 per dip per month). Table 3.6 shows the variation in larval density of *An. arabiensis* in different seasons.

Table 3.6: Density of larvae of *Anopheles arabiensis* collected by dipping method in Algerif West village, Khartoum State from June 2010 – May 2011.

Month	Total No. larvae	Average No. of larvae per dip
June 2010	00	00
July	48	4.8
August	150	15
September	523	52.3
October	234	23.4
November	48	4.8
December	23	2.3
January2011	08	0.8
February	00	00
March	00	00
April	00	00
May	00	00
Total	1034	103.4

3.3.7 Seasonal Abundance of Indoor Resting Density of Adult Females *An. arabiensis* in Algerif West Village, Khartoum State

Figure 3.8 show the density of indoor resting females and larvae of *An. arabiensis*, in Algerif West between June 2010 and May 2011. High density was recorded in the wet rainy season (122) from July to October 2010 followed by low density in the cool dry season (42), and the lowest density was recorded in the hot dry season (06).

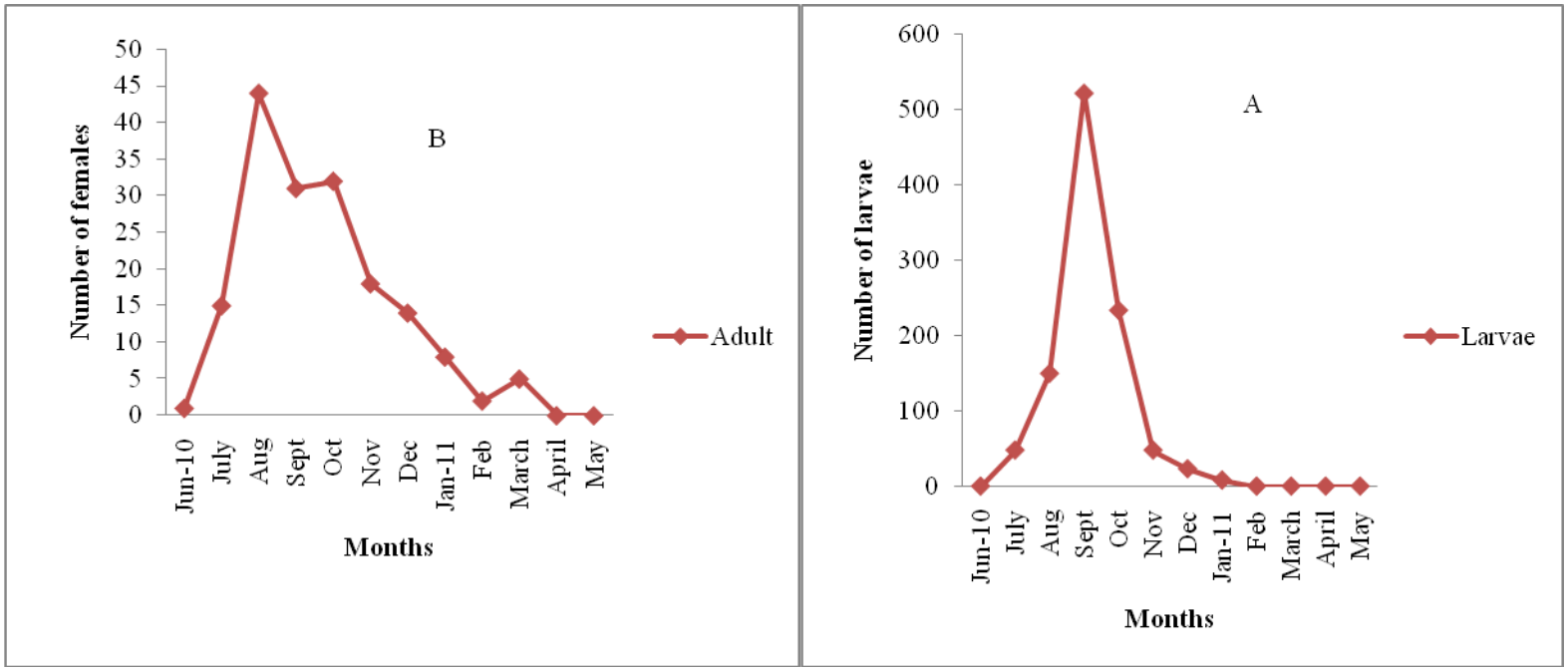


Figure 3.8: Monthly variations of the numbers of A= larvae and B= adult mosquitoes in Al gerif West village, Khartoum State during the study period from June 2010 to May 2011.

3.3.7.1 Sella's Stages of the *An. arabiensis* Population in Algerif West Village

As seen in Table 3.7, examination of the abdomens of adult females *An. arabiensis* collected in Algerif West revealed that they remained gonoactive as a result of regular blood feeding throughout the study period. All feeding stages were present in the samples examined.

A total of 170 females *An. arabiensis* were collected in the indoor resting sites, 12.35% were found to be unfed, 33.53% were fed, 24.71% were half gravid and 29.41% were gravid. The data of Sella's stages gave a fed to gravid high ratio of 1: 1.38 suggesting that the duration of gonotrophic cycle 2 to 3 days in September and low one 1:0.3 in December gonotrophic cycle 1 to 2 days.

Table 3.7: Frequency of Sella's feeding stage of the total pyrethrum spray monthly catches of female *An. arabiensis* in Algerif West village, Khartoum State during the period June 2010 – May 2011

Months	No. females	Unfed	Fed	Half gravid	Gravid	Fed/Half+ Gravid
June 2010	01	01	00	00	00	00
July	15	04	05	04	02	0.83
August	44	06	15	11	12	0.62
September	31	00	18	05	08	1.38
October	32	08	10	08	06	0.71
November	18	00	06	05	07	0.5
December	14	01	03	02	08	0.3
January2011	08	01	00	03	04	00
February	02	00	00	01	01	00
March	05	00	00	03	02	00
April	00	00	00	00	00	00
May	00	00	00	00	00	00
Total	170	21	57	42	50	4.34
%	-	12.35	33.53	24.71	29.41	-

3.3.7.2 Reproductive Conditions of the *An. arabiensis* Population Algerif West Village

The proportions of Christopher's stages of indoor resting females of *An. arabiensis* in Algerif West during the study period are shown in Table 3.8 and Figure 3.9 Dissection of the ovaries revealed various Christopher's stages of ovarian development, invariably representing catches of successive months of wet rainy and cool dry seasons, while in the hot dry season early stages II, late stage II and stage V were not found.

Table 3.8: Frequency and proportion of Christophers stages of the total pyrethrum spray monthly catches of females *An. arabiensis* in Algerif West village, Khartoum State during the period June 2010 – May 2011.

Season	StageI	Early StageII	Late StageII	Stage III	Stage IV	Stage V	Total
Rainy July-Oct	12 (9.84%)	24 (19.67%)	25 (20.49%)	25 (20.49 %)	15 (12.30%)	21 (17.2%)	122
Cool Nov - Feb	02 (4.76%)	03 (7.14%)	06 (14.29%)	11 (26.19 %)	11 (26.19%)	09 (21.43%)	42
Hot March - June	01 (16.6%)	00	00	03 (50%)	02 (33.33%)	00	06

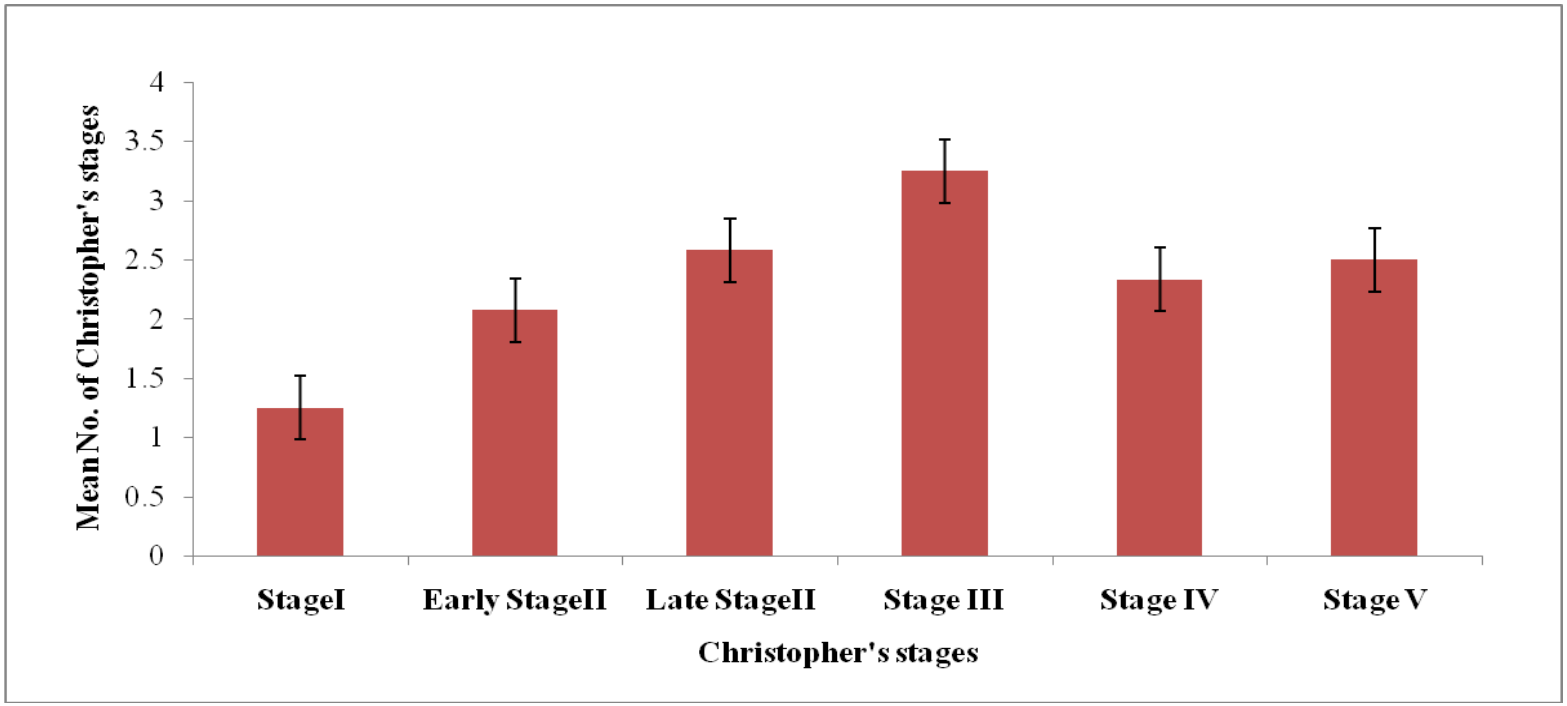


Figure 3.9: The proportion of Christopher's stages of *An. arabiensis* in Algerif West village, Khartoum State during June 2010 – May 2011.

3.3.7.3 Parity Rate of *An arabiensis* Population in Algerif West

Table 3.9 illustrates the monthly parous rate in Algerif West village, Khartoum State. The highest rate was 100% in January and the lowest value of 61.1% was in September as shown in Figure 3.10. However population samples were very low and therefore does not give a clear picture of the parity rates. Parous rate has an insignificant negative correlation negative correlated insignificantly with temperature (r = -0.38, P = 0.21) and positive insignificant with rainfall (r = 0.38, P = 0.22), but highly correlated with humidity (r = 0. 613, P = 0. 034). In cool dry season the parous rate = 60.4%, zero in hot dry and 74.27% in wet rainy season.

Table 3.9: Monthly parous rate of females *An. arabiensis* collected by pyrethrum spray method in Algerif West village, Khartoum state during the period from June 2010 – May 2011

Month	No. mosquitoes dissected	No. parous	No. Nulliparous	Parity rates %
June 2010	0	0	0	0
July	5	4	1	80
August	21	15	6	71.4
September	18	11	7	61.1
October	13	11	2	84.6
November	6	4	2	66.6
December	4	3	1	75
January2011	1	1	0	100
February	0	0	0	0
March	0	0	0	0
April	0	0	0	0
May	0	0	0	0

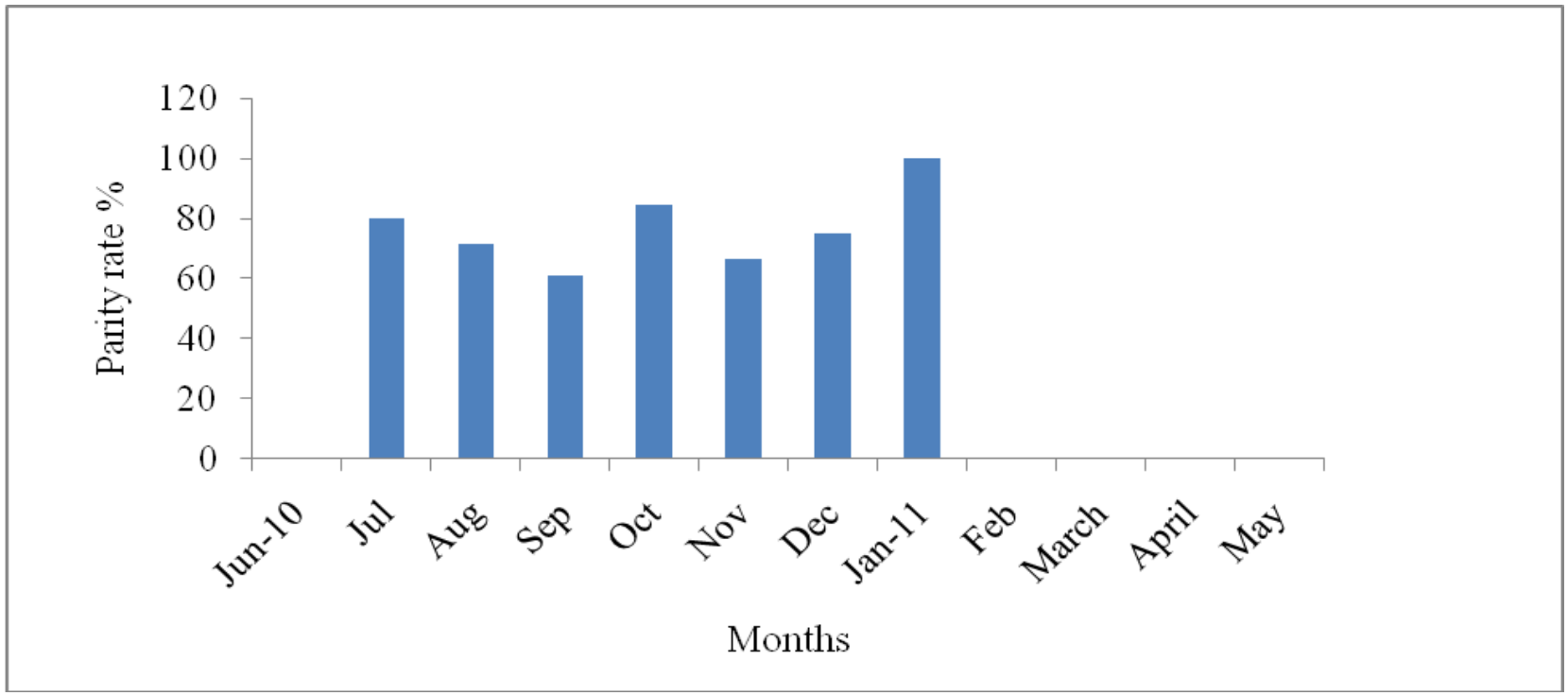


Figure 3.10: Parous rates of *An. arabiensis* in Algerif West village, Khartoum State during the period from June 2010 to May 2011.

3.3.7.4 Sporogonic Cycle in the *An. arabiensis* Population in Algerif West Village

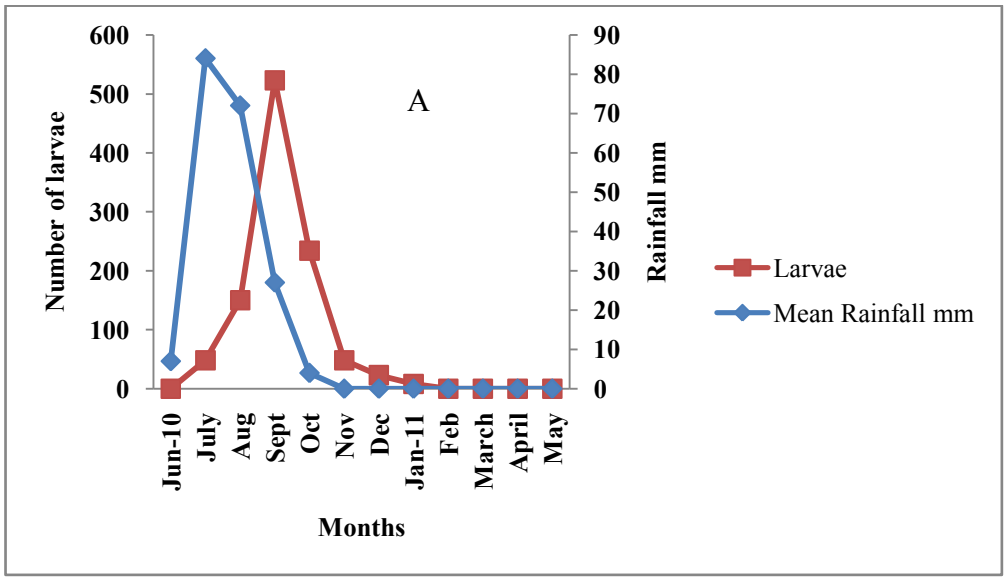
Table 3.10 shows the values of the sporogonic cycles of *P. falciparum* in the study area during the study period. The monthly mean temperatures varied between the months, and accordingly so do the duration of sporogonic cycles. The sporogonic cycles ranged from 5.15 to 16.08 days. The highest sporogonic cycle value was computed for January 2011 (16.08 days) and the lowest in May 2011 (5.15 days).

Table 3.10: Monthly duration of the sporogonic cycle of *Plasmodium falciparum* in *Anopheles arabiensis* calculated from the monthly average mean temperature of Khartoum during the period June 2010 –May 2011.

Month	Mean temp. (°C)	Sporogonic cycle (days)
June 2010	36.3	5.46
July	32	6.93
August	31.4	7.11
September	32.4	6.85
October	34.2	6.09
November	31.6	7.20
December	26.5	10.57
January2011	22.9	16.08
February	27.7	9.48
March	28.6	8.80
April	32.7	6.64
May	37.5	5.16

3.3.7.5 Effect of Climatic Factors on the Population Dynamics of *An. arabiensis* in Algerif West Village

Figures 3.11 and 3.12 shows the correlation between the prevalence of monthly total number of larvae and adults of *An. arabiensis* in Algerif West village, respectively, during the study period. Breeding sites were encountered in the study area throughout the study period, so one assumed that the climatic conditions represented the main factors that determined the seasonal abundance of *An. arabiensis*. The total number of females showed an insignificant positive correlation with temperature (r= 0.034, P = 0.92) and rainfall (r= 0.55, P = 0.06), but highly correlated with humidity (r =0.77, P= 0.003). Larvae, however, showed an insignificant positive correlation with rainfall(r = 0.26, P= 0.4) and humidity (r = 0.54, p = 0.06) (Figure 3.11).



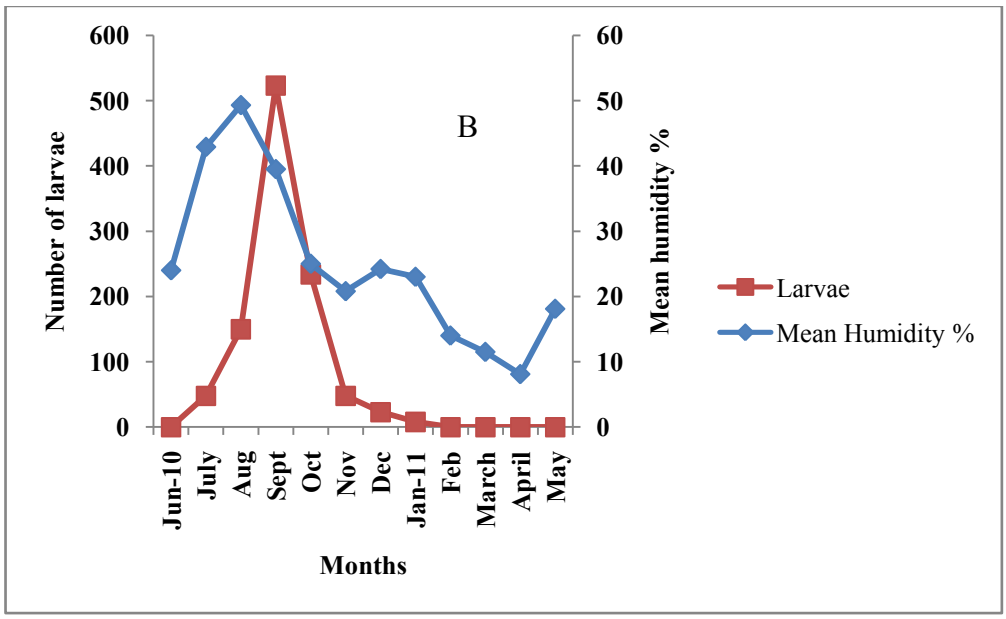


Figure 3.11: The relationship between number of larvae A= rainfall and B= humidity in Algerif West village, Khartoum State

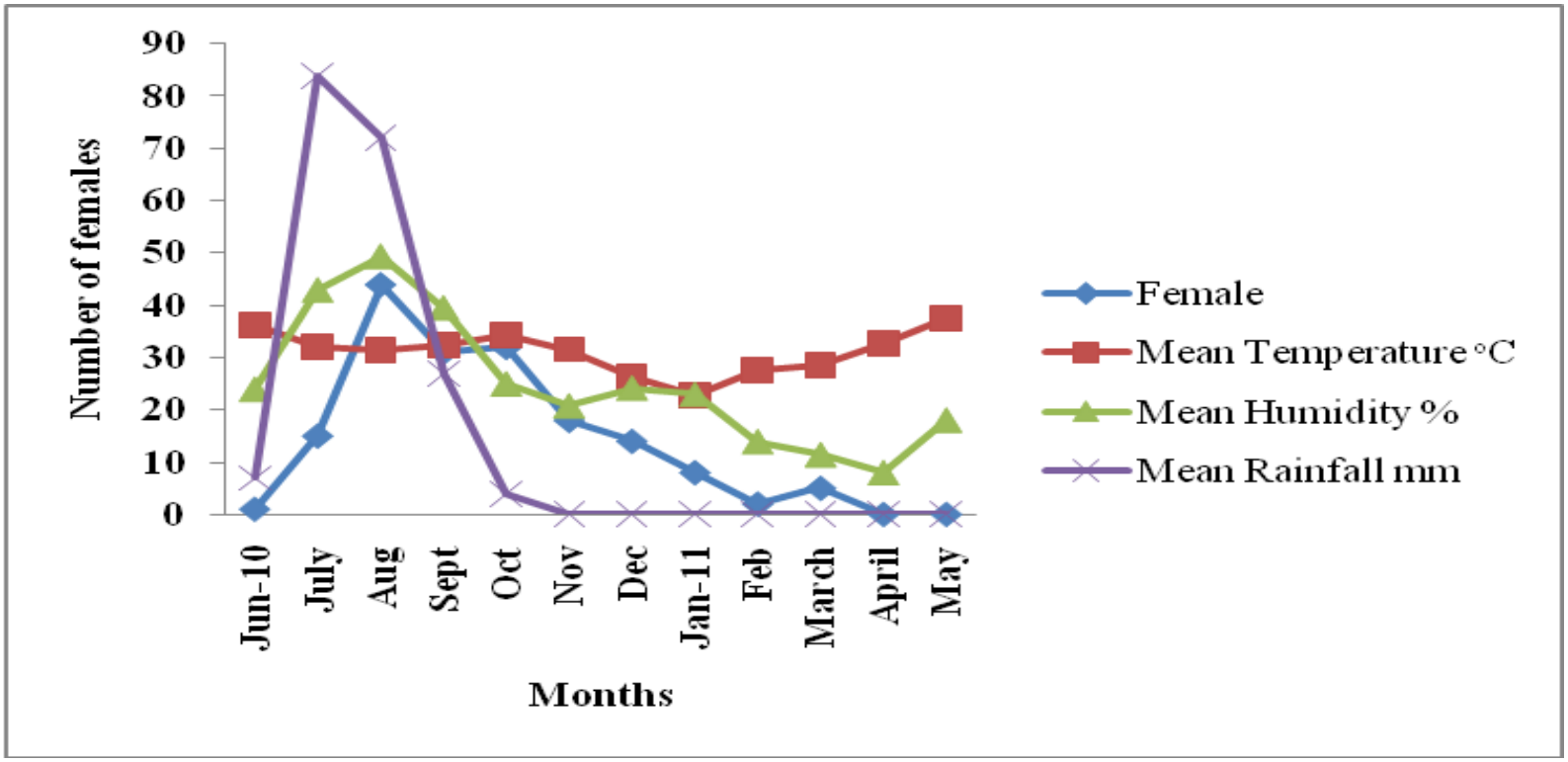


Figure 3.12: The effect of climatic factors on the distribution of *An. arabiensis* in Algerif West village, Khartoum State

3.4 Discussion

Malaria is still a major health problem in Sudan and their transmission is high due to the extensive distribution of *Anopheles arabiensis*, the main malaria vector in the country. Understanding the ecology of the adult and larval habitats is essential in designing targeted malaria control programmes. The mosquito density is a major determinant of malaria transmission and depends largely on the longevity of the adult mosquitoes and the availability of suitable breeding places (Agyepong *et al.*, 1995). The most obvious observation in the present study is that *An. gambiae* complex forms by far the majority of the *Anopheles* fauna. In Abu Algoni village, all adult collected were identified as *An. arabiensis*. Except for some *An. rufipes* individuals encountered, all trapped adults were from the same species. In Algerif West village, except for *An. arabiensis*, no other *Anopheles* species was found. Overall, the prevalence and abundance of both larval and adult populations exhibited a seasonal pattern and were influenced by changes in the climatic conditions. It was also noted that *An. arabiensis* was the only member of the *An. gambiae* complex found in the study areas.

3.4.1 Larval Habitat

The abundance of the larvae of *An. arabiensis* in Abu Algoni village during the wet rainy season indicated that their breeding sites were numerous including rain pool, animals hooves and heavy truck-tracks. In the dry season irrigation ditches, seepages, water leakage from broken pipe, and water containers served as the main breeding sites. All breeding sites are exposed to sunlit or half shaded. In the present study the reduction in the larval density during the post rainy season was an unexpected result as the environmental conditions were good for breeding. It was found that simple control measures such as the construction and maintenance of a

drainage system plus intervention using larvicides were quite enough to wipe out all the larval population. The population density of *An. arabiensis* was recorded during all months of the year except in June. The major peak was in October and the minor ones in February and April. El-Tayeb (2001) who studied malaria control method in an endemic area of El Rahad, north Kordofan State showed that the peak population density of *An. arabiensis* occurred at the end of the rainy season. Imbahale *et al.*, (2011) who conducted a longitudinal study on the abundance of *Anopheles* larvae in western Kenya, published that the significant larvae breeding habitats are drainage duct, hoof prints, tire tracks, rice paddies and leaking or broken watering taps, with a regular run of water. These are similar with the findings of the present study where larval habitats, permanent or semi permanent, included canals, animals hooves, water from leaking or broken pipes, and rain pools. Fillinger *et al.* (2004) who studied the practical importance of permanent and semi permanent habitats in an effort to control the aquatic stages of *Anopheles gambiae sensu lato* mosquitoes in western Kenya found that semi-permanent and permanent habitats were the main source of both anophelines and culicines.

3.4.2 Species Composition

Information about the species composition, biology and behaviour of vector anophelines are necessary to understand their epidemiological role in malaria transmission, and resulting from that an effective malaria control programme (Hunt *et al*., 1998). Studies carried out in central Sudan, based on morphological and cytogenetic experiments, showed that *An. arabiensis* is the predominant species in that area (El Gaddal *et al*., 1985; Petrarca *et al*., 2000).

Throughout the present study increased predominance of *An. arabiensis* larvae and adults were observed in Abu Algoni and Algerif West villages. This is in

line with a report from Tasneem (2010) who recently surveyed the *Anopheles* populations in Sennar State. She identified about 92% of the collected *Anopheles* individuals as *An. arabiensis*, and considered it as the main malaria vector. In the present study, this species accounted for more than 99% of the total anophelines collected in Abu Algoni, also a village of Sennar State. Thus, *An. arabiensis* is the only malaria vector present in the surveyed areas in the present study. Azrag (2007) also found *An. arabiensis* to be the main vector in North Sudan. Similarly, findings by Hamza (2003) and Himeidan *et al.*, (2004) showed that *An. arabiensis* is the main malaria vector in Kassaala State. Recently *Seidahmed et al.*, 2012 and Yagoop *et al*., 2013 confirmed that *An. arabiensis* is the main malaria vector in Khartoum and El Rahad Central Sudan respectively.

3.4.3 Variations in Population Abundance in Relation to Climatic Factors

Algerif West village area is characterized by a low density of *An. arabiensis* restricted only to the rainy season. This reduction is due to the effect of efficient control activities in Khartoum State (Malaria-Free Khartoum programmes). Obala *et al.* (2012) studied *Anopheles gambiae* and *An. arabiensis* population densities and infectivity in Kopere village, Western Kenya and found that the population densities of the malaria vectors peaked in June and May 2002 and 2003, respectively. They observed that the lowest vector densities occurred in September 2003. Their findings seemed to disagree with the current study which showed that the population density of *An. arabiensis* was recorded during all the months of the year except in June, with major peak in October 2010 and minor peaks recorded in February and April 2011, respectively. This density variation may be due to ecological variations and climatic differences between the two

countries. Mwanziva *et al*. (2011) studied the malaria transmission intensity and the vector population structure in northern Tanzania, and revealed that only *An. arabiensis* was found in this area during both the wet and dry seasons, and the seasonal abundance is rain dependant. Similarly Kulkarni *et al.* (2006) conducted an entomological study of *An. arabiensis* in, Northeastern Tanzania. They revealed that the density tended to increase at the onsett of rainfall, consequently assumming an important role in the transmission of malaria at the end of the lengthy rainny period with its relative increase in density. Mwangangi *et al.* (2010) studied the larval abundance and diversity of *An. arabiensis* in central Kenya. They revealed that the larval abundance was positively associated with the short and long rains in Kiamachiri (r = 0.759) and short rains in Mbui Njeru (r = 0.602). On the contrary, larval abundance was negatively correlated with both the long and short rains in Murinduko (r = -0.267). This study agreed with the present study which showed that the number of *An. arabiensis* larvae in Abu Algoni village had a highly significant correlation with rainfall (r = 0.813), while in Algerif West village, the number of *An. arabiensis* larvae had a moderate correlation with rainfall (r = 0.256), attributable to the rain levels in Abu Algoni villagebeing higher than Algerif West village. Obala *et al.* (2012) showed that temperature was the only climatic component which had a significant correlation with the vector densities (r = 0.626), and the lack of positive correlation between the mosquito densities, rainfall, relative humidity (RH) and maximum temperatures, probably accounted for 51% of the variance in the data. In the present study, females *An. arabiensis* in Abu Algoni village showed a negative correlation with temperature(r = -0.09), while positively correlated with rainfall (r = 0.66) and humidity(r = 0.80). In Algerif West village, females *An. arabiensis*

showed an insignificant positive correlation with temperature (r= 0.034) and rainfall (r= 0.55), but highly correlated with humidity (r =0.77). Humidity may have an effect on the temperature of the larval habitats. In the present study when humidity is high, the temperature of the larval habitat is low and vice versa. This difference may be attributed to climatic changes that affected the breeding habitats as well as insecticide resistance. El-Tayeb (2001) showed that the peak population density of *An. arabiensis* occurred at the end of the rainy season. According to this study there is a direct correlation between the amount of rainfall and density of *An. arabiensis*. The seasonal abundance of *An. arabiensis* explained that the majority of the vectors were collected throughout the rainy season which might be associated with the availability of extra breeding habitats created by rainfall. In this case, the incidence of malaria will be high throughout the rainy season than in the dry season. There was a reduction in female during August although in this month the rain was heavy, and probably flooded the breeding sites and washed out the eggs and larvae, consequently reducing the abundance of adult *An. arabiensis.* Adeleke *et al*. (2008) studied mosquito larval habitats and public health implications in Abeokuta, Ogun State, Nigeria. They observed that intense breeding of mosquitoes occured in both dry and wet seasons, whereas in the present study the incidence was higher in wet than dry season.

3.4.4 Age –Grading

One of the major determinants of vector-borne disease transmission is vector survival. For instance, vector *Anopheles* are capable of transmitting *Plasmodium* only after they are at least 10 days old because the parasites take at least that long to develop and become infective in the mosquito (Beier, 1998). The conventional

method used by scientists to determine the approximate physiological age of a female mosquito is to look at the morphological transformations in the reproductive system (Polovodova, 1941; Gillies, 1958, 1961; Gillies and Wilkes, 1965; Detinova, 1962) and to assess disease transmission potential (Garret, 1964; Dye, 1992).

Mosquito age composition plays an important role in malaria transmission. Parous females that have previously blood fed, have a higher possibility of being infected with malaria parasites. Lemasson *et al.* (1997) conducted a study comparing the behavior and vector competence of *An. gambiae* and *An. arabiensis* in Senegal during 1994-1995. They observed that *An. gambiae* had a higher parity rate than *An. arabiensis*, but data collected during 1995-1996 showed that the parity rate of *An. gambiae* was significantly lower than *An. Arabiensis* (Lemasson *et al.*, 1997). These findings are similar to the current study where the parity rate was high in *An. arabiensis* in the two study sites in Sudan. Ndiath *et al.* (2008) studied the dynamics of transmission of *Plasmodium falciparum* by *An. arabiensis* and the molecular forms M and S of *An. gambiae* in Dielmo, Senegal. They found that the mean parity rate was 70.9% for *An. arabiensis*, 68.7% and 80.1% for *An. gambiae* M and S forms, respectively. These findings are in line with the present study where the mean parity rate was 77.19% in Abu Algoni village and 63.85% in Algerif West village. The low parity rate in Algerif West village maybe due to the effective vector control programme in this area. In contrast Himeidan (2000) who studied the biology and behaviour of *An. arabiensis* in New Halfa eastern Sudan recorded a parous rate of 32.23%. He explained that the low level of parity rate might be due to the application of insecticide during the study period.

Dukeen and Omer (1986) studied the ecology of the *An. arabiensis* by the Nile in northern Sudan. They found that out of a 1398 mosquitoes collected 2.2% (30) unfed, 54.6% (763) freshly fed and late fed, and 43.3% (60) were gravid. In the current study, out of a 1358 females collected, 6.7% (91 females) were unfed, 21.94% (298) were fed, 34.61% (470) were halfgravid and 37.04% (503) were gravid. High proportion of gravid, half gravid and fed females in the present study concluded that continuous breeding and blood-feeding mean high persistent transmission during the year. Tchuinkam *et al.*(2010) found that the duration of the gonotrophic cycle in *An. gambiae* in Western Cameroon, was 2-3 days in the lowlands, 3-4 days on the plateau and 6-7 days uphill in Santchou, Dschang and Djuttitsa respectively. as the same was also observed by Kulkarni *et al.* (2006). They recorded a longer gonotrophic cycle estimated at 5 - 6 days at higher altitudes, based on the annual mean fed/gravid ratio, in relation to the seasonal changes in resting behavior. This estimate takes for granted equal access of indoor and outdoor mosquito populations, and they are consistent with an earlier estimate of a 3 day cycle in *An. arabiensis* in an irrigated area situated at a lower altitute in northern Tanzania (Ijumba *et al.*, 2002). The latter is in line with the present study where the gonotrophic cycle is estimated at 2 to 3 days in the two study sites. The fluctuation of fed/gravid ratio during the study period may be related to the climatic conditions, which favoured the survival of females *An. arabiensis* resting inside houses.

In Abu Algoni village there were reduction in Christophers stage II and III during the hot dry season which was characterized by the absence of breeding places. Algerif West village area was characterized by a low mosquito production restricted to the rainy season due to the effect of control measures that were applied during this time of the year.

Sporogonic cycle of *Anopheles arabiensis* duration in the present study was between 6 to 16 days depending on the temperature. When the temperature is high up to 36°C, the duration for the development of the sporozoites within the mosquito will be shorter and therefore transmission from vector to host will be much quicker. A similar finding was observed by Eling *et al.* (2001) where the hot tropical temperatures influenced the growth of the *P. falciparum* in *Anopheles* mosquito. They stated that the sporogonic cycle is dependent on the ambient temperature and proceeds faster at higher temperatures. In contrast, the cycle will take longer (16 days) at low temperatures (23°C) (Boyd, 1949; Noden *et al.*, 1995; Eling *et al.*, 2001). This explains the importance of temperature in the transmissions of malaria.

CHAPTER 4

POPULATION STRUCTURE OF *Anopheles arabiensis* POPULATIONS IN SELECTED AREAS OF SUDAN BASED ON MITOCHONDRIAL AND NUCLEAR RIBOSOMAL DNA

4.1 Population Genetics of Anophelines Based on mtDNA and Nuclear rDNA Studies

Population genetics studies are essential in the control and management of malarial incidence (Tabachnick and Black, 1995). Through the population structure data, the topographic spreading and possible reproductive segregation can be elucidated (Lanzaro and Tripet, 2003). Furthermore, such studies permit gene flow estimations within and between populations and identification of the biological and environmental factors that influence their movement (Lanzaro and Tripet, 2003). Several markers are widely utilised for this purpose particularly the haploid and maternally inherited mtDNA (Avise, 2004). To complement these markers, the hypervariable microsatellite or simple sequence repeat (SSRs) markers are also often applied in population genetics studies of medically important mosquitoes in the last few decades. Investigations of population genetics in the mosquitoes of medical importance within the last decade have relied mainly on mtDNA and microsatellite markers (Besansky *et al.,* 1997; Birungi and Munstermann 2002; Nyanjom *et al*., 2003; Temu and Yan, 2005; Kent *et al.*, 2007; Venkatesan *et al.*, 2007; Muturi *et al.*, 2010; Vicente *et al.*, 2011; Nghabi *et al.,* 2011).

Gene flow is the movement of genes within and between populations (Ferris *et al*, 1983). It is facilitated by the movement of gametes, individuals or groups of individuals from one place to another (Slatkin, 1985). Natural selection, random

genetic drift and mutations are the major factors which lead to inter and intragenetic differences between and within species and populations (Ferris *et al.*, 1983; Slatkin, 1995). The present knowledge of precise factors influencing gene flow in natural populations is far from complete, but population genetics theories may offer several underlying explanations (Slatkin, 1987). Gene flow can be estimated using either a direct or indirect approach (Slatkin, 1995). Direct gene flow estimates can be assessed through the examination of dispersal within a defined range of time and space (Taylor *et al*., 2001; Edillo *et al.,* 2002). The direct method is conducted through mark–release recapture testing or evaluation of the offspring or sperm from field collected mosquitoes. The indirect model is based on the evaluation of allele frequencies, usually obtained through molecular methods on proteins or DNA. The effect is cumulative acting over all temporal and spatial scales (Lanzaro *et al.*, 1998; Walton *et al.*, 1998; Donnelly and Townson, 2000). These techniques rely on determining the standardized variance in allele frequencies among populations (F_{ST}). This would allow an estimation of the number of migrants per generation (Nm) using the equation, $F_{ST}= (1/4Nm+1)$ (Wright, 1931) where N = population size and m = the average rate of immigration.

4.1.1 Population Genetics of *Anopheles gambiae* Complex Based on Mitochondria DNA

Mitochondrial DNA (mtDNA) is relatively simple to amplify for the reason that it is present in multiple replicates in the cell. Its gene contents are highly conserved across animals, with very little duplication, no intron, and very short intergenic regions (Gissi *et al.*, 2008). Fast mutation rates in mtDNA, particularly in specific genes result in significant variation in sequences within and between species

providing ample within-species variance which is useful for phylogenetic investigations (Tamura and Nei, 1993; Mohanty *et al.*, 2009).

In anophelines, mtDNA sequences have been applied to determine associations at different taxonomic levels of divergence, for example at population, species, genus and also at higher taxonomic levels (Krzywinski *et al.*, 2001; Krzywinski and Besansky, 2003; Loaiza *et al.*, 2010). This is due to the differing rates of mutation among genes. For instance, the D-loop, cytochrome b, NDI and ND5 genes (Besansky *et al.*, 1997; Donnelly *et al.*, 2004; Marshall *et al.*, 2008) are frequently utilised for population genetics studies due to their higher mutational rates while 16S rRNA (Shouche and Patole, 2000) is utilised for higher level rankings. It is also very effective in estimating time of separation of main lineages (Krzywinski *et al.*, 2006; Moreno *et al.*, 2010) and for resolution of sibling species (Goswami *et al.,* 2006; Dusfour *et al.*, 2007).

Besansky *et al.* (1997) showed that within *An. arabiensis* populations across Africa, no population subdivision was detected even at distances of 600 km based on partial gene sequences from the cytochrome b, NDI and ND5 genes. Evidence that populations of *An. gambiae* and *An. arabiensis* show low levels of population differentiation across their range as detected using microsatellite loci and mitochondrial ND5 gene has been attributed to the recent population range expansion of these species (Donnelly and Townson, 2000; Donnelly *et al.*, 2001).

The lack of mitochondrial difference between populations and species within *An. gambiae* complex has led to conclusions of gene flow across great geographic expanses and contemporary genetic introgression among species within the complex (Beard *et al.,* 1993; Besansky *et al.,* 1994; 1997; Caccone *et al.,* 1996; Lehmann *et*

al., 1997; 2000 and Donnelly *et al.*, 2001; 2004). In the present study, the ND5 gene was chosen because past studies involving Kenyan populations of both *An. gambiae* and *An. arabiensis* established that this region was polymorphic {Besansky *et al.*, 1997; Kamau *et al.*, 1999). In addition, a common gene would facilitate easier comparisons.

4.1.2 Taxonomic and Population Genetics of the Anopheline Mosquitoes Based on the Nuclear rDNA Gene

The potential presence of cryptic species necessitates correct species identification and determination of the evolutionary and phylogenetic relationships of the *Anopheles* for the control of malarial transmission. Therefore, precise taxonomic investigation is vital. Early classification of the Anophelines was based on conjectural taxonomic interpretations of a partial number of morphological similarities and molecular phylogenetics methods (Harbach *et al*., 1997). Phylogenetic study is a useful approach for taxonomic and sytematics studies (Mohanty *et al.*, 2009; and Morgan *et al.*, 2009). However, the phylogenetics of *An. gambiae* complex has not been completely elucidated. Besansky *et al.* (2003) hypothesized that the *An. gambiae* and *An. arabiensis* developed from the same ancestral origin based on the *white* and *tox* mutations and allozyme studies of *G6pd* and *xdh* genes. The tree they constructed was the converse of the previous general accepted phylogenetics that had placed the two principal vectors on distant branches (Besansky *et al.,* 2003). A study by Coluzzi *et al.* (1979) revealed that *An. gambiae* is one of the most recently diverged cluster of complex species. Therefore, the ability to conclude evolutionary associations in this group may be problematical even with new molecular techniques. For this reason delineating the evolutionary position of *Anopheles* subfamily require additional data.

Sequences of the nuclear ITS gene which form part of the rDNA cluster is an important tool to accumulate such data. There have been several reports of rDNA intragenomic variation in *Anopheles* (Wilkerson *et al*., 2005; Fairley *et al*., 2005), but its occurrence and scale is not well studied. One explanation for such high intragenomic variation in is the possibility that rDNA arrays are connected to different sex chromosomes. In some species, they may be found in only the female (Collins *et al.*, 1989) but in others in both sex chromosomes (Marchi and Pili, 1994). In *An. gambiae* and *An. arabiensis* the rDNA locus is only on the X chromosome, while in other member species of the *An. gambiae* complex, it is in both sex chromosomes (Collins *et al.*, 1989). Therefore, when recombination is limited in either sex chromosome, incomplete homogenization could occur. Population genetics study have also been reported for the ITS gene. Gentile *et al.* (2002) failed to detect population subdivision within African *An. arabiensis* populations over a wide geographic range based on the ITS1 and ITS2 genes. The study found that populations were only represented by a single major haplotype with some minor variants.

The aim of the study reported in this Chapter was to estimate genetic variability and population structure, for any evidence of subdivision and restricted gene flow within *An. arabiensis* and to determine the relationships of *An. arabiensis* within the *An. gambiae* complex species based on two genes; mitochondrial ND5 and nuclear ITS.

4.2 Materials and Method

4.2.1 Study Sites

The mosquitoes were collected from six localities in Sudan representing different ecological zones separated by the River Nile and its tributaries from June 2010 to May 2011. Three localities were located in Khartoum State, namely 1. Mygoma (My) 2. Al Haj Yousif (Hj) 3. El Gerif West (Gw). My and Hj are nearest to each other and east of the Blue Nile. In addition Hj is North east of Helt Koko, where animals are bred for milk production in a rich green area on the West bank of the Blue Nile. The fourth and fifth sites are in Kassala State namely 4. Alhalang Shemal (H.sh), located on the east bank of the AlGash River area of non-agricultural land and 5. Alkrmota (Kr) located on the west bank of the Al Gash River in the centre of an agricultural area which is surrounded by groves of fruit and vegetables in all directions. The sixth population is in the Sennar state (Se), and was collected from 6. Abu Algoni (Se), a farming area which is located on the west bank of the Blue Nile River **(**Table 4.1).

Table 4.1 Sampling localities and sample sizes (N) of *An. arabiensis* populations.

Regions	Populations	Latitude North	Longitude East	ND5 (N)	ITS (N)
Khartoum	Alhaj Yousiif (Hj)	15° 37′14.61″	32° 38′ 19.99″	20	15
	Mygoma (My)	15° 36′ 54.42″	32° 35′ 59.57″	17	15
	Algerif West (Gw)	15° 33′38.42″	32° 35′ 6.89″	16	15
Sennar	Abu Algoni (Se)	13° 33′ 38.97″	33° 34′ 13.44″	16	18
Kassala	Krmota (Kr)	15° 26′ 13.31″	36 ° 23′ 30.88″	17	15
	Alhalang Shemal (H.sh)	15° 28′ 12.45″	36° 23′ 27.24″	17	14
Total				103	92

4.2.2 Molecular Laboratory Analysis

Genomic DNA was extracted from single mosquitoes following QIAGEN protocol (QIAGEN Sciences, Maryland 20874, USA). The mitochondrial ND5 DNA was amplified using the specific PCR primers 19CL F: 5'- CTT CCA CCA ATT ACT GCT ATA ACA G-3'; and DM3A R: 5'-AGG ATG AGA TGG CTT AGG TT-3' of *An. gambiae* mtDNA (Besansky *et al*., 1997). PCR was carried out in a 25 µL reaction containing 18µL sterile H_2O, 2.5 µL 10X PCR Buffer with MgCl2, 1.0 Ml dNTPs (2 mM), 0.3uL MgCl2 (25 mM), 1.5uL forward and reverse primers, 0.2 µL *Taq* DNA polymerase (5 U/µL) (iNtRON, Gyeonggi-do, Korea) and 0.2 µL template DNA. The cycling conditions conducted in the Mastercyler Thermal Cycler (Eppendorf) were 4 min denaturation at 94ºC, followed by 35 cycles of 30 sec denaturation at 94ºC, 40 sec annealing at 53ºC and 30 sec extension at 72ºC with a final cycle extension at 72ºC for 10 min. ITS gene was amplified using the specific primers 18S F: 5'-AGG TGA ACC TGC GGA AG-3' and 28S R: 5'-AGG GGG TAG TCA CAC ATC-3' of *An. gambiae* ITS gene (Gentile *et al.*, 2001) with the same ND5 PCR conditions. The amplified PCR products from each reaction were mixed with 2µL loading dye, electrophoresed through a 2% agarose and stained with ethidium bromide to assess for purity and quantity. Amplified fragments were visualized by illumination with ultraviolet light. The PCR products were purified by the Promega kit (Promega Corporation, Madison, WI, USA) subsequent the procedure suggested by the company. After that the products were sent for DNA sequencing to a service provider, (First BASE Laboratories Sdn Bhd, Selangor, Malaysia) for both ND5 and ITS genes.

4.2.3 Data Analysis

4.2.3.1 Nucleotide Alignment

The ND5 and ITS sequences were aligned separately for both genes. Clustal W implemented in MEGA 5.0.5 (Tamura *et al.,* 2011) was used for editing and aligning the sequences. The sequences were then translated into protein on the same programme to detect any occurrence of stop codons which would indicate presence of numts. Collapse 1.2 (Posada, 2004) was used to build a haplotype datasheet and the allocation of the haplotype between all populations was manually summarized from DnaSP program Rozas *et al.* (2003). Within and between population variations were analysed based on these haplotypes.

4.2.3.2 Genetic Diversity

The programme MEGA 5.05 was also utilised to calculate nucleotide variable and parsimony informative sites, number of haplotypes, number of transitions and transversions, synonymous and non-synonymous amino acid substitutions and nucleotide frequencies. Intra and interpopulation genetic divergence of haplotypes based on Kimura 2-parameter genetic distance were also estimated in the same programme. Using Arlequin 3.11 (Excoffier *et al.*, 2005), three estimations of diversity measurement were calculated to describe DNA polymorphism at each sampling site; haplotype gene diversity, nucleotide diversity and theta S. Haplotype gene diversity (Hd) estimates the probability of uniqueness of a haplotype in a given population. Nucleotide diversity (π) measures the mean number of pairwise nucleotide differences among individuals in a population. Theta S (θs) (Watterson, 1975) calculates the number of segregating sites among haplotypes in a population.

4.2.3.3 Population Differentiation and Structuring

F statistics i.e F_{ST} population pairwise comparisons that determine genetic differentiation in and between sites were calculated in Arlequin 3.11 (Excoffier *et al*., 2005) to estimate the level of differences among populations and spatial population structuring. The analysis used Kimura 2-Parameter distance method and statistically significant pairwise comparisons were tested with 1000 permutations procedure. Analysis of molecular variance (AMOVA) was conducted to infer the relative attribution of variance among groups (F_{CT}) (Khartoum, Kassala and Sennar), among populations within groups (F_{SC}) (My, Hj, and Gw –Khartoum; H.sh and Kr –Kassala; and Se –Sennar) and within populations using Arlequin 3.11 (Excoffier *et al*., 2005). Using the DnaSP program, gene flow estimates (Nm) based on both haplotype-based and sequence-based statistics were derived as in Nei (1973) and Hudson *et al.* (1992).

4.2.3.4 Phylogeography and Phylogenetic Studies among the *Anopheles gambiae* complex and among the *An. arabiensis* Populations

The ND5 nucleotide sequences of six members of the *An. gambiae* complex; 1. *An. gambiae* (DQ440687.1, DQ440689.1, DQ440773.1) 2. *An. melas* (JX117756.1, U10123.1, U10129.1, JX117667.1) 3. *An. bwambae* (AF116828.1) 4. *An. quadriannulatus* (U10133.1, U10132.1), 5. *An. merus* (U10130.1, U10131.1) and 6. *An. arabiensis* (hj12, Hj 15, Hj16, from this study). *Anopheles stephensi* (AY842526.1) retrieved from the GenBank database (www.ncbi.nim.nih.gov) were also included in the analysis as the outgroup. For ITS gene, sequences originating from *An. arabiensis* (Burkina_Faso) AF470146.1, *An. arabiensis* (Nigeria) DQ287730.1, *An. arabiensis* (Nigeria) DQ287753.1, *An. arabiensis* (Nigeria) DQ287768.1, *An. arabiensis* (Benin) AF470152.1, *An. arabiensis* (Benin)

AF470154.1, *An. arabiensis* (Benin) AF470153.1, *An. arabiensis* (Madagascar) AF470117.1, *An. arabiensis* (Kenya) AF470182.1, *An. arabiensis* (Gambia) AF470164.1, *An. arabiensis* (Gambia) AF470163.1, *An. arabiensis* (Gambia) AF470168.1 were retrieved from GenBank. Sequence alignment was performed by using MEGA 5.05 software through exclusion of all positional gaps and missing data. The sequences were then trimmed to obtain equal lengths for all the species. As a result, a total of 729 bp of ND5 and 885bp of ITS sequences were used in the final dataset. Evolutionary divergence between all sequences pairs of the *An. gambiae* complex were calculated using the Maximum Composite Likelihood model based on the Kimura 2-parameter model (Kimura, 1980**).** Neighbor-Joining (NJ) (Saitou and Nei, 1987) and Maximum Parsimony (MP) (Eck and Dayhoff, 1966) methods were implemented. The Neighbour joining (NJ), a distance-based and Maximum parsimony (MP), a character-based method were calculated to build the phylogenetic trees with the least evolutionary steps or with the least total branch length. The NJ method was based on the Kimura 2-Parameter (Kimura, 1980) evolutionary distance with confidence level at each node measured by 1000 bootstrap replications (Felsenstein, 1985). The most parsimonious MP tree was generated through the close-neighbour-interchange (CNI) with an initial tree by random addition of sequences at 20 replicates, set at search level of three. To view the most frequent topology, an MP consensus tree with 50% cut-off was generated. On the other hand, the NJ tree was constructed based on F_{ST} values, to depict the phylogenetic relationships among the populations.

4.2.3.5 Demographic History

Estimations of departure from neutral expectation were examined through Tajima's D (Tajima, 1989a) and Fu's Fs (Fu, 1997) statistics for each population

from Sudan using Arlequin 3.11 (Excoffier *et al.,* 2005). These departures could arise as a consequence of historical population range expansion or mutation-drift disequilibrium. Deviation from neutrality due to population bottleneck or expansion, directional selection or introgression is detected by Tajima's D, based on mutation (segregating sites) frequency. The null hypothesis for Tajima's D test is neutral evolution in an equilibrium population, which means no selection bias and the population has not experienced growth or size reduction (Tajima, 1989b). A recent directional selection (selection sweep) or recent population growth with excess of rare alleles would result in a negative value while balancing selection or population sub-structuring or recent bottleneck (Tajima, 1989b) would generate positive value of Tajima's D. Fu's Fs statistics is based on Ewens, sampling distribution (Ewens, 1972) and haplotype distribution. It is very efficient in detecting past population size fluctuation (Ramos-Onsins and Rozas, 2002)**.**

4.2.3.6 Isolation-by-Distance (Mantel Test)

Mantel test (Sokal, 1979) was used to investigate the correlation between geographical and genetic distances among populations using Arlequin 3.11 (Excoffier *et al.,* 2005).

4.2.3.7 Minimum Spanning Network

To conduct the Minimum Spanning Network (MSN), haplotypes were imported to Nexus (PAUP 3.0/MacClade) format. The MSN calculation based on median-joining was used to construct the MSN phylogenetic networks and tree, infer ancestral and potential types, evolutionary branching and variants in Network version 4.6.1.0 Software (Tobias & Siavash, 2012).

4.3 Results

4.3.1 NADH Dehydrogenase Subunit 5 (ND5) Gene Analysis

All 103 individuals of *An. arabiensis* from the six sampling areas were successfully sequenced for the ND5 gene producing a 981bp fragment. A final segment of 823 bp was obtained after alignment and editing of ambiguous sequences. These 103 sequences of 823 bp length revealed 16 segregating sites with the generation of 19 haplotypes (Table 4.2). Sample sizes ranged from 16 to 20 with an average of 17 individuals per population. As observed in the table, there appears two sets of major haplotypic groups; 1. Hap1- Hap12 and 2.Hap13-Hap19 with some slight derivation. Hap9 of Kr seems to be the link between the two groups.

4.3.1.1 Nucleotide Composition

The 823 bp segment of 103 sequences of the partial ND5 gene from six localities revealed 16 segregating sites defining 19 haplotypes,with mean haplotype diversity, Hd: 0.80, mean number of variable sites: 7 and nucleotide diversity: 0.034. The average nucleotide composition was C: 10.26%, T: 44.18%, A: 30.98% and G: 14.58%. All unique sequences have been deposited in GenBank with accession number KC610567-KC610669. There were 8 nonsynonymous substitutions out of 19 haplotypes. The nonsynonymous substitutions were: G ⟷ A at positions 162, 225, 288, 613 and T ⟷ C at positions 21, 24, 36, 45 (Table 4.2). Three substitutions result in amino acid changes namely at positions 117 and 7 (Ala replaced by Pro) in Hap02 and Hap 05 respectively and at position 205 (Serine replaced by Glycine). Different rates of transitional and transversional substitutions were observed **(**Table 4.3).

Table 4.2: Haplotype frequency and nucleotide polymorphic sites encoded by 19 haplotypes in *An. arabiensis* generated by partial mtDNA ND5 gene.

	Nucleotide variable position															
							1	2	2	2	3	3	4	5	6	6
	1	2	2	3	4	4	6	1	2	8	4	9	0	4	0	1
	9	1	4	6	2	5	2	6	5	8	0	3	8	9	3	3
hap01	G	T	T	T	C	T	G	T	A	A	G	T	C	A	C	A
hap02	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
hap03	.	C	.	.	.	.	.	.	.	.	.	.	T	.	T	.
hap04	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
hap05	C	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
hap06	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
hap07	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
hap08	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
hap09	.	.	.	.	.	.	.	A	.	G	.	C	T	.	.	.
hap10	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
hap11	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.
hap12	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	.
hap13	.	C	.	.	.	.	A	.	.	G	.	.	T	.	.	.
hap14	.	C	C	.	.	C	A	.	.	G	.	.	T	.	.	.
hap15	.	C	.	.	.	.	A	.	.	G	.	.	T	.	T	.
hap16	.	C	.	.	G	.	A	.	.	G	.	.	T	.	T	.
hap17	.	C	.	.	.	.	A	.	.	G	.	.	T	T	.	.
hap18	.	C	.	.	.	.	A	.	G	G	.	.	T	.	.	.
hap19	.	C	.	.	.	.	A	.	.	G	.	.	T	.	T	G

Table 4.3: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	**T**	**C**	**G**
A	-	*1.88*	*0.44*	**10.81**
T	*1.32*	-	**10.91**	*0.62*
C	*1.32*	**46.82**	-	*0.62*
G	**22.95**	*1.88*	*0.44*	-

Each entry shows the probability of substitution (r) from one base (row) to another base (column). Rates of different transitional substitutions are shown in **bold** and those of transversional substitutions are shown in *italics*.

4.3.1.2 Haplotype Distribution

Haplotype distribution varied throughout the regions studied and showed that 68.42% were population specific, 5.26% regional specific while 26.32 % of the totals were shared in more than one region respectively. Of the 19 haplotypes, 13 were population specific (private alleles) namely Hap02, Hap03, Hap04, Hap05, Hap07, Hap09, Hap10, Hap12, Hap14, Hap16, Hap17, Hap18 and Hap19 (Table 4.4). For example My had private alleles Hap02 and Hap14. The six non private haplotypes were Hap01, Hap06, Hap08, Hap11, Hap13 and Hap15. Hap01 was the most common (25.42%), observed in all populations and regions except in the Kassala populations. Hap06, Hap08, Hap13 and Hap15 were found in all regions but not represented in every population. However, Hap11 was only restricted to the two Kassala populations.

Table 4.4: Haplotype distribution across six populations of *An. arabiensis* and ratio of observed transition versus transversion substitution in each population inferred from partial mtDNA ND5 gene.

Region	Khartoum			Kassala		Sennar		
Pop / Hap	My	Hj	Gw	Kr	H.sh	Se	Total	Hap freq
hap01	7	10	2			7	26	0.252
hap02	2						2	0.019
hap03					2		2	0.019
hap04					2		2	0.019
hap05				2			2	0.019
hap06			2	2		2	6	0.058
hap07			2				2	0.019
hap08			2		2	2	6	0.058
hap09				4			4	0.039
hap10			4				4	0.039
hap11				3	2		5	0.049
hap12		2					2	0.019
hap13	4			1	2	4	11	0.107
hap14	2						2	0.019
hap15	2	8		3	5	1	19	0.184
hap16					2		2	0.019
hap17				2			2	0.019
hap18			2				2	0.019
hap19			2				2	0.019
Total	17	20	16	17	17	16	103	
TS:TV	7:1	5:0	8:1	6:4	4:1	5:1	5.8:1.3	

Populations: My = Mygoma, hj = AlhajYousif, Gw = Algerif West, Kr = Krmota, H.sh =AlHalang Shemal, Se = Abu Algonia (Sennar).

4.3.1.3 Genetic Diversity

The number of variable sites ranged from 5-9, and number of haplotype ranged 3-7 per population. Overall, high levels of haplotype diversity but low nucleotide diversity was observed. Haplotype diversity (Hd) ranged from 0.611 in Hj to 0.9 in Gw, while nucleotide diversity (π) from 0.003 in H.sh to 0.004 in Kr (Table 4.5).

4.3.1.4 Demographic History

As shown in Table 4.5 all populations showed positive but insignificant value for Tajima's D neutrality tests from neutral expectation, except for Hj which indicates balancing selection or population substructuring or recent bottleneck for this population (Tajima, 1989). Gw and H.sh population showed negative but nonsignificant values for Fu'sFs while other populations showed positive but were nonsignificant except for Hj which showed positive significance.

Table 4.5: Genetic diversity for ND5 sequences in *An. arabiensis* showing sample size (N), number of variable sites (#V), number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π), theta (S) and neutrality tests of Tajima's D and Fu'sFs for each population and each group.

Genetic diversity							**Neutrality test**	
Pop	N	#V	H	Hd	π	Theta (S)	Tajima's D	Fu'sFs
My	17	8	5	0.78	0.004	2.37	0.947 P > 0.10	1.563 P >0.10
Gw	16	9	7	0.90	0.004	2.71	0.344 P> 0.10	-0.559 P>0.01
Hj	20	5	3	0.61	0.003	1.41	2.516 P < 0.01	4.186 P <0.02
Se	16	6	5	0.76	0.03	1.81	0.978 P > 0.10	0.709 P>0.01
H.sh	17	5	7	0.88	0.003	1.48	1.586 P > 0.10	-1.271 P >0.05
Kr	17	9	7	0.89	0.004	2.67	1.035 P > 0.10	0.08 P >0.10
average	17.2	7	5.7	0.80	0.003			

Populations: My = Mygoma, Hj = AlhajYousif, Gw = Algerif West, Kr = Krmota, H.sh = AlHalang Shemal, Se = Abu Algonia

4.3.1.5 Population Differentiation and Structuring

F_{ST} pairwise differences ranged from -0.02 to 0.20. All F_{ST} values were significant except between Kr and H.sh, My and Hj and My and Se. According to Wright's values $F_{ST} = 0$ indicates no genetic divergence, $F_{ST} = 0 – 0.05$ indicates low genetic differentiation, $F_{ST} = 0.05 – 0.15$ indicates moderate difference and $F_{ST} = 0.15 – 0.25$ indicates high genetic differentiation. As shown in Table 4.6. No genetic divergence was found between My and Se. Low genetic differentiation was observed between Kr and H.sh. Moderate values were observed between My and Hj; Gw and Se; and Gw and Kr. High genetic differentiation were observed between pairwise Kr and Hj, H.sh and Hj and unexpectedly also between Gw and Hj which are geographically closely related populations. Thus, overall the F_{ST} analysis generated two groups; 1. Kr, H. Sh, Gw and 2. Se, My and Hj.

Table 4.6: Population differentiation of *An. arabiensis* using distance method F_{ST} pairwise difference

	Kr	My	Se	Hj	Gw	H.sh
Kr	*	431.75	334.01	427.72	443.21	12.88
My	0.14	*	292.07	3.93	15.55	416.55
Se	0.14	- 0.02	*	311.55	323.10	429.31
Hj	0.20	0.07	0.10	*	5.38	448.59
Gw	0.09	0.12	0.09	0.20	*	426.5
H.sh	0.04	0.11	0.13	0.16	0.10	*

F_{ST} estimated using pairwise distance shown below diagonal
Geographical distance shown above diagonal.

To determine the hierarchical genetic apportion for *An. arabiensis* in Sudan, an AMOVA approach was applied. The AMOVA results showed a high proportion of total variation within population (88.06%), but moderate proportions of variation was attributed to difference among populations within groups (9.75%) and low among groups within total (2.19%) – Table 4.7. However, all analyses are significant.

Table 4.7: Results of AMOVA *An. arabiensis* populations ND5 gene**.**

Source of Variation	d.f.	Variance Components	Percentage of Variation	Fixation Indices	*P value*
Among groups	2	0.010	2.19	F_{CT}: 0.10	0.00
Among populations within groups	3	0.044	9.75	F_{SC}: 0.119	0.00
Within populations	97	0.398	88.06	0.022	0.018

Genetic differentiation, genetic distance and gene flow were estimated among all *An. arabiensis* populations from Sudan (Table 4.8). Gene flow ranged from 1.63 to infinity. High gene flow was found between My and Hj; My and Gw which are geographically close populations and surprisingly between My and Se; and Gw and Se which are distantly positioned. As expected the lowest gene flow was found between Gw and H.sh; and Se and H.sh, which are distant populations. However, unexpected low gene flow was also observed between Kr and H.sh which are at near geographical distance (Table 4.8). The genetic distance between populations ranged from 0 to 0.307, the genetic distance between Gw and Se; and My and Se = zero while it was high between Gw and H.sh.

Table 4.8: Gene flow (Nm) and genetic distance between populations

	Kr	H.sh	My	Hj	Gw	Se
Kr	0	0.096	0.074	0.109	0.106	0.084
H.sh	**5.21**	0	0.171	0.132	0.307	0.307
My	**6.79**	**2.92**	0	0.019	0.018	0.00
Hj	**4.57**	**3.79**	**26.07**	0	0.058	0.036
Gw	**4.72**	**1.63**	**27.96**	**8.57**	0	0.00
Se	**5.94**	**1.63**	**inf**	**13.79**	**inf**	0

Nm (bold) shown below diagonal
Genetic distance shown above diagonal.

4.3.1.6 Isolation-by-Distance (Mantel Test)

The Mantel test calculated based on the mean pairwise genetic distance versus geographical distance showed low and nonsignificant correlation (R^2 = 0.229, P >0.05) between the two parameters. The scatter plot of the Mantel test is illustrated in Figure 4.1.

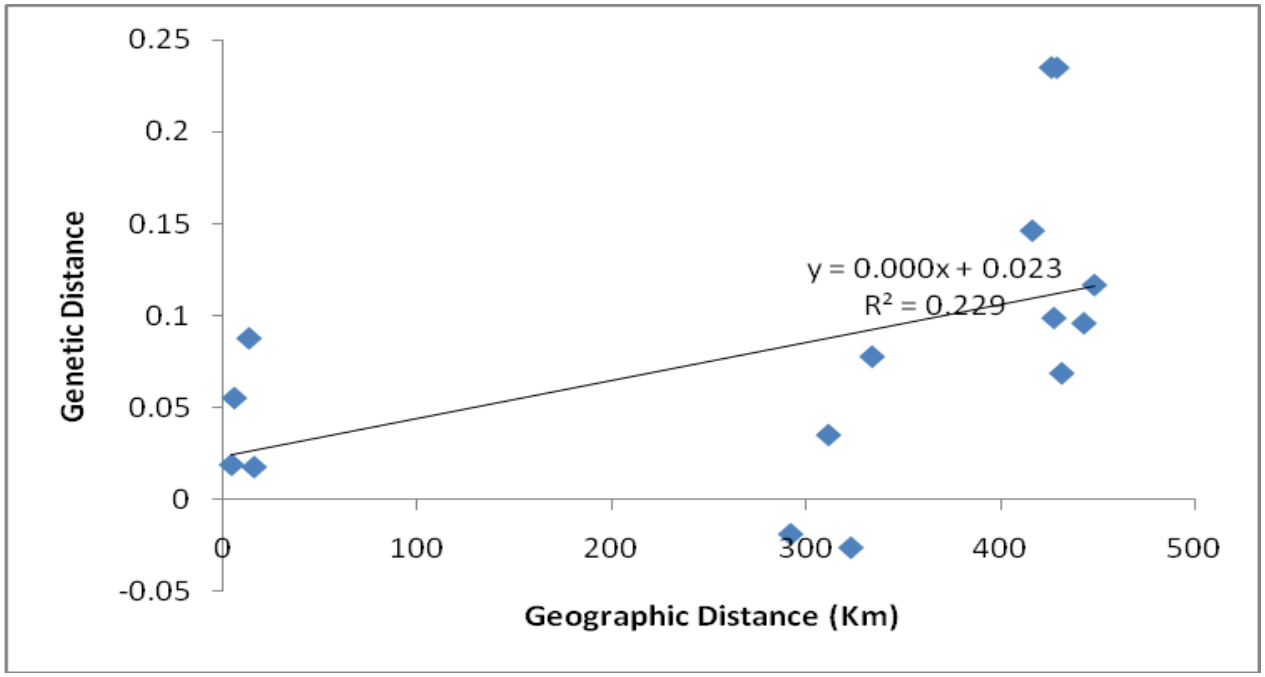


Figure 4.1: Scatter plot showing the relationship between geographic distance and genetic distance

4.3.1.7 Phylogeography and Phylogenetic Relationships Among Sudanese Haplotypes

MP tree divided the populations into two major clades (Figure 4.2). The first clade was moderately supported at 80% while the second clade highly supported at 100%. Several population specific haplotypes were observed namely Hap 02 and Hap 14 (My); Hap 07, Hap10, Hap18 and Hap19 (Gw); Hap12 (Hj); Hap3, Hap4 and Hap16 (H.sh); Hap5, Hap 9 and Hap17 (Kr). However, there was considerable overlapping among haplotypes of populations even those involving Kr and Se which were generally the most distant populations based on other analyses.

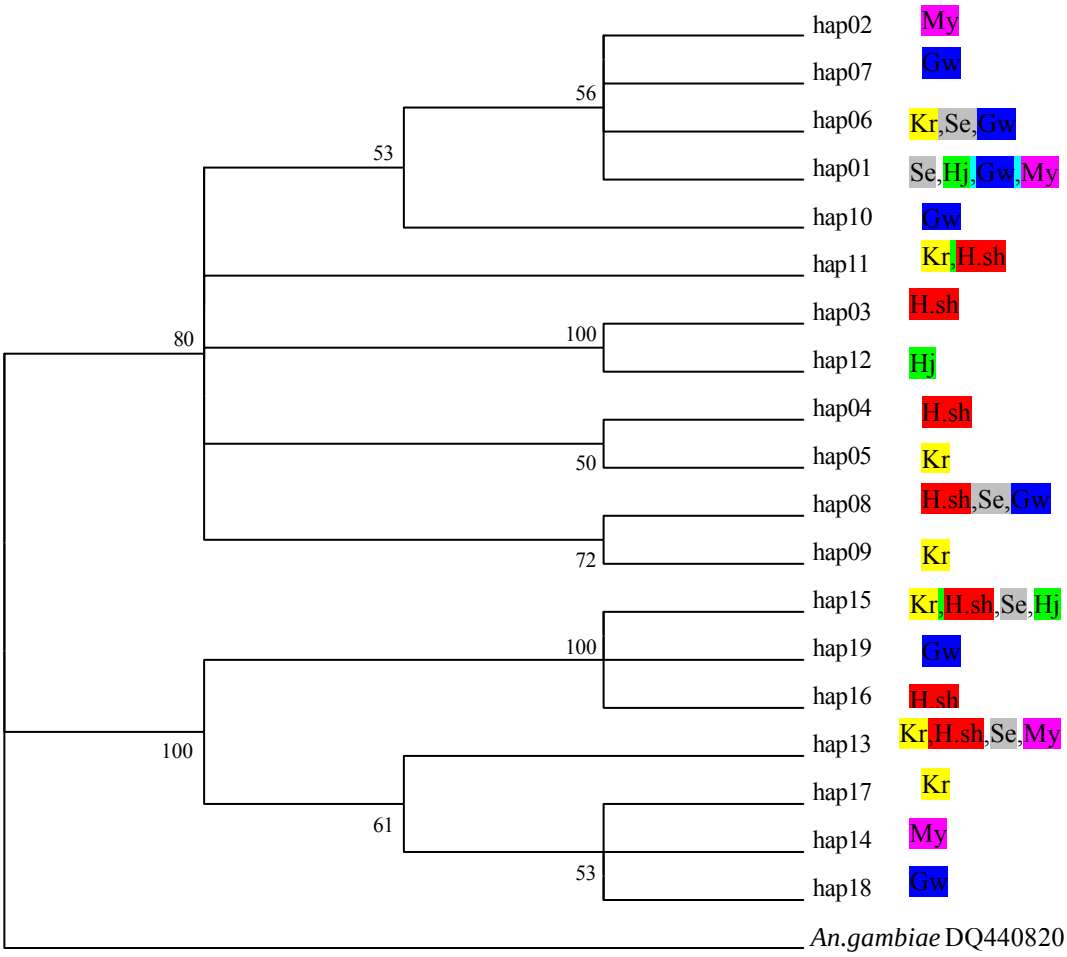


Figure 4.2: The Maximum Parsimony tree of *An. arabiensis* haplotypes inferred from ND5 gene. Bootstrap value denoted at each branch (value < 50% is not shown). Populations: My = Mygoma, Hj = AlhajYousif, Gw = Algerif West, Kr = Krmota, H.sh = AlHalang Shemal, Se = Abu Algonia.

Neighbor-Joining tree based on F_{ST} values consisted of two clades; Clade I includes Kr, H.sh and Gw, while Clade II includes Hj, My and Se (Figure 4.3).

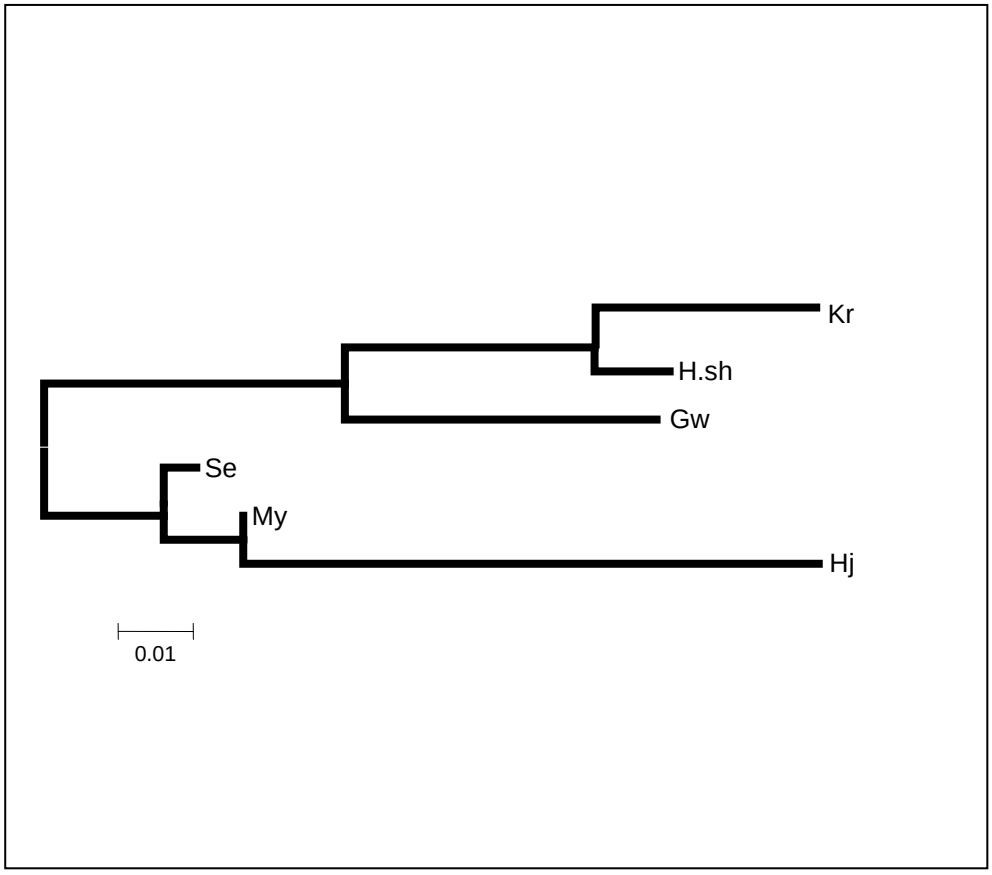


Figure 4.3: Phylogenetic Neighbour-joining tree of Sudanese populations based on pairwise F_{ST}

4.3.1.8 Phylogenetic Relationships Among Sudanese and Selected African GenBank ND5 Haplotypes

The analysis showed unresolved clades. Only two branches on the MP tree are well supported at 100%. The NJ tree was similar but not presented here. However, there was a great deal of admixture among the populations from Ethiopia, Malawi and Sudanese populations from the present study. Thus, no population structuring was observed even on a large scale. For instance, several haplotypes from Ethiopia and Malawi clustered with haplotypes from Kr namely Hap5 and Hap9 (the most genetically distant Sudanese population) while others from the same region (countries) cluster with haplotypes from other Sudanese regions (Figure 4.4).

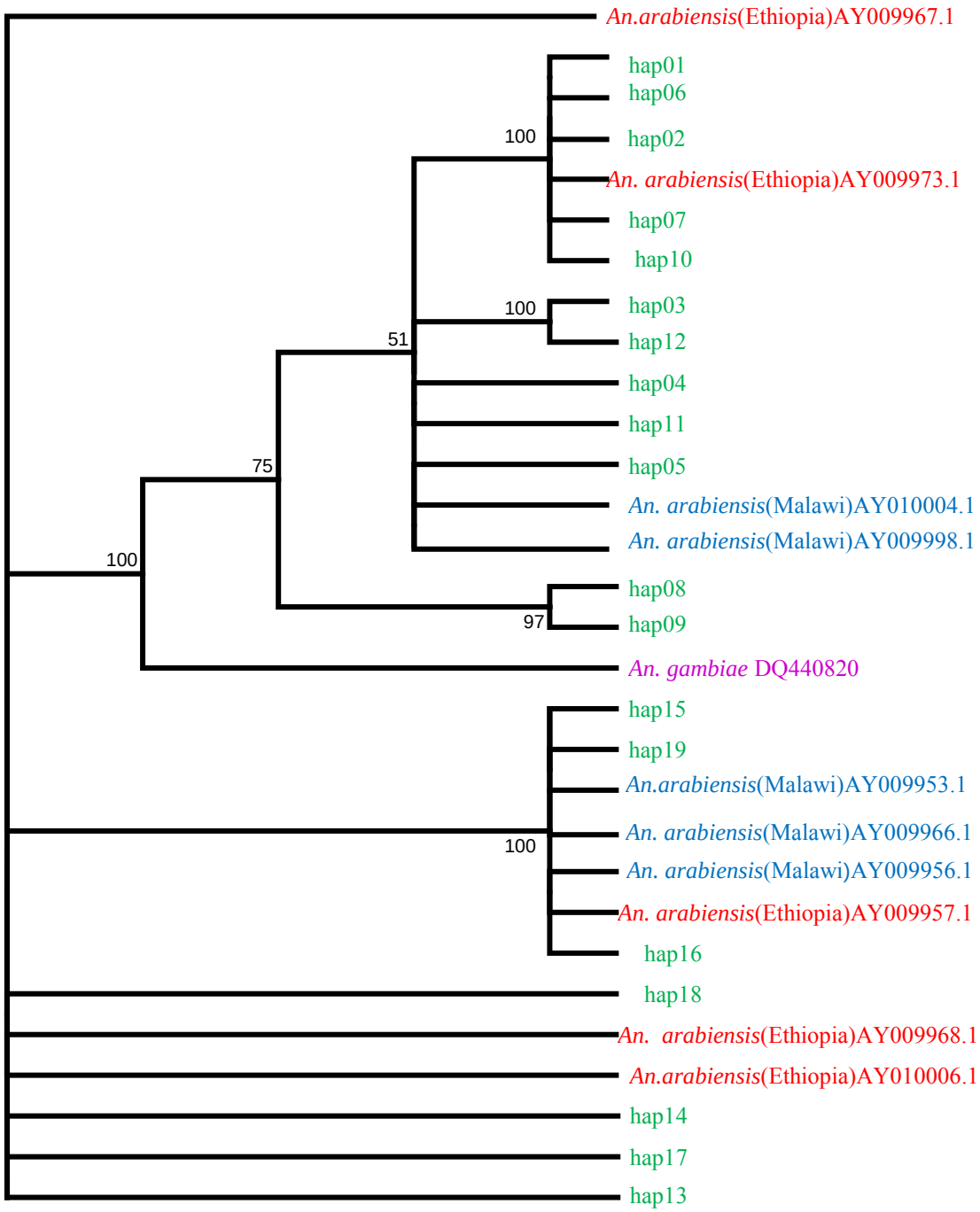


Figure 4.4: Phylogenetic MP tree of *An. arabiensis* among Sudanese and selected African ND5 haplotypes

4.3.1.9 Phylogenetic Relationships Among *Anopheles gambiae* complex (GenBank) and Sudanese Samples.

Neighbour Joining and Maximum Likelihood methods generated similar trees for phylogenetic relationships among selected anopheline mosquitoes (Figure 4.5). The estimates of evolutionary divergence between sequences of the *An. arabiensis* and other members of the complex are presented in Table 4.9. The evolutionary divergence is lowest between *An. arabiensis* and *An. gambiae* (0.005), followed by *An. bwambae* and *An. quadriannulatus* (0.012) ,*An. merus* and *An. quadriannulatus* (0.017) and *An. merus* and *An. bwambae* (0.019). The divergences between other pairwise species comparisons were much greater (0.376-0.744).

Table 4.9: Estimates of Evolutionary Divergence among members of *An. gambiae* complex

Species	1	2	3	4	5	6
An. arabiensis hj16						
An. gambiae DQ440687	0.005					
An. melas JX117756.1	0.521	0.534				
An. bwambae AF116828.1	0.376	0.388	0.709			
*An.quadriannulatus*U10133.1	0.393	0.405	0.717	0.012		
An. merus U10130.1	0.393	0.405	0.744	0.017	0.019	

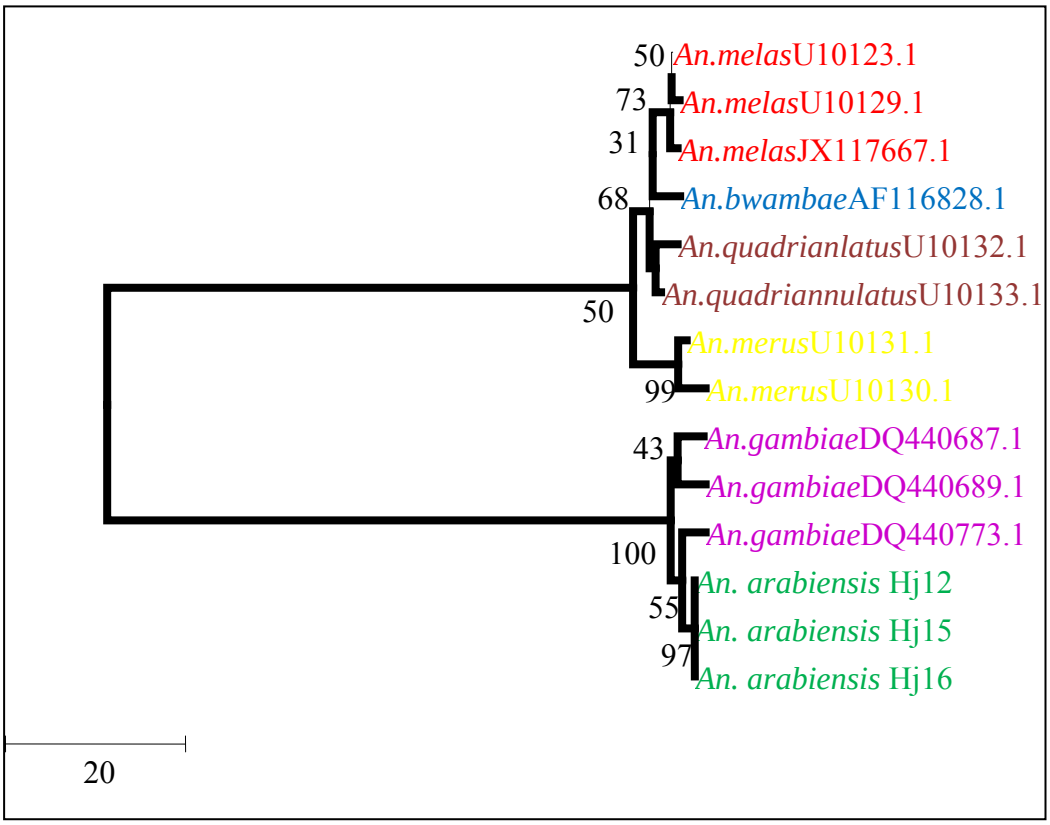


Figure 4.5: Phylogenetic tree of *An. gambiae* complex based on present study and Genbank sequences.

4.3.1.10 Minimum Spanning Network of ND5 Gene

The minimum spanning network (MSN) depicts haplotype geneology (Figure 4.6). The size of the circle parallels the frequencies of haplotypes. The area of coloured region represents the contribution of each population for a particular haplotype. Median vectors (mv) are shown in red diamond dots which represent sample that should be present but could not be detected during sampling. Numbers between two haplotypes represent the mutation sites. The minimum spanning network of *An. arabiensis* revealed two highly divergent clades with one common central haplotype each, regarded as the ancestral haplotype. The central haplotypes Hap01 and Hap13 represent the inferred ancestral haplotypes for clade 1 and clade 2 respectively. Hap01, Hap 13 and Hap 15 are the dominant haplotypes in populations Se, My, Kr, and H.sh with high frequency in Hj population from which lower frequency haplotypes radiated. All populations are represented in both major clades with varying frequencies.

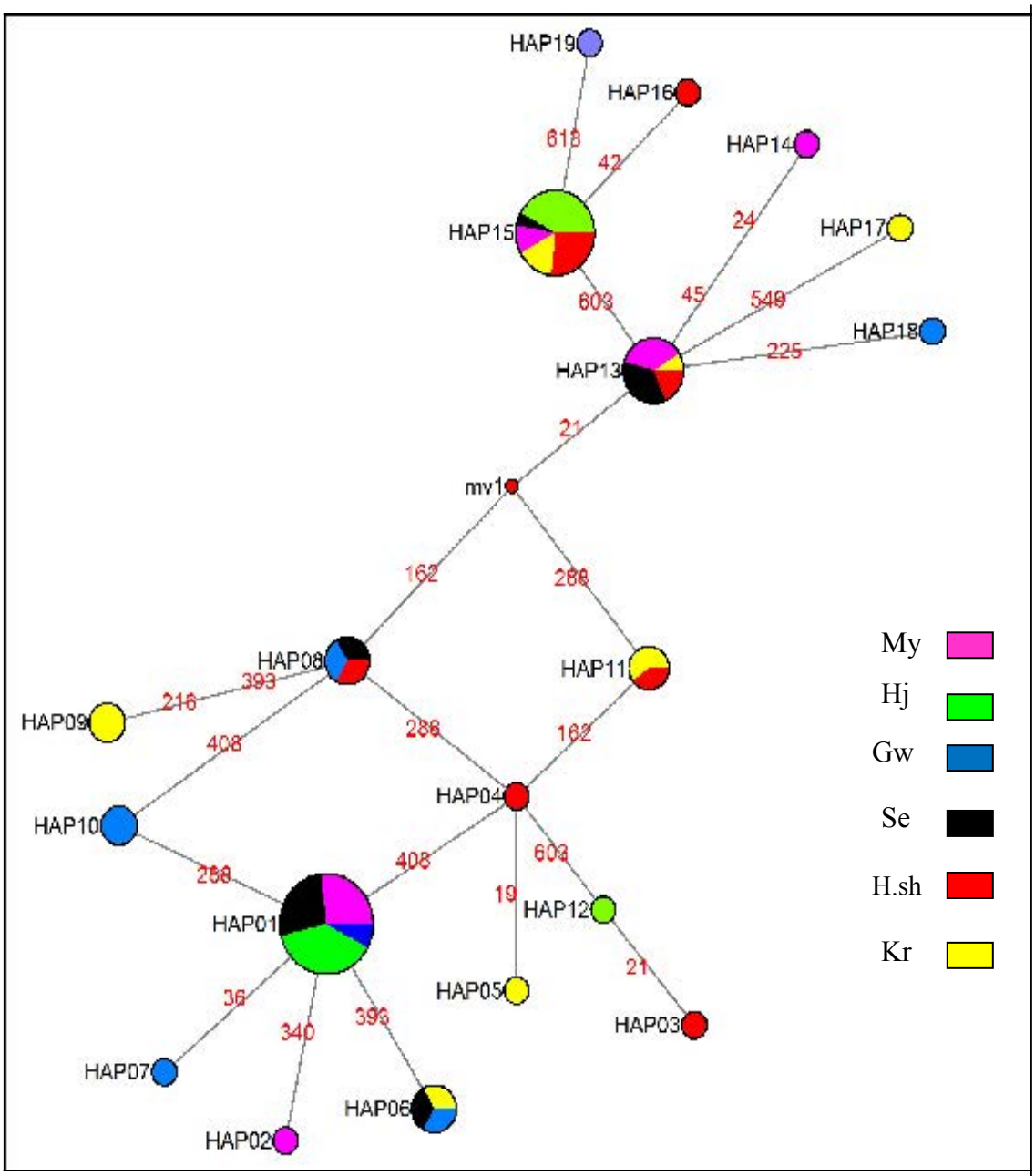


Figure 4.6: Minimum spanning network diagram inferred from partial mtDNA ND5 gene

4.3.2 Internal Transcribed Spacer (ITS) Gene Analysis

All 92 individuals of *An. arabiensis* from the six sampling areas were successfully sequenced for the ITS gene producing a 989 bp fragment. A final segment of 939 bp was obtained after alignment and editing of ambiguous sequences. Sample size varied from14 in H.sh to 18 individuals in Se population.

4.3.2.1 Nucleotide Composition

Final alignment of sequences revealed a total of 26 segregating sites, haplotypes numbers of h = 20, mean haplotype diversity, Hd: 0.37, mean number of nucleotide differences: 0.71and nucleotide diversity: 0.0076. The average nucleotide composition was C 27.05%, T 23.32%, A 25.56% and G 24.07%. There were 16 nonsynonymous substitutions leading to amino acid changes (Table 4.10). Nonsynonymous : G ↔ A at position 65, 133, 843, T ↔C at position 787 and 893 G ↔T at position 55, 83, 127, 851, 868, 872, 877, and C ↔A at position 87, 104, 107, 541. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics as in (Table 4.11).

Table 4.10: Haplotype frequency and nucleotide polymorphic sites encoded by 20 haplotypes in *An. arabiensis* generated by ITS gene.

	Nucleotide variable position																									
					1	1	1	1	1	1	5	7	8	8	8	8	8	8	8	8	8	8	8	8	9	9
	5	6	8	8	0	0	1	2	2	3	4	8	3	3	4	4	5	5	6	6	7	7	8	9	1	2
	5	5	3	7	4	7	4	7	8	3	1	7	5	7	0	3	1	9	5	8	2	7	0	3	6	4
Hap1	G	G	G	A	C	C	G	C	T	G	C	C	C	G	G	G	G	G	A	G	G	G	G	C	T	A
Hap2	.	.	T	C	.	.	.	T	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
Hap3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
Hap4	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
Hap5	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap6	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
Hap7	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap8	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap9	.	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
Hap11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
Hap12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
Hap13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
Hap14	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.	.	T
Hap15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.
Hap16	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap17	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap18	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	.	.	.
Hap19	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	T	.	.

Table 4.11: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	**T**	**C**	**G**
A	-	*7.8*	*9.04*	**8.82**
T	*8.54*	-	**8.05**	*8*
C	*8.54*	**6.95**	-	*8*
G	**9.42**	*7.8*	*9.04*	-

Each entry shows the probability of substitution (r) from one base (row) to another base (column).Rates of different transitional substitutions are shown in **bold** and those of transversional substitutions are shown in *italics*.

4.3.2.2 Haplotype Distribution

The common haplotype, namely Hap01 was observed in all populations and contributed 79.34% of the total haplotypes (Table 4.12). The other haplotypes were all private haplotypes and population specific. Thus, Hap02, Hap06-07, Hap16, Hap18 were only found in Hj; Hap03-05 and Hap08 only detected in My; Hap09-14, Hap17, Hap20 were population specific confined to Sennar population; Hap15 was particular to H.sh, while Hap19 was found mainly in Gw. Most of the haplotypes only differed by one or two nucleotides from the reference Hap01 unlike in ND5 where there were two main groups of haplotypes.

Table 4.12: Haplotype distribution across six populations of *An. arabiensis* and ratio of observed transition versus transversion substitution in each population inferred from partial rDNA ITS gene.

Hap	Hj	Gw	My	Se	Kr	H.sh	Total	Hap freq
hap01	10	14	11	10	15	13	73	0.7934
hap02	1						1	0.0109
hap03			1				1	0.0109
hap04			1				1	0.0109
hap05			1				1	0.0109
hap06	1						1	0.0109
hap07	1						1	0.0109
hap08			1				1	0.0109
hap09				1			1	0.0109
hap10				1			1	0.0109
hap11				1			1	0.0109
hap12				1			1	0.0109
hap13				1			1	0.0109
hap14				1			1	0.0109
hap15						1	1	0.0109
hap16	1						1	0.0109
hap17				1			1	0.0109
hap18	1						1	0.0109
hap19		1					1	0.0109
hap20				1			1	0.0109
Total	15	15	15	18	15	14	92	
TS:TV	2:6	1:2	2:2	4:10	0	1:1		

Populations: My = Mygoma, hj = AlhajYousif, Gw = Algerif West, Kr = Krmota, H.sh =AlHalang Shemal, S = Abu Algonia

4.3.2.3 Genetic Diversity

The number of variable sites ranged from zero to 14, and haplotype number ranged 1-9 per population. The genetic diversity revealed generally low levels of haplotype diversity except for Se, Hj and My but low nucleotide diversity. Haplotype diversity (Hd) ranged from 0.000 in Kr to 0.706 in Se, while nucleotide diversity (π) from 0.000 in Kr to 0.002 in Se (Table 4.13).

4.3.2.4 Demographic History

Hj and Se populations showed negative and significant value for Tajima's D neutrality test indicating recent directional selection (selection sweep) or recent population growth with excess of rare alleles. My, Gw, H.sh and Kr were negative but insignificant. Hj, Se, My and Gw populations showed negative and significant values for Fu'sFs while H.sh population showed insignificant value.

Table 4.13: Genetic diversity for ITS sequences in *An. arabiensis* showing sample size (N), number of variable sites (#V), number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π), theta (S)and neutrality tests of Tajima's D and Fu'sFs for each population

Genetic diversity							**Neutrality test**	
Pop	N	#V	H	Hd	π	Theta(S)	Tajima's D	Fu'sFs
My	15	3	5	0.476	0.0006	0.923	-1.286 P > 0.10	-2.525 P < 0.05
Gw	15	3	2	0.133	0.0004	0.923	-1.685 P > 0.05	-2.581 P < 0.05
Hj	15	8	6	0.571	0.001	2.460	-2.086 P < 0.05	-2.610 P < 0.02
Se	18	14	9	0.706	0.002	4.070	-2.144 P < 0.01	-4.287 P < 0.05
H.sh	14	2	2	0.143	0.0003	0.629	-1.480 P > 0.10	-1.475 P > 0.10
Kr	15	0	1	0.000	0.000	0.000	0.000 NA	0.000 P=N.A

Populations: My = Mygoma, Hj = AlhajYousif, Gw = Algerif West, Kr = Krmota, H.sh = Al Halang Shemal, Se = Abu Algonia

4.3.2.5 Population Differentiation and Structuring

The F_{ST} pairwise difference ranged from - 8.2 x $10^{-4}$to 1.786 x 10^{-2} indicating no genetic differentiation among the six populations. The AMOVA results (Table 4.14) showed a high proportion of significant variation within populations (93.82%), but none or low insignificant variation attributed to differences among populations within groups (Hj, My and GW (Khartoum); Kr and H.sh (Kassala) and Se (Sennar) (0.70%) and among groups - Khartoum, Sennar and Kassala groups (5.48%).

Table 4.14: AMOVA results of *An. arabiensis* populations based on ITS gene**.**

Source of Variation	d.f.	Variance Components	Percentage of Variation	Fixation Indices	*P value*
Among groups	2	0.011	5.48	F_{CT}:0.055	0.156
Among populations Within groups	3	0.01	0.70	F_{SC} : 0.07	0.155
Within populations	86	0.187	93.82	0.062	0.015

4.3.2.6 Phylogeography and Phylogenetic Relationships Among Sudanese and Selected African GenBank ITS Sequences

Haplotypes from Sudan (this study) and GenBank sequences from Nigeria, Benin, Madagascar, Burkina, Gambia and Kenya were analysed. The estimates of evolutionary divergence between sequences of the *An. arabiensis* ranged between 0 to 5 as presented in Table 4.15. Both MP and NJ (results not shown) trees showed homogeneity of 99% for the Sudan and most of the GenBank sequences of African populations as shown in Figure 4.7. However, the Gambian and Kenyan haplotypes clustered in a different highly supported clade.

Table 4.15: Internal Transcribed Spacer (ITS) nucleotides diversity among *An. arabiensis* populations from Sudan.

	S5	S7	S17	My7	My11	My15	Gw7	Gw11	Gw15	Kr7	Kr11	Kr15	Sh7	Sh9	Sh14	Hj7	Hj11	Hj15
S5																		
S7	4.0																	
S17	3.0	1.0																
My7	4.0	2.0	1.0															
My11	3.0	1.0	0.0	1.0														
My15	4.0	2.0	1.0	2.0	1.0													
Gw7	3.0	1.0	0.0	1.0	0.0	1.0												
Gw11	3.0	1.0	0.0	1.0	0.0	1.0	0.0											
Gw15	3.0	1.0	0.0	1.0	0.0	1.0	0.0	0.0										
Kr7	3.0	1.0	0.0	1.0	0.0	1.0	0.0	0.0	0.0									
Kr11	3.0	1.0	0.0	1.0	0.0	1.0	0.0	0.0	0.0	0.0								
Kr15	3.0	1.0	0.0	1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0							
Sh7	3.0	1.0	0.0	1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0						
Sh9	3.0	1.0	0.0	1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0					
Sh14	5.0	3.0	2.0	3.0	2.0	3.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0				
Hj7	4.0	2.0	1.0	2.0	1.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.0			
Hj11	3.0	1.0	0.0	1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0		
Hj15	5.0	3.0	2.0	3.0	2.0	3.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	3.0	

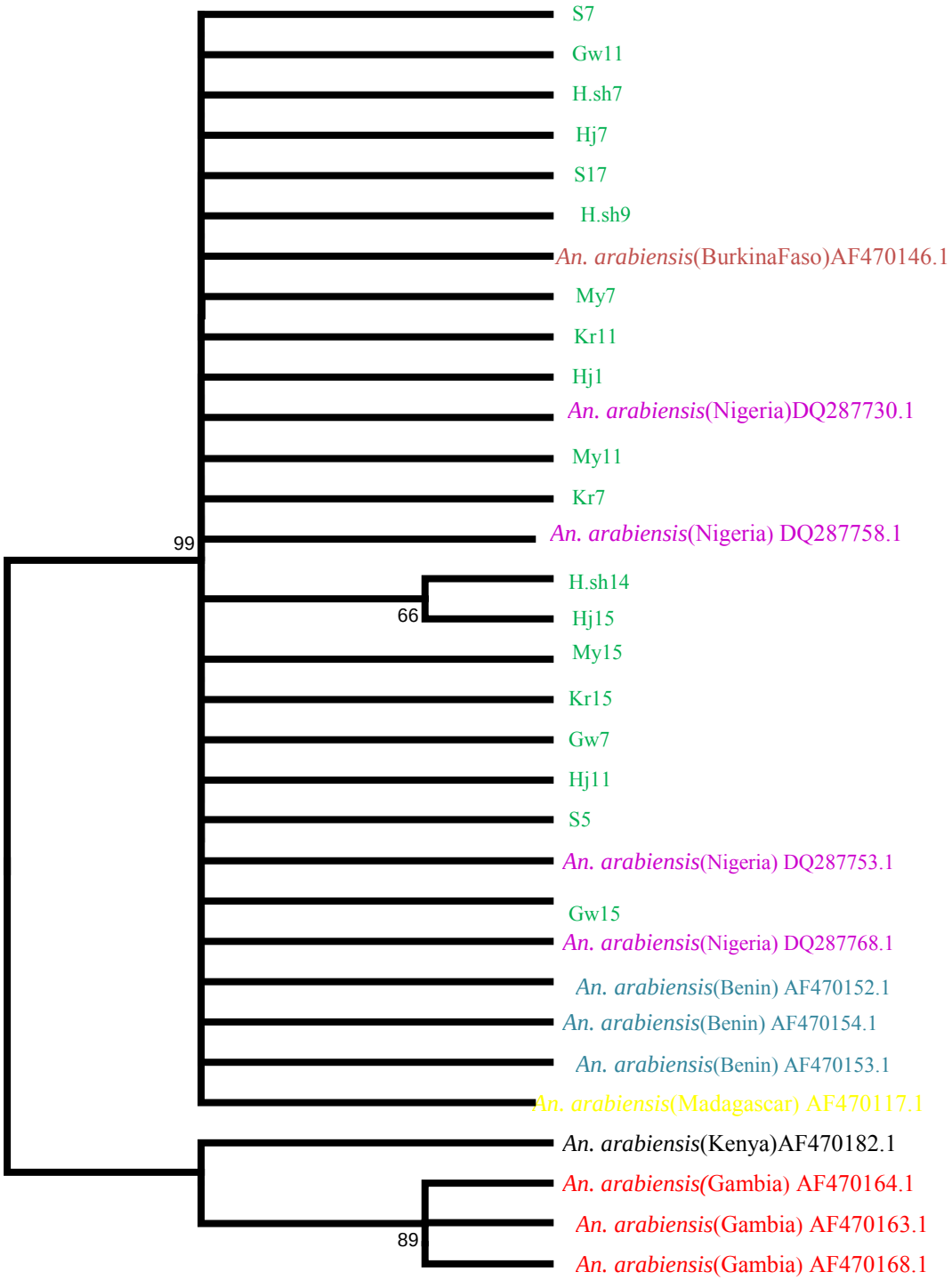


Figure 4.7: Phylogenetic MP tree of *An. arabiensis* among Sudanese and selected African ITS haplotypes

5.3.2.7 Minimum Spanning Network Based on ITS Gene

The results of haplotype geneology are displayed in the minimum spanning network (MSN) (Figure 4.8). The MSN of *An. arabiensis* revealed one main presumably, ancestral haplotype, Hap01 from which lower frequency haplotypes radiated unlike in the ND5 analysis where two main clades were formed.

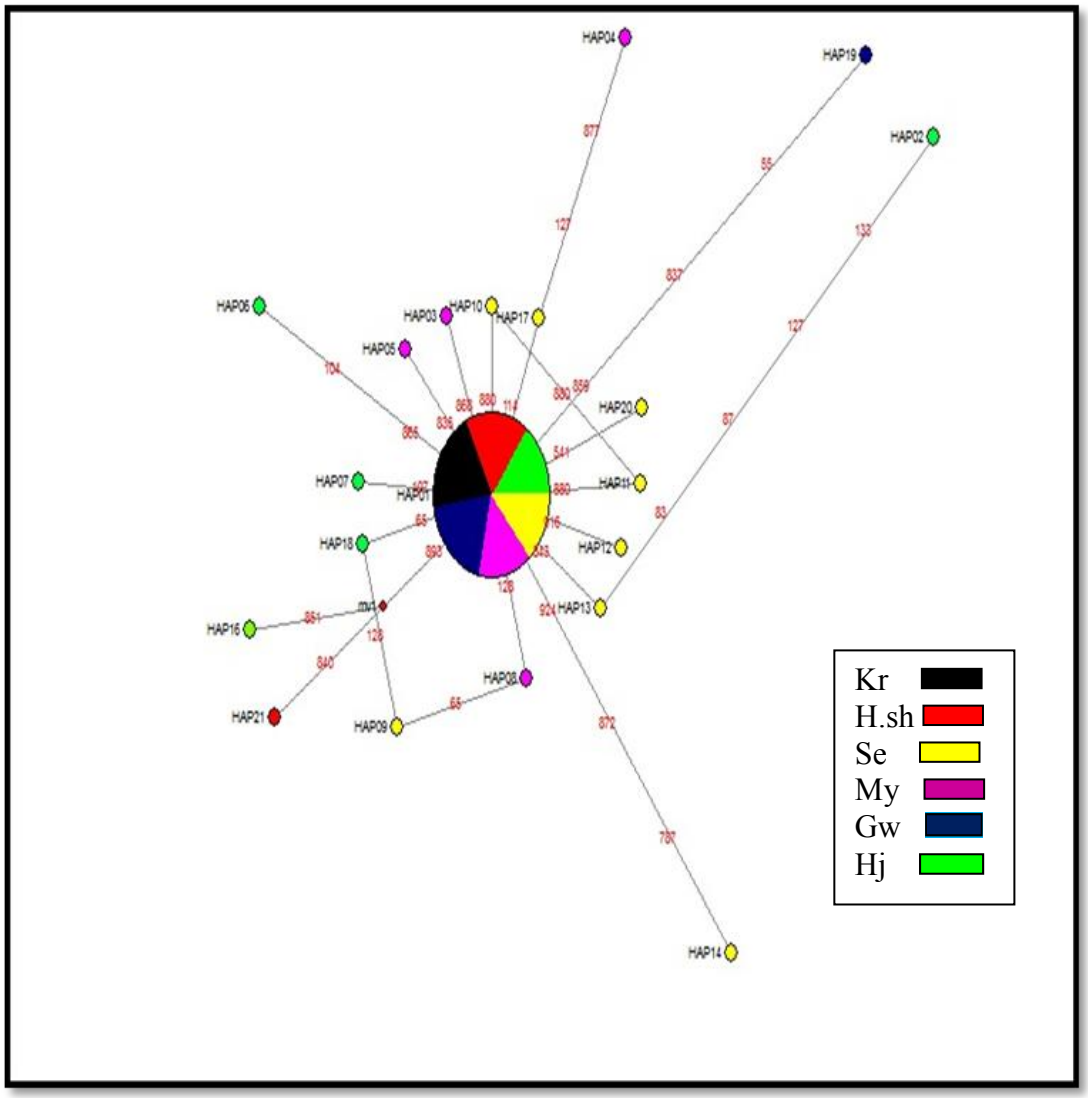


Figure 4.8: Minimum spanning network diagram inferred from partial ITS gene. Diagram in large circle pattern represents haplotypes from all populations, vertical line with small circle pattern are haplotypes from one region. mv =median vector. Numbers are nucleotide mutation sites joining two haplotypes.

4.4 Discussion

A good knowledge of the molecular evolution and phylogenetics of the *An. gambiae* complex will assist in the understanding of its origin, evolution, classification and epidemiology (Foley *et al*., 1998; Thelwell *et al*., 2000). This is essential in strategising the control of malaria (Besansky *et al*., 2003). Knowledge of species identification, distribution, whether vector or not, their roles in the malaria transmission and insecticide resistance gene spreading through populations are important in vector control.

4.4.1 NADH Dehydrogenase Subunit 5 (ND5) Gene Analysis

4.4.1.1 Nucleotide composition

The nucleotide diversity in ND5 gene was high ranging from 0.003 to 0.004 with a mean of 0.0034, similar to Besansky *et al*.(1997) who estimated nucleotide diversity of 0.0038 and 0.0046 in *An. gambiae* and *An. arabiensis* respectively in their study conducted in Kenya; Senegal; and South Africa. Solorzano *et al.* (2010) who studied genetic diversity of *Ae. vexans* in New Orleans using ND5 gene reported that the haplotype diversity ranged from 0.887 to 0.975 in agreement with the current study which revealed haplotype diversity ranging from 0.61 to 0.9. This high haplotype diversity indicates that the species is native, or has been established for a long period of time in their habitat (Hartl and Clark, 1997). However, Maia *et al.* (2009) observed decreased levels of genetic variation in *Ae. albopictus* from, Brazil, based on analysis of the ND5 with mean values of haplotype and nucleotide diversities of 0.187 ± 0.059 and 0.0004 ± 0.00014, respectively. In contrast, the current study showed mean haplotype and nucleotide diversities of 0.831 and 0.0034 respectively. Their observation was attributed to a small founder population of only a few individuals (Davies *et al.*, 1999; Martocq and Villablanca, 2001), which led to a

decrease in genetic variability as a result of genetic drift. This was coupled by the increase of population in the suburbs of the city (Fé *et al*., 2003). This recent expansion in Manaus resulted in homogenisation of the gene pool through high gene flow (*Nm* values). Therefore, the low genetic structuring was attributed to an inadequate period for accumulation of genetic differences between populations of *Ae. albopictus* in Manaus, and not due to the widespread contemporary gene flow.

Nucleotide substitution was found in *An. arabiensis* population in Sudan similar to that reported by Temu and Yan, (2005) in *An. gambiae* and *An. arabiensis* collected from four study sites in Western Kenya, the Great Rift Valley and coastal Kenya. This indicated subdivision which supported the ancestral background. The maximum parsimony tree in this study divided the populations into two subpopulations. This also supports the subdivision of *An. arabiensis* in Sudan. In the present study the six populations based on F_{ST} NJ tree were divided in two groups, cluster 1. Kr, H.sh (Kassala) with Gw (Khartoum), cluster 2. Hj, My (Khartoum), Se (sennar) support the ancestral background. In group 1 comprising of Gw, and Kassala populations of Kr, H.sh this may be due to the movement of Ethiopians refugees through Kassala to Gw in Khartoum. The *Anopheles* is transported with their belongings and thrives in the similar environments. Group 2 comprising of Hj with My is similar as would be expected of populations which are close by. In Hj, My and Se, the mosquitoes are found all the year round with high density in the rainy season. This similarity may also come through human movement for trading needs-selling and buying goods, animals and crops.

4.4.1.2 Haplotype distribution

In the current study, several haplotypes were shown be shared between two or more populations and even between regions in Sudan similar as Besansky *et al.*

(1997) who observed several shared haplotypes between the Kenyan populations of *An. gambiae* and *An. arabiensis*. Temu and Yan, (2005) also reported similar sharing between haplotypes of *An. arabiensis* populations from Western Kenya, the Great Rift Valley and coastal Kenya. Marshall *et al.* (2008) also reported shared haplotypes between molecular forms of *An. gambiae* from the islands of Sao Tome and Principe. Donnelly *et al.* (2004) stated that shared haplotypes between species in the *An. gambiae* complex may reflect non-contemporary processes such as incomplete lineage sorting between species or historical introgression events.

In the current study five out of 19 haplotypes were shared between populations which were geographically distant for example, Hap 01 was shared between My, Hj, Gw (Khartoum) and Se (Sennar) populations. Hap15 was shared between My, Hj (Khartoum), Kr, H.sh (Kassala) and Se (Sennar) populations. This sharing of haplotypes showed that they were of the same ancestral origins which have shared a widespread demographic history.

4.4.1.3 Genetic diversity

Maia *et al.* (2009) found low genetic diversity (π =0.00044) in *Ae. albopictus* populations from Manaus, Amazonas State, Brazil (present study,π = 0.00335). They suggested that this may be due to an inadequate time for accumulating genetic differences among populations of *Ae. albopictus* in Manaus, and not due to the widespread gene flow occurring among them. Similarly, Birungi and Munstermann (2002) reported genetic diversity (π = 0.0002) was lower compared to the present study in *Ae. albopictus* populations from the south and southeast coast of Brazil, utilising ND5 gene. Kambhampati and Rai (1991) and Kambhampati *et al.* (1991) also observed low genetic variation in 17 populations of *Ae. albopictus* from Asia.

They suggested a recent expansion of *Ae. albopictus* in this region to account for the observation.

However, when there are no barriers to gene flow, populations would randomly mate and become panmictic. In contrast, when gene flow is low, the populations undergo genetic drift. Solorzano *et al.* (2010) revealed high levels of gene flow with Nm value ranging from 29 to 32 between populations of Orleans and Chalmette in the United States. Similar high gene flow, among several populations from Sudan was observed and this is believed due to human transportation through daily or frequent movement between the villages and cities for medical needs, trading of animals and vegetables that have resulted in passive long-distance migration of the *An. arabiensis* populations. Microsatellite loci revealed significant levels of gene flow between the *An. arabiensis* populations and between *An. gambiae* populations from Asembo Bay in Western Kenya and Ghana in West Africa suggesting a high capability for the dispersal of genes such as those accountable for insecticide resistance or refractoriness to malaria among these populations (Kamau *et al.*, 1999). In Sudan insecticide resistance had been reported in most parts of the country as a result of spreading of this gene due to interaction and the movement from one to another place in the country. The unexpected low gene flow between Kr and H.sh which are geographically near to each other may be because of the presence of the Al Gash River which acts as a physical barrier between them.

4.4.1.4 Demographic history

Tajima's test did not detect a significant deviation from neutrality in five of the six populations investigated in this study suggesting that the nucleotide substitutions of ND5 gene are consistent with the neutral evolution theory and gene

flow is extensive in the *An. arabiensis* populations. Attributed to the insecticide resistance reported in Sudan similar reported by Donnelly *et al.* (2000) who studied evidence for extensive genetic differentiation among populations of malaria vector *An. arabiensis* in eastern Africa. The sixth population, Hj significantly deviated from neutral expectation indicating balancing selection or population substructuring or a recent bottleneck event (Tajima, 1989b). Maia *et al.* (2009) also established that Tajima's *D* neutrality test was non-significant for all populations analyzed from Manaus, Amazonas State, Brazil, concluding that these populations were in genetic equilibrium (Kimura, 1983). Similarly, Birungi and Munstermann (2002) also reported that Tajima's *D* test was also non-significant in their study on ND5 gene in Brazil and United States. Fu's *Fs* neutrality test, which assesses population growth, also did not show significant values, and therefore *Ae. albopictus* populations in Manaus were believed to be expanding. The populations structure in their study maybe due to ecological and climatic factor in the different populations. The sixth population of the present study, Hj, significantly deviated from neutral expectation indicating balancing selection or population. This due to effective control programmes applied in this places, while others populations showed insecticides resistance or high development than Hj.

4.4.1.5 Isolation by distance

In the present study no isolation by distance (Mantel test), specifically no correlation of genetic distance with geographic distance, was found among the six populations from Sudan. Similarly, Solorzano *et al.*, 2010 found 'no isolation by distance' across the three parishes of New Orleans that they investigated and suggested that there was no geographic barrier to movement of mosquitoes. In the same manner, Kamgang *et al.* (2011) who studied genetic structure of the tiger

mosquito, *Ae. albopictus*, in Cameroon found no observable relationship between genetic and geographic distances suggesting that the genetic structure has been shaped by additional biotic or biotic factors. In contrast, based on the COI gene Azrag (2007) observed that there was isolation by distance between study sites of *An. arabiensis* in northern Sudan. This meant that the River Nile restricted gene flow in this species. In addition, Carnahan *et al.* (2002) observed a strong correlation between geographic and genetic distance across consistently favourable habitat in Mali. This revealed that geographic barriers are not the only the reason for a decrease in gene flow in *An. gambiae s.s*. The circumstances in Mali, West Africa were evidently different than those in East Africa. This may be due to the different habitats and climatic factors between Mali and East Africa. Ayala & Coluzzi, (2005) and Costantini *et al.* (2009) established that the species within *An. gambiae* complex are not isolated by geographic barriers. Therefore various overlap may expand to the microspatial range, where different species can be chronologically breeding. Although hybrids are extremely rare, reproductive isolation is not complete and is measured to have occurred as a by-product of ecologically based divergent selection linked with alternative larval breeding sites. In this study there was no isolation by distance in the six populations, this may be due to different larval habitats, rainfall, temperature and humidity in the six populations. As well as the River Nile and its tributaries not acts as geographic barriers between this populations.

4.4.1.6 Phylogenetic divergence among *Anopheles gambiae* complex

Mitochondrial ND5 gene is a powerful tool in elucidating the level of genetic and phylogenetic divergence between closely related species (Krzywinski *et al.*, 2001). The results of the present study on *An. gambiae* complex inferred that *An. arabiensis* and *An. gambiae* are the most closely related taxa based on the

evolutionary sequence divergence of the two species as had been documented in previous studies. This is in conformity with the morphological, behavioural, and ecological similarity between the two species described by Besansky *et al.* (2003). Furthermore, Otarigho and Falade (2013) in their study about molecular evolution and phylogenomics of the *Anopheles gambiae* complex using COI gene showed that *An. arabiensis* and *An. gambiae,* the two major vectors of malaria are genetically similar.

4.4.2 Internal Transcribed Spacer (ITS) gene analysis

The rDNA internal transcribed spacers (ITS1 and ITS2) are generally divergent between species and homogeneous within species. In the present study the ITS sequences obtained from *An. arabiensis* showed 99% identity among populations in Sudan and most of the selected GenBank sequences. This is in agreement with Bower *et al.* (2008) whose investigation on *An. farauti* sensu stricto in Australia revealed a high level of homogeneity. This was attributed to the intraspecific concerted evolution of the rDNA ITS1and population differentiation observed in the ITS1, which indicates a higher rate of turnover relative to the adjacent 'core' region. Marrelli *et al.* (2006) studied the second ITS (ITS2) of nuclear ribosomal DNA as a tool for Latin American anopheline taxonomy. They revealed that ITS sequences were effective in differentiating vector mosquito species; however, information on intra-specific and geographic variation is limited.

The intraspecific variation in the ITS2 region of *An. arabiensis* in the six populations ranged from 0 to 5%. These variations were mainly in the form of single base mutation and this occurred rapidly, sufficient to reduce variation within regional populations. Similarly, Manguin *et al.* (1999) studied population structure of the primary malaria vector in South America, *An. darlingi*. They found homogeneity of

ITS2 gene among all populations with low intraspecific variation of 0.74%. The majority of this variation was in the form of insertion-deletions involving a few base pairs or single base point mutations. The almost identical ITS sequences across all of South America suggested a single panmictic breeding population.

There were differences between results of the ITS and ND5 genes. It was obvious that the former was not effective in resolving population genetics of the *An. arabiensis* populations in Sudan. The discordance between ITS and ND5 analyses is comparable to the contradictory pattern observed among the rDNA markers and many other genomic markers (Lehmann *et al.*, 2003; della Torre *et al.*, 2005). Sperling (1994) postulated that X-linked differences tend to be linked with species limits and it appears that such is the case among *An. gambiae* complex (Stump *et al.*, 2005). The ND5 and ITS rDNA sequences have different mutational rates and/or may track two separate colonization events, thus accounting for the differences observed. Concerted evolution occurs in rDNA due to its repetitive structure (Arnheim 1983) and therefore new alleles can spread much faster through a population than other markers (Arnheim 1983; Collins and Paskewitz 1996). The ease by which rDNA variants occur and convert other copies into themselves would tend towards homogenisation. The internal transcribed spacer (ITS2) is known to be efficient in detecting cryptic species (Marrelli *et al*., 2006; Kronefeld *et al.,* 2012). However, the present study could not detect any new species or strain of *An. arabiensis* in Sudan.

CHAPTER 5

POPULATION STRUCTURE OF *Anopheles arabiensis* BASED ON MICROSATELLITE MARKERS

5.1 Introduction

Anopheles arabiensis is a highly adaptable species with capability to feed on multiple host species, both indoors and outdoors and acclimatize to a wide range of larval habitats (Mutriu *et al.*, 2008; Mwangangi *et al.*, 2008). At present, indoor residual spraying (IRS) and insecticide treated bednets (ITNs) are the main methods extensively used for vector control worldwide. These methods have proven valuable in reducing malaria burden (Lengeler, 2004; Fegan *et al.*, 2007), but their usefulness have been threatened by increasing prevalence of insecticide resistance in the most important malaria vectors as well as the phenotypic flexibility of *An. arabiensis*. Thus, there is an urgent need for effective and sustainable alternatives to these traditional vector control strategies.

The genetic makeup of a vector population and its gene frequencies may be influenced by many evolutionary forces e.g. mutation, founder effect, genetic drift, migration of individuals with novel genotypes into or out of a population. Furthermore, genes may differ in their fitness among individuals in such a way that natural selection will only favour the best fit individuals with best fit genes (Hedrick, 2005) resulting in predominance of specific alleles.

Microsatellites are genetic markers of short tracts of tandemly repeated DNA sequences. These markers have become the genetic marker of choice for studying the population genetics of many eukaryotic species including mosquitoes. For instance,

they have been widely utilised in such studies in the *Anopheles gambiae* complex (Zheng *et al.*, 1996; Nyanjom *et al*., 2003; Temu and Yan, 2005; Kent *et al.*, 2007; Azrag, 2007; Muturi *et al.*, 2010; Nghabi *et al,* 2011; Deitz *et al.*, 2012). Furthermore, they could be developed into PCR-based molecular markers that are very useful for small organisms with limited extractable DNA (Aransay, 2003).

Due to their extensive length polymorphism, microsatellites can be used as DNA markers for studying relationships of individuals within and between populations. They are widely utilised because of their many advantages; single locus codominant markers, high mutation rates and thus high variability, ease of scoring and broad genome distribution. Furthermore, several loci can be joined in a multiplex to provide fast and economical repeated sampling of the genome (Liua and Cordes, 2004; Selko and Toonen, 2006). Due to their generally high-mutation rates (resulting in high allelic diversity), they are very suitable for studying small or recently bottlenecked populations than markers with lower mutation rates such as allozymes that cannot detect recently diverged species (Hedrick, 1999; Conn and Mirabello, 2007). These high mutation rates of microsatellites have been hypothesized to be a consequence of a number of mechanisms occurring during the process of DNA replication and repair; counting errors during recombination, unequal crossing-over and polymerase slippage during DNA replication (Schlötterer and Tautz, 1992) or repair (Strand *et al.*, 1993). Mutation replication slippage is considered the major mutational mechanism for microsatellite allele variability (Ellegren, 2000a). This could occur during replication where errors during duplex formation may cause misalignment, leading to insertion or deletion of a number of repeat units. The characteristically high variability and abundance of microsatellite markers lends them very useful for various genetic analyses. Reliable estimates of microsatellite

mutation rates are often required for such analyses. However, the factors determining mutation rates are yet to be completely understood (Ellegren, 2000a, b; Kayser *et al.*, 2000; Sibly *et al*., 2001, 2003).

Although microsatellite markers are generally considered species-specific, cross amplification can successfully occur among closely related taxa. Selko and Toonen, (2006) revealed that in sibling species, homologous microsatellite loci could be amplified using the same primers but this may sometimes result in reduced amplification rate depending on the genetic relatedness of the species. In addition, the allelic diversity often decreases when primers are used in non-source species.

To date, one hundred and fifty polymorphic microsatellite loci have been characterized in *An. gambiae*s. s.l. (Zheng *et al.*, 1993; Zheng *et al.*, 1996) which has been widely used to explain the population structure and gene flow within and between members of the *An. gambiae* complex (Kamau *et al.*, 1999; Kamau *et al.*, 2007; Moreno *et al.*, 2007). The majority of these studies have mostly been conducted on *An. gambiae* and not as many on *An. arabiensis*. Present literature on *An. arabiensis* has revealed the lack of subpopulation differentiation in relation to larval habitat utilization (Kamau *et al.*, 2007). Lack of annual bottlenecks in response to changes in the environment has also been documented (Simard *et al.*, 2000; Kent *et al.*, 2007). Large effective population size and/or recent range expansion as opposite to group migration (Donnelly and Townson, 2000; Simard *et al.*, 2000) have been attributed to the widespread gene flow. This is based on several experimental studies which have reported a short flight range for this malaria vector species (Thomson *et al.*, 1995) among villages in Gambia. On the other hand, there is evidence in support of population structuring (Simard *et al.*, 1999) from West Africa

and eastern outer islands (Donnelly and Townson, 2000) in Eastern Africa. Furthermore, Kamau *et al.* (1999) and Kent *et al.* (2007) also showed limited gene flow in west and south east of the Rift Valley and in Southern Zambia, respectively. Geographic distance and habitat alterations have been suggested as the main contributors of genetic isolation.

Anopheles arabiensis has changeable deme sizes ranging from as low as 25 km (Donnelly and Townson, 2000) to a few 1000 kilometres as reported by Kent *et al.* (2007). It was observed that in the Mwea Rice Scheme of Central Kenya, *An. arabiensis* mosquito densities decrease with increasing distance from the Scheme (Mutero *et al.*, 2004b, Muturi *et al.*, 2006). On the contrary, the human blood index (Muturi *et al.*, 2008) in addition to malarial transmission (Mutero *et al.*, 2004b) by this species was significantly lower inside than in the outer areas of the rice scheme.

With respect to the above study, agricultural practices can alter local environment (Karaca *et al.*, 2008) as well as affect the number and diversity of larval habitats. All these factors influence mosquito reproductive fitness, survivorship and fertility (Afrane *et al.*, 2006). Such alteration may change malarial transmission indices (Ijumba and Lindsay, 2001) and can lead to subpopulation differentiation (Toure *et al.*, 1994, 1998) as was observed in this agricultural scheme. But on the other hand the lack of evident geographical barriers that might have restricted gene flow between mosquito populations in the surrounding areas had led to the generation of a single panmictic population. A number of studies, example one that was conducted by Dolo *et al.* (2004) in the irrigated area of Sahel in Mali have suggested that the existence of mosquito colonies in an adjoining non-irrigated area

during the dry season is maintained through migration of a few individuals from the irrigated areas.

Several comparative population studies between *An. arabiensis* and *An. gambiae* have shown a higher level of genetic differentiation in the latter species. Significant genetic differentiation, F_{ST} = 0.072- 0.100 were observed for *An. gambiae* populations between western Kenya and coastal Kenya using microsatellite markers. Lehmann *et al.* (1999; 2003) suggested the Great Rift Valley as a major gene flow barrier for this species. However, nonsignificant genetic differentiation was identified for *An. arabiensis* population from the two areas using the same loci (Kamau *et al.*, 1999). Similarly, Donnelly and Townson (2000) noted nonsignificant genetic differentiation of *An. arabiensis* populations in Malawi and Sudan. It thus appeared that different mechanisms of gene movement were in operation between the two species. Considering *An. gambiae*, these studies were discordant with another study on *An. gambiae* s.s. (mean F_{ST} = 0.006) which was genetically undifferentiated across the 6,650 km2 of the Kilombero valley landscape southern Tanzania. This suggested that the genetic differentiation was not due to physical barriers or distance. One plausible explanation is that there was environmental diversification even within the Kilombero valley (Nghabi *et al.*, 2011). Thus, the differentiated populations of *An. gambiae* could have been maintained by some degree of reproductive isolation.

With respect to *An. arabiensis,* several studies have reported varying levels of genetic differentiation. Nyanjom *et al.*, (2003) detected low F_{ST} but statistically significant genetic structure for *An. arabiensis* populations in Ethiopia and Eritrea. Similarly, Azrag (2007) also documented low but significant difference between populations of *An. arabiensis* in Northern Sudan using microsatellite markers. On the

other hand Simard *et al.* (1999) reported high levels of genetic differentiation in two island populations of *An. arabiensis* populations that were 240 km apart in the Indian Ocean (F_{ST} 0.080–0.215). Ng'habi *et al*. (2011) detected high levels of genetic differentiation among *An. arabiensis* populations (mean F_{ST} = 0.066) in Kilombero valley southern Tanzania.

Therefore the objective of this investigation was to study the population structure and gene flow among *An. arabiensis* populations in Sudan based on microsatellite markers, which may assist in developing control strategies.

5.2 Materials and Methods

5.2.1 Study Areas

A total of 200 specimens of *An. arabiensis* were collected from six different localities (Fig 3.2) as previously described in Chapter 4. Sample size ranged from 32 individuals in Hj to 34 in My, with an average of 33 individuals per population. The pairwise distance between the six localities ranged from 3.93- 569.25 Km. DNA extraction method was as described previously (Chapter 3)**.**

5.2.2 Microsatellite PCR Amplification

Seven published *An. gambiae* microsatellite loci primers (Zheng *et al*., 1996) were used to amplify the DNA samples. The PCR reactions were performed in a gradient thermal cycler (MJ Research PTC-200 Peltier Thermal Cycler) for 30 cycles. The PCR mix contained 1 uL of genomic DNA, 5X PCR buffer (Promega, Madison, WI), 15 pmol of each fluorescent labelled (NED, HEX or FAM) forward primer, 200 mMof each dATP, dCTP, dGTP and dTTP, 1.2 uL of 25mm $MgCl_2$ and 0.5 U *Taq* DNA polymerase (Promega, Madison, WI) in a 20 uL total reaction volume. Details of primer sequences, repeat motif and fluorescent labelling are given

in Table 5.1. Singleplex PCR amplification was conducted for loci AGXH678, AG2H290, AG2H603, AG2H143, AG3H29, AG3H45, AG3H158 (Table 5.1). The thermal cycling conditions were; an initial hold at 95ºC for 2min, followed by 30 cycles of 94˚C for 30sec, 55˚C for 30s and 72˚C for 30s and a final extension at 72˚C for 5 min. Satisfactory PCR products as detected in a 2% agarose gel were sent to the service provider (First BASE Laboratories Sdn Bhd, Selangor, Malaysia) into two primer multiplex sets for fragment analysis. Set A contained a mixture of AGXH678, AG2H290 and AG3H45, while set B was a mixture of AG2H603, AG2H143, AG3H29 and AG3H158. Loci AGXH678, AG3H29, AG3H45 and AG3H158 are found outside the inversion regions of the chromosome, while AG2H603 and AG2H143 loci are found within fixed inversion of chromosome 2La, and AG2H 290 in the 2R polymorphic inversion.

Table 5.1 Microsatellite loci (Zheng*et al.*, 1996) selected for population genetic analysis of *An. arabiensis* individuals collected from different sites in Sudan.

Locus	Repeat motif	Primer (5' – 3')	Observed allele size range	Chromosome number and position
AGXH678	(AG)7	**F** CCTCTCCCCAGAATCGGTAC R AAGAGCAGAAACAACCGCAG	153-169	XL outside inversion)
AG2H290	(AG)9	**F** GGCGTGTGCTGTGCTCCC R ACGCAATTTTTGCCTCAGCG	93-99	2R Polymorphic
AG2H603	(AG)8	**F** TGCACCGTTGATGCACATGC R GTGGACGATGTGAAAGATAAGG	107-119	2L inside fixed inversion 2La
AG2H143	(TC)9	**F** CGTACGAGTGAGTGAGTTGG R CAAAAATAGCATCACGGCCG	152-167	2L inside fixed inversion 2La
AG3H29	(TGA)	**F** TGTTGCCGGTTTGTTGCTGA R ATGTTCCAGAGACGACCCAT	144-146	3R outside inversion 29C
AG3H45	(TC)4+7+4	**F** AAAAGTGGTGACCGAGTGAC R ATCTTCAACACTTCAGCACG	150-163	3L outside inversion 45C
AG3H158	(AG)12	**F** CTGGCACGATCAATCAATCG R ACGATGGTGTACACGTAACG	83-89	3R outside inversion

F = forward primer labelled with fluorescent dyes, R = reverse primer.

5.2.3 Data Analysis

Allelic data scoring of alleles was carried out as in Arif *et al., (*2010). Screening of all the genotypic data was executed using Micro-Checker v2.2.3 (Van Oosterhout *et al*., 2004) to check for presence of null alleles and stuttering or large allele dropouts. The Monte Carlo simulation (bootstrap) method was applied to generate expected homozygote and heterozygote frequencies of alleles. The HWE was used to calculate expected allele frequencies and the frequency of any null allele detected with significance level at $P< 0.05$ obtained through 1000 permutations. To ensure compatibility with different software analyses the raw data was converted into several specific data formats for using CONVERT (Glaubitz, 2004). Significant relationship between alleles at any two loci was tested using the likelihood ratio test of linkage disequilibrium based on Expectation-Maximization (EM) algorithm (Slatkin and Excoffier, 1996). This was applied to all pairwise comparisons of loci using Arlequin version 3.11 (Excoffier *et al*., 2005) with 10000 permutations followed by false discovery rate (FDR) adjustment (Benjamini and Hochberg, 1995) at 95% significant level.

Population genetic diversity was measured by allelic richness (A_R) and number of alleles (N_A). Inbreeding coefficient (F_{IS}) for each locus and population (Weir and Cockerham, 1984) was estimated in FSTAT v.2.9.3 (Goudet, 1995). Allelic richness is defined as the number of alleles per locus adjusted for different sample size. To test for global deviation from HWE in a population, the F_{IS} was estimated based on the difference between observed and expected heterozygosity. A value of -1 denotes no inbreeding while +1 denotes total identity. Mean genetic heterozygosity, observed (H_O) and expected (H_E) heterozygosities per locus and population were estimated over all loci. Testing of deviation from Hardy-Weinberg

equilibrium (HWE) was conducted using the exact tests with 10000 steps in Markov chain and 10000 dememorization steps in Arlequin version 3.11 (Excoffier *et al*., 2005). Multiple testing of HWE was adjusted using False discovery rate (FDR) corrections with a global significance level of 0.05

Estimates of population differentiation, using Wrights F_{ST} and Slatkin R_{ST} (Goodman, 1997) over all loci were conducted. F_{ST} is a measure of genetic difference among groups. It is based on the infinite allele model (IAM) which hypothesizes that each new allele is generated at a given rate, μ (Francois and Nicolas, 2002). Slatkin R_{ST} is based on the stepwise mutational model (SMM). The model assumes that new alleles are generated by adding or deleting a single repeat unit of the microsatellite, with an equal probability μ/2. Consequently, alleles that vary most in sizes is expected to be more distantly related than alleles of similar sizes

AMOVA was carried out at different hierarchical levels on all populations using Arlequin 3.11 (Excoffier *et al*., 2005) as described in Chapter 4. A Mantel test as explained in Chapter 4 was also carried out to determine the association between genetic and geographic distances.

The program BOTTLENECK V 1.2.02 (Piry *et al.*, 1999) was used to detect whether the populations had experienced recent effective population size reduction. Two-phase (TPM) models, infinite allele (IAM) and stepwise mutation (SMM), and deviation from HWE was estimated using a two-tailed Wilcoxon sign-rank test followed by FDR adjustment. Qualitative descriptor of allele frequency ("mode-shift" indicator) was also performed in BOTTLENECK to discriminate "shifted mode" populations (bottleneck) from stable populations (Luikart *et al*., 1998a).

Assignment of individuals to their respective source populations based on multilocus genotypic data was determined in STRUCTURE version 2.3 (Pritchard *et al.,* 2000). An assumption of correlated allele frequency among populations (Falush *et al.,* 2003) and admix model was used with the burnin period and MCMC length, each at 10000 and 10 iterations, respectively. An examination of k=1 to 10 was performed and the true number of groups, k was determined (Evanno *et al.* (2005) based on the highest peak of $\Delta k = k$ which is considered the most probable number of groups for the entire dataset. Then, posterior probability of any admixed individuals belonging to the original population was calculated to assign them back to the original population. The probabilities of genotype assignment into each individual group were performed across replicates using CLUMPP version 1.1.2 (Jakobsson and Rosenberg, 2007) and the graphical presentation was carried out using Structure Harvester (Earl *et al.,* 2012). Finally, based on genetic distance a Neighbour-joining tree was constructed to determine the phylogenetic tree among the six populations using MEGA 5.0.5 (Tamura *et al.,* 2011).

5.3 Results

All 200 individuals from six populations of *An. arabiensis* in Sudan were successfully genotyped and scored for all seven microsatellites loci. No evidence for scoring error due to null alleles, large allele dropout or stuttering was detected after assessing with Microchecker software.

5.3.1 Allelic Frequency Distribution and Linkage Disequilibrium

A locus was considered to be polymorphic if the most frequent allele was at a frequency of less than 0.99 or less than 0.95 (Ayala, 1982). All microsatellite markers of *An. arabiensis* populations were found to be polymorphic in at least one

population. However, locus AG3H29 was least polymorphic, being monomorphic in most populations, may be due to presence of null alleles. Loci AG2H143 and AG3H45 were highly polymorphic with number of allele per locus ranging from 6-12 and 4- 10 respectively. The total number of alleles per locus ranged from 2 to 12 with an average of 7.6. The mean A_R ranged from a minimum of 3.5 in Hj to a maximum of 6.8 in Se and the mean observed heterozygosity of alleles per locus ranged from 0.55 to 0.67, while means of expected heterozygosity ranged from 0.55 to 0.62 for *An. arabiensis* as shown in Table 5.2 and Figures 5.1a and 5.1b.

Table 5.2 Allelic richness (A_R), number of alleles (N_A), and the mean allele number of observed (H_O) and expected heterozygosity (H_E) at each locus per population.

Locus	Pop	Gw	H.sh	Hj	My	Se	Kr	Total
	N	33	34	32	34	33	34	200
AGXH678	A_R	2	6	1	8	7	7	8
	N_A	3	4	2	5	5	4	7
AG2H290	A_R	1	3	2	3	3	3	3
	N_A	2	4	3	4	4	4	4
AG2H603	A_R	3	6	4	4	4	4	6
	N_A	4	7	5	5	5	5	7
AG2H143	A_R	6	12	6	10	12	12	15
	N_A	4	5	4	4	7	7	13
AG3H29	A_R	–	1	–	–	–	1	1
	N_A		2				2	2
AG3H45	A_R	10	10	4	9	10	10	13
	N_A	6	5	3	7	6	5	13
AG3H158	A_R	4	4	4	4	5	6	6
	N_A	3	3	3	3	5	5	7
Mean	A_R	4.3	6	3.5	6.3	6.8	6.1	7.4
	N_A	3.7	4.3	3.3	4.7	5.3	4.6	7.6
	H_O	0.55	0.55	0.6	0.63	0.67	0.63	0.61
	H_E	0.57	0.55	0.56	0.62	0.59	0.61	0.58

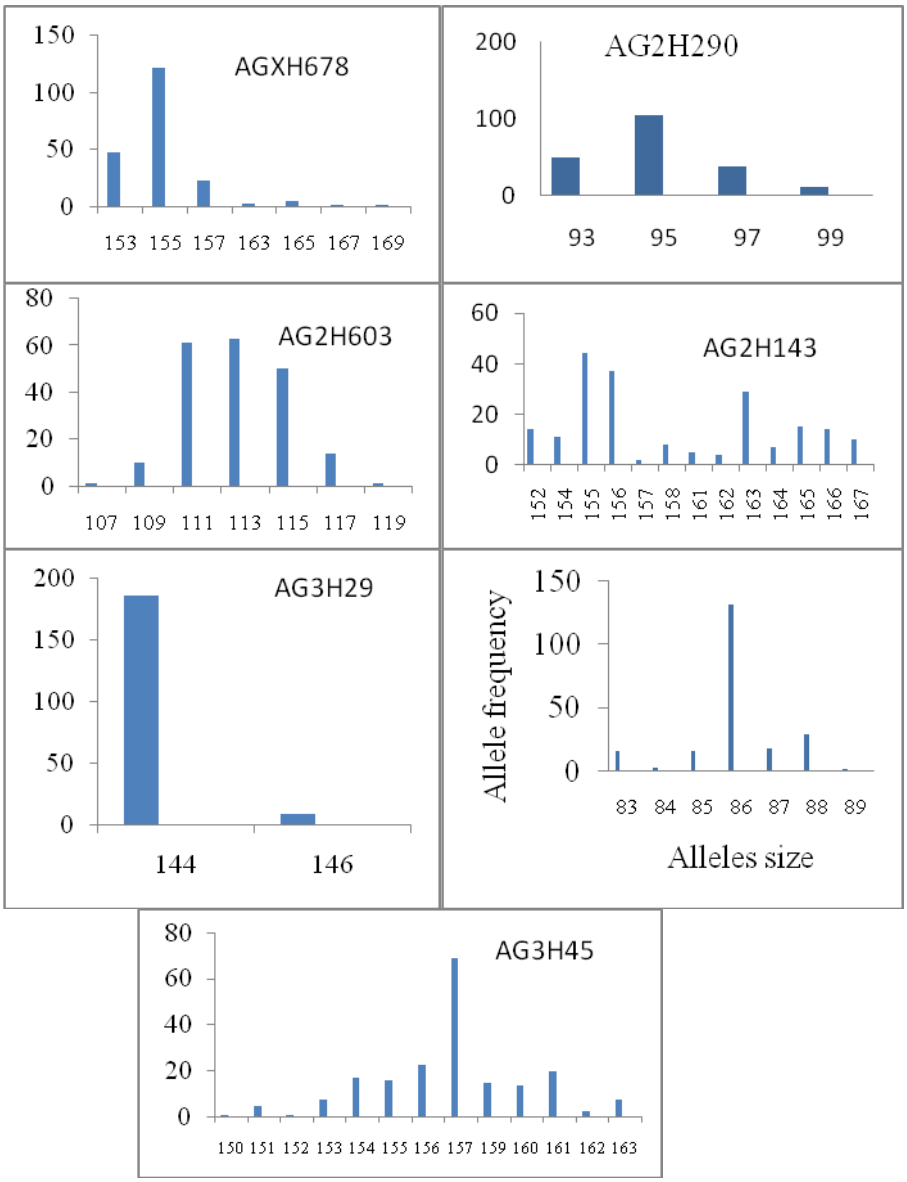


Figure 5. 1a: Allele frequencies per each locus over all populations. Allele size is given on the X-axis, frequency is given on the Y-axis..

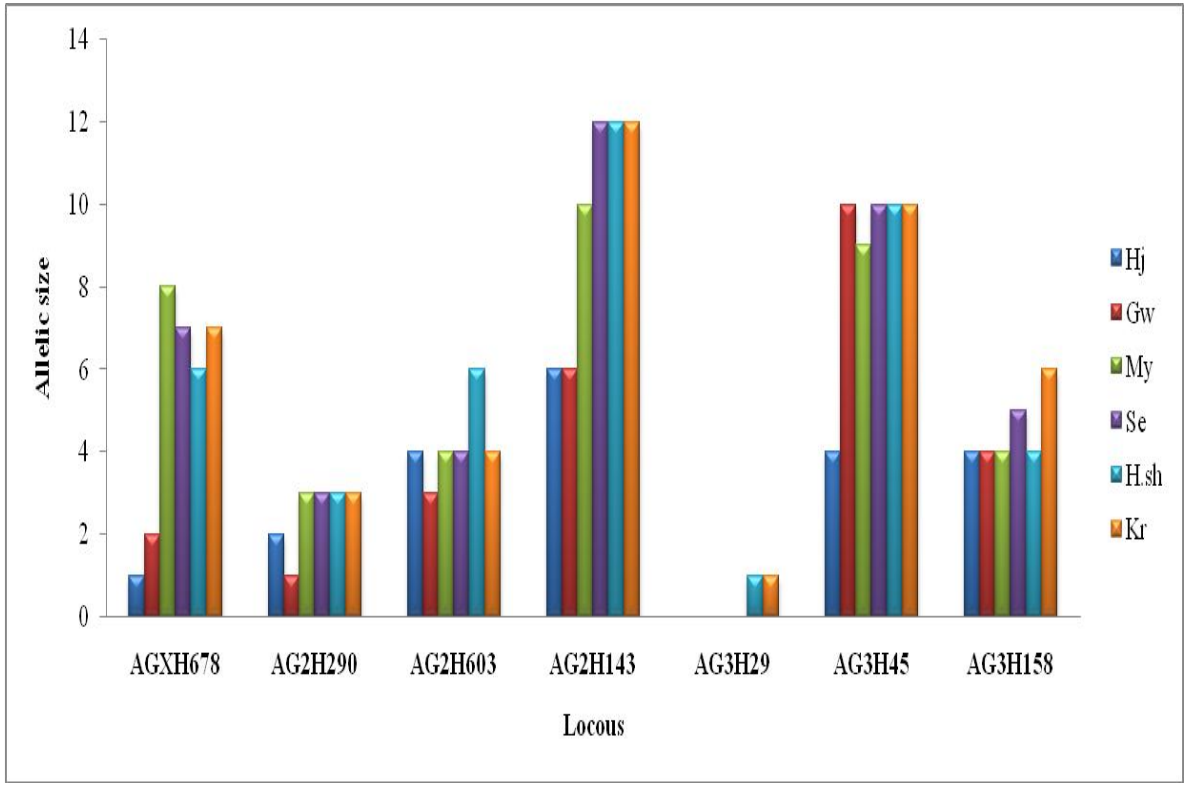


Figure 5.1.b: Number of alleles observed in each locus for each population.

Test for linkage disequilibrium revealed that 14 pairwise comparisons (11.11% out of 126 pairwise comparisons) after Bonferroni correction were significantly deviated from the non-random association of alleles at two or more loci with the highest linkage disequilibrium detected from pairwise loci comparisons in Kr (5 pairs in loci AGXH678, AG2H290, AG2H143, AG3H45 and AG3H158), followed by My (4 in loci AGXH678, AG2H290, AG2H603 and AG2H143), H.sh (2 pairs in loci AG2H290, AG2H603), Hj (1 pair in AG3H158) and Se (2 pairs in loci AGXH678 and AG2H143). No linkage disequilibrium was observed in Gw.

5.3.2 Hardy-Weinberg Equilibrium (HWE) and F_{IS}

Each locus was tested separately for significant departure from HWE. Observed heterozygosity varied from 0.12 to 0.94 while expected heterozygosity ranged from 0.11 to 0.76 (Table 5.3). Deviations from HWE were found in 21 out of 42 tests in the six populations. These were observed in locus AGXH678 (Gw and H.sh), locus AG2H290 (H.sh, Hj, My and Kr), locus AG2H603 (Gw, H.sh, Hj, My and Kr) locus AG2H143 (Gw, Hj, My and Se), locus AG3H45 (H.sh, Hj, My, Se and Kr), AG3H158 in Kr. There was no consistent pattern according to locus or population and fairly equal numbers of heterozygote deficiencies and heterozygote excess were observed. This could be due to population subdivision rather than the existence of null alleles based on the Microchecker results. Locus AG3H29 showed no deviations from Hardy-Weinberg equilibrium and this locus was monomorphic in all populations except in H.sh and Kr populations. Inbreeding coefficient (F_{IS}) over all loci showed that most of the populations had high excess of heterozygosity. F_{IS} negative values indicate no inbreeding. High F_{IS} positive values mean presence of inbreeding.

Table 5.3: Population genetic diversity as measured by observed (H_O) and expected (H_E) heterozigosities and F_{IS}.

locus	Pop	Gw	H.sh	Hj	My	Se	Kr
	No	33	34	32	34	33	34
AGXH678	H_O	0.49	0.85	0.44	0.63	0.58	0.64
	H_E	0.53	0.65	0.48	0.63	0.60	0.53
	F_{IS}	0.09	-0.32	0.08	0.01	0.04	-0.20
	P	0.03*	0.00*	0.71	0.18	0.16	0.19
AG2H290	H_O	0.61	0.50	0.53	0.56	0.70	0.67
	H_E	0.49	0.63	0.60	0.61	0.70	0.65
	F_{IS}	-0.26	0.21	0.12	0.08	0.01	-0.03
	P	0.27	0.00*	0.03*	0.00*	0.10	0.00*
AG2H603	H_O	0.61	0.65	0.69	0.84	0.79	0.75
	H_E	0.70	0.68	0.73	0.76	0.72	0.77
	F_{IS}	0.14	0.05	0.06	-0.11	-0.10	0.03
	P	0.00*	0.00*	0.00*	0.00*	0.12	0.00*
AG2H143	H_O	0.58	0.62	0.88	0.67	0.55	0.58
	H_E	0.65	0.68	0.61	0.51	0.50	0.63
	F_{IS}	0.12	0.09	-0.44	-0.30	-0.08	0.07
	P	0.001*	0.20	0.00*	0.00*	0.02*	0.38
AG3H29	H_O		0.12				0.08
	H_E		0.11				0.08
	F_{IS}	N.A	-0.05	N.A	N.A	N.A	-0.03
	P		1				1
AG3H45	H_O	0.52	0.53	0.75	0.69	0.58	0.94
	H_E	0.50	0.63	0.55	0.74	0.70	0.76
	F_{IS}	-0.04	-0.35	0.16	-0.32	-0.27	-0.05
	P	0.73	0.00*	0.01*	0.00*	0.00*	0.00*
AG3H158	H_O	0.49	0.59	0.31	0.66	0.58	0.72
	H_E	0.53	0.44	0.37	0.50	0.46	0.69
	F_{IS}	0.09	-0.35	0.16	-0.32	-0.27	-0.05
	P	0.22	0.09	0.07	0.09	0.20	0.00*

H_O=observed heterozygosity, H_E= expected heterozygosity

F_{IS}= inbreeding coefficient, high positive values indicate deficiency of heterozygotes, while small or negative values indicate excess of heterozygotes.

P = significance of deviation from HWE ($P<0.05$), *= significant deviation from HWE.

5.3.3 Genetic Structure

A hierarchical AMOVA performed after defining into three groups (Khartoum, Sennar and Kassala) revealed that 2.75% of the total genetic variance (F_{CT}) was contributed by 'among groups variation' while 13.61% (F_{SC}) was 'among populations within group variation' (Table 5.4). 83.63% of the genetic variation was attributed between individuals within population i.e. intrapopulation variation. All hierarchical levels i.e. F_{SC} and F_{CT} and within population revealed significant variation as in mitochondrial ND5 gene.

Table 5.4: Results of AMOVA of *An. arabiensis* populations inferred by microsatellite markers

Source of Variation	d.f.	Variance Components	Percentage of Variation	Fixation Indices	*P value*
Among groups	2	0.06	2.75	F_{CT} :0.16	0.000
Among populations within groups	3	0.30	13.61	F_{SC} :0.14	0.000
Within population	394	1.84	83.63	0.03	0.003

Significant differentiation among populations, (F_{ST}) was observed between all *An. arabiensis* pairwise comparisons for all loci (Table 5.5). F_{ST} ranged from 0.06 to 0.24. According to Wright's values when $F_{ST} = 0$ there is no genetic divergence, $F_{ST} = > 0 – 0.05$ indicates low genetic differentiation, $F_{ST} = > 0.05$-0.15 indicates moderate differentiation and $F_{ST} = > 0.15$- 0.25 indicates high genetic differentiation. Pairwise differentiation values, F_{ST} of the list below with other populations were as follows:

1. Kr ranged from 0.17 to 0.24
2. Hj ranged from 0.11 to 0.24
3. Gw ranged from 0.08 to 0.22
4. My ranged from 0.06 to 0.21
5. Se ranged from 0.06 to 0.19
6. H.sh ranged from 0.08 to 0.17

All the above showed that each of the population was highly differentiated from at least one other population. But if Kr is excluded and in most cases, Se as well, the comparisons will indicate moderate genetic differentiation for all. Moderate genetic differentiation was observed between *An. arabiensis* populations from My (Khartoum State) and Se (Sennar State) ($F_{ST=}0.06$) and Gw (Khartoum State) and H.sh (Kassala state) ($F_{ST=}0.08$). High genetic differentiation was observed between *An. arabiensis* populations from Kr and other populations $F_{ST}= 0.17$-0.24. Therefore Kr population was more genetically isolated from the rest. However, the analogous pairwise estimates of R_{ST} ranged between 0.00 to 0.84. High R_{ST} value was found between Hj and Gw (0.84) and lowest values between Kr and H.sh, My and H.sh and Se and H.sh.

Table 5.5: Population differentiation of *An. arabiensis* as estimated using Wright's pairwise F_{ST} and Slatkin $R_{ST.}$

	Gw	H.sh	Hj	My	Se	Kr
Gw	*	0.33	0.84	0.30	0.38	0.31
H.sh	0.08	*	0.51	0.01	0.03	-0.00
Hj	0.11	0.12	*	0.39	0.56	0.45
My	0.13	0.11	0.11	*	0.07	0.01
Se	0.18	0.16	0.17	0.06	*	0.01
Kr	0.22	0.17	0.24	0.21	0.19	*

R_{ST} above diagonal F_{ST} below diagonal.

Gene flow (Nm) calculated from mean F_{ST} statistics ranged from 1.5 – 9.05 suggesting high gene flow between populations (Table 5.6). High migration rate and therefore high gene flow was detected among Se (Sennar State) with My and Gw (both Khartoum State); Hj and My (both Khartoum State). Very little gene flow was observed between Kr (Kassala State) and other populations.

Table 5.6: Gene flow estimates between populations of *An. arabiensis* in Sudan

0	Gw	H.sh	Hj	My	Se	Kr
Gw	0	**426.5**	**5.38**	**15.55**	**323.1**	**443.21**
H.sh	5.89	0	**448.59**	**416.55**	**429.31**	**12.88**
Hj	4.06	3.51	0	**3.93**	**311.55**	**427.72**
My	3.48	4.07	3.81	0	**292.07**	**431.75**
Se	2.22	2.71	2.47	9.05	0	**334.01**
Kr	1.69	2.30	1.50	1.88	1.97	0

Geographical distance (km) between sites (above diagonal- in bold)
Nm, gene flow between *An. arabiensis* populations (below diagonal)

When considering the loci outside the inversion (AGXH678, AG3H29, AG3H45 and AG3H158), F_{ST} statistics ranged from 0.026 to 0.32 with mean F_{ST}= 0.13 as in Table 5.7 and showed higher genetic differentiation between-population according to Wright's F_{ST}. For loci inside inversion (AG2H603; AG2H290 and AG2H143), F_{ST}ranged from 0.019 to 0.20 with mean F_{ST}= 0.16. This shows that irrespective of their position, the loci outside or inside the chromosomal inversions or pooling together showed similar values for *An. arabiensis* populations.

Gene flow (Nm) for loci outside the chromosomal inversions ranged from 1.04 to 43.52. The highest gene flow was between My and H.sh with Nm= 43.52, while little gene flow was found between Kr and Hj with Nm= 1.04. For loci inside the fixed chromosomal inversion, 2La, a range of Nm= 1.38 to 19.99, the highest gene flow was between Se and My with Nm = 19.99, while little gene flow was detected between Se and Kr with Nm= 1.38.

Table 5.7: Pairwise F_{ST} and Nm for loci outside and inside inversion

	F_{ST}(mean)	Nm
Outside inversion	0.026 – 0.32 (0.13)	1.05 – 43.52
Inside inversion	0.019 – 0.20 (0.16)	2.00 – 25.48

The genetic distance (Table 5.8) ranged from 6% between My and Se (although are geographically distant) to 32% between Kr and Hj. The genetic distance was high between Kr and H.sh. Thus, although the detailed magnitudes of population differentiation vary among population comparisons in different analyses, in summary Kr is most distant or differentiated from other populations and followed by Se to a certain extent for several pairwise comparisons.

Although there was significant differentiation, F_{ST}, between the different populations, Mantel tests showed no significant correlation (r^2= 0.09, P> 0.05) between genetic differentiation measured as linearized F_{ST} ($F_{ST/(1-F_{ST})}$ and geographic distance (km) (Figure 5.2). Thus genetic and geographic distances are not correlated.

Table 5.8: Pairwise genetic distance between each population

	Gw	H.sh	Hj	My	Se	Kr
Gw	0					
H.sh	0.09	0				
Hj	0.12	0.14	0			
My	0.14	0.13	0.13	0		
Se	0.23	0.19	0.20	0.06	0	
Kr	0.28	0.21	0.32	0.27	0.24	0

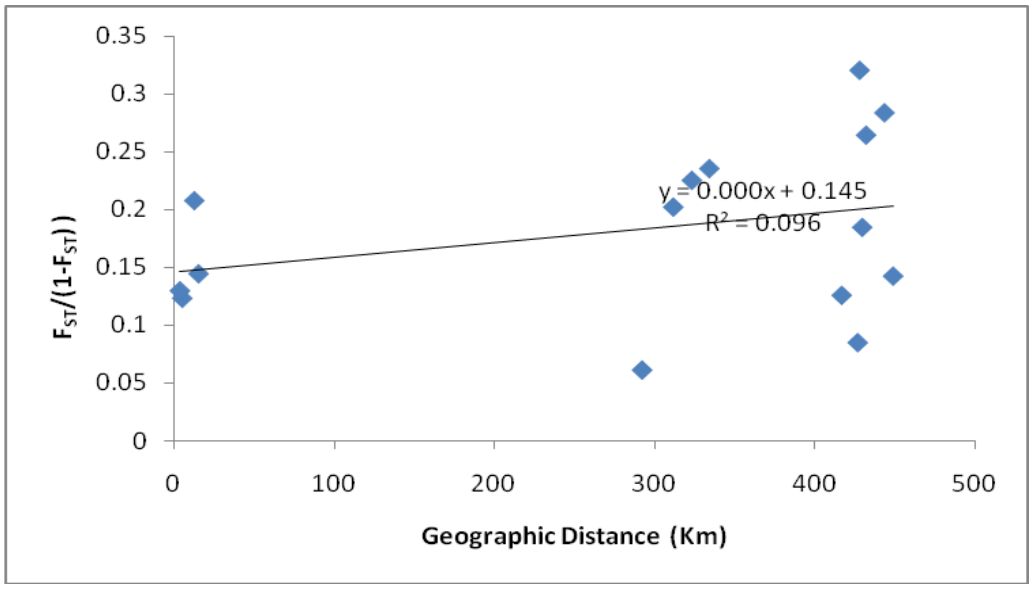


Figure 5.2: Mantel test showing the scatter plot of the relationship between linearized and geographic distance (km) among *An. arabiensis* populations.

5.3.4 Bottleneck Population

The Wilcoxon test (Table 5.9) indicated that all populations excluding Se were significant for IAM mutation-drift equilibrium ($\alpha<0.05$), but with normal L-shaped distribution (Figure 5.3). This shows that these populations had not experienced population bottleneck. However, population Hj with shifted mode suggest recent population size reduction. TPM analysis showed insignificance for all populations except Hj while the SMM analysis showed insignificance for all populations.

Table 5.9: Bottleneck analysis of *An. arabiensis* populations from six areas in Sudan

Area	IAM	TPM	SMM	Mode-shift
Hj (n=32)	0.016	0.031	0.109	shifted
Gw (n=33)	0.031	0.438	0.438	Normal
My (n=34)	0.047	0.438	0.844	Normal
Se (n=33)	0.078	0.563	0.563	Normal
H.sh (n=34)	0.039	0.375	0.299	Normal
Kr (n=34)	0.023	0.055	0.688	Normal

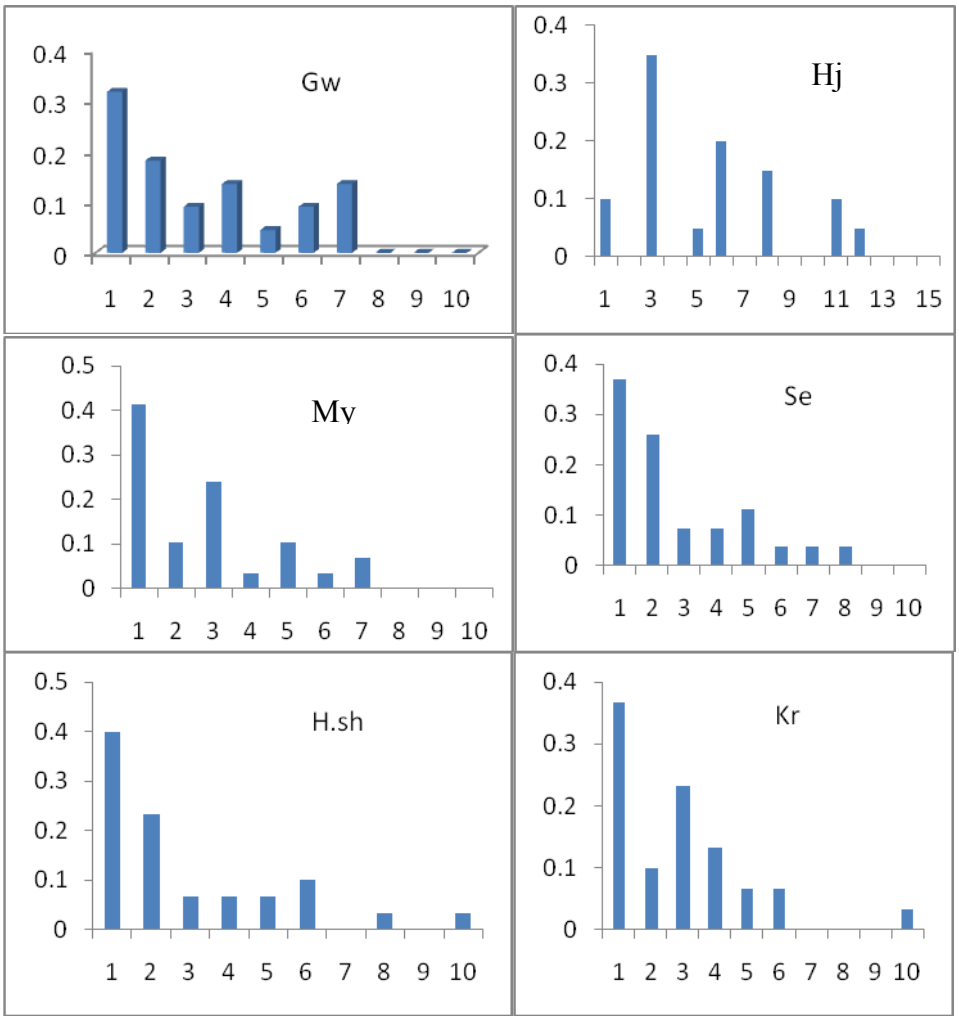


Figure 5.3: Allelic frequency distribution for each population with shifted mode in Hj while other populations were L- shaped; X axis is the number of alleles and Y axis is allele frequency.

5.3.5 Population Structure

Based on the programme STRUCTURE after calculations of the delta K and plotting its value against the assumed number of populations (K= 10), showed a peak at K =2 revealing two main clusters (Figure 5.4). The dataset was further analyzed by assigning individuals between the two suggested clusters. Figure 5.5 explains the analysis for assignment of the most likely K (K = 2). Each colour represents the true assigned population. Each perpendicular bar represents a single individual. Population codes are labeled along the X-axis while the Y-axis represents the probability of assignment of an individual to each cluster. Cluster A (green color) includes Hj, Gw, My and H.sh populations and Cluster B (red color) includes Se and Kr populations. This is generally in agreement with the F_{ST} analyses where Kr and to a lower degree, Se are distant from the other populations although My is closely related to the latter. However, in the earlier analysis F_{ST}, Kr and Se were also genetically distant.

However, according to the phylogenetic tree only Kr is distant from the other populations. The phylogenetic tree showing the microsatellite genetic distance between *An. arabiensis* populations from Sudan showed that populations Hj, GW and H.sh, My and Se are genetically strongly related, while Kr appeared to be comparatively differentiated from other populations (Figure 5.6).

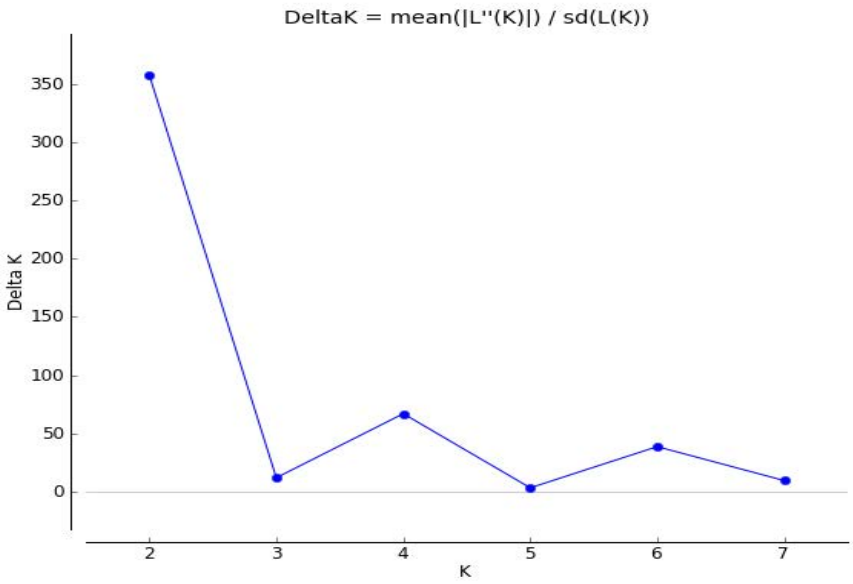


Figure 5.4: Bayesian cluster analysis using STUCTURE.HARVESTER Graphical representation of the data set for the most likely K (K = 2),

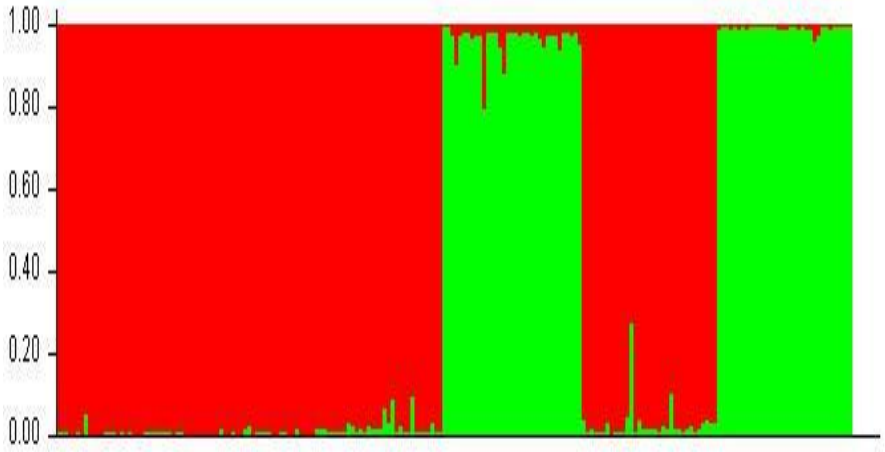


Figure5.5: Bayesian cluster analysis using STUCTURE. Graphical representation of the data set for the most likely K (K = 2), where each color corresponds to a suggested cluster. Subpopulation A (red color) includes Hj, Gw, My and H.sh populations and subpopulation B (green color) includes Se and Kr populations.

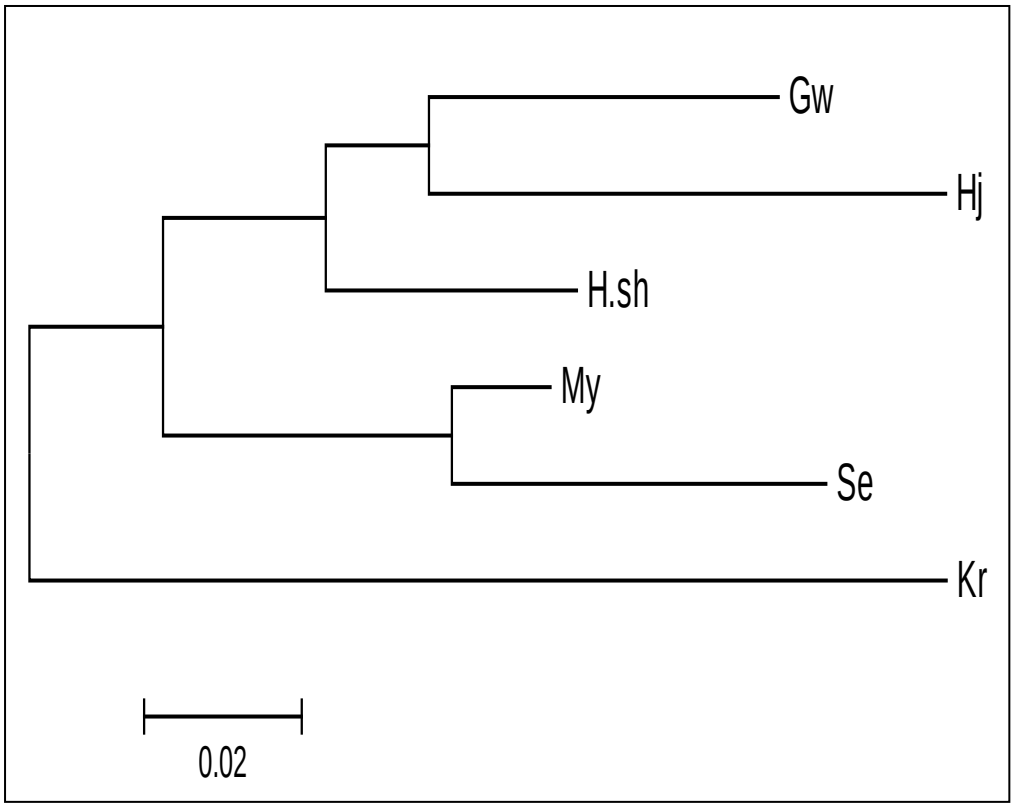


Figure 5.6: Phylogenetic tree based on Neighbour Joining method showing the genetic relationships among the *An. arabiensis* populations of Sudan

5.4 Discussion

5.4.1 Allele Frequency and Linkage Disequilibrium

In this study, a set of seven microsatellite markers, specific for *An. gambiae* (Zheng et al. 1996) was used to analyse the population genetics among six *An. arabiensis* populations in Sudan. Allele number per locus ranging from 2 to 12 with an average of 7.6 alleles per locus was concordant to a study by Muturi *et al.*, (2010) who found allele number per locus ranging from 5 to 12 in *An. arabiensis* populations in a rice growing area of Central Kenya. They suggested that the level of allelic polymorphism provided powerful measures to identify population subdivision.

In the current study, the mean observed heterozygosity of alleles per locus ranged from 0.55 to 0.67 for *An. arabiensis*. Donnelly and Townson (2000) who investigated evidence for extensive genetic differentiation among populations of this malarial vector in Eastern Africa found values of 0.65-0.78. This is also similar to those observed in Central Kenya (Muturi *et al*., 2010) of 0.558-0.608 and Madagascar, but higher than those observed in outer eastern Africa Islands of Reunion and Mauritius (Simard *et al*., 1999;Simard *et al*., 2000).

The estimated means of expected heterozygosity (0.55-0.62) are within the range of those obtained by Lehmann *et al.* (2003) who studied the population structure of *An. gambiae s.s*. in Africa. He observed a mean expected heterozygosity of 0.57-0.71. Temu *et al.* (2004) reported microsatellite DNA polymorphism and heterozygosity in the *An. funestus* in east and southern Africa ranging from 0.64-0.78. Vicente *et al.* (2011) documented genetic variation of the malaria vector *An. Atroparvus* in southern Europe ranging from 0.61-0.73.

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Linkage disequlibrium is the non-random association between different loci (Slatkin, 1995). 11.11% out of the overall pairwise comparisons among *An. arabiensis* populations were statistically significant (P<0.05). All populations after Bonferroni correction were significant which suggest the existence of population subdivision. However, in the absence of forces such as genetic drift, population mixing, mutation and natural selection, linkage disequilibrium is predicted to approach zero for an ideal population, (Ohta, 1982). Nghabi *et al*. (2011) using different microsatellite markers for *An. arabiensis* in Southern Tanzania observed high linkage disequilibrium and which they suggested to be the existence of population subdivision. Significant linkage disequilibrium was observed in this study as a reason of heterozygote deficits and this recommend either departure from random mating, for that reason inbreeding or selection for certain genotypes following the changes of ecological and environmental conditions. The population subdivision in this study was also detected by ND5 gene and this could be caused by 1. high linkage disequilibrium and signature of departures from H-W equilibrium between loci, 2. high levels of gene flow between *An. arabiensis* populations, 3. Bayesian probability distribution at K = 2. This obviously shows that *An. arabiensis* gene pool in these populations is not homogeneous.

5.4.2 Population differentiation and population structure

The population pairwise F_{ST} values ranged from 0.06 to 0.24. These values divided the six populations into two groups as defined by F_{ST} values of; Group 1 comprising of Kr and Se with intra and inter group F_{ST} values of 0.16- 0.24 (high genetic differentiation)and Group 2 comprising of H.sh, My, Hj and Gw with pairwise intra-group F_{ST} values of 0.08-0.13 (moderate genetic differentiation). This was in agreement with the Bayesian cluster analysis performed with STRUCTURE

showing that the most likely *K* value identified was *K*=2.The K 1 group includes Hj, Gw, My and H.sh populations and K 2 include Se and Kr populations.

The F_{ST} from pooled loci reported in this study was inconcordance with the reported F_{ST} values for *An. arabiensis* from Ethiopia and Eritrea (Nyanjom *et al*., 2003) and Northern Sudan (Azrag, 2007). Both studies detected low F_{ST} but statistically significant genetic structure in *An. arabiensis* populations. However, Donnelly and Townson (2000) did not detect significant genetic structure for *An. arabiensis* populations within Malawi and Sudan. Chen *et al.* (2004) also detected a low, but significant, genetic structure of *An. gambiae* in Lake Victoria islands (F_{ST} = 0.019) and among the six villages in the mainland (F_{ST}= 0.010).

A high level of genetic differentiation was found in *An. arabiensis* between certain populations in the current study in agreement with that observed between the Reunion and Mauritius Islands (F_{ST} ranged from 0.080 to 0.215), located 240 km apart in East Africa (Simard *et al*., 1999). Similarly, Ng'habi *et al*. (2011) detected high levels of genetic differentiation among *An. arabiensis* populations (mean F_{ST} = 0.066). This was not in agreement with *An. gambiae s. s*.,(mean F_{ST} = 0.006)which was genetically undifferentiated across the 6,650 km2 of the Kilombero valley landscape in southern Tanzania. Kayondo *et al.* (2005) studied genetic structure of *An. gambiae* populations among islands in north-western Lake Victoria, Uganda with F_{ST} ranging from 0.014–0.105 and concluded that these populations were significantly genetically differentiated. Similar to F_{ST} values of 0.20 and 0.30 as described by Vicente *et al.*, (2011), Kamau *et al.*, (1998) observed F_{ST} values of 0.25 while Walton *et al.*, 2001, who studied genetic population structure and introgression in *An. dirus* mosquitoes in South-east Asia, reported F_{ST} value of 0.21-0.39. In the

present study, Kr was highly differentiated from the other populations may be due to its ecology which was far from agriculture areas compared to the others which were near or within agriculture areas. Kr population is presumably reproductively isolated due to nonrandom mating or ecologically isolated as an effect of the movement of the people from Eithyopia to Kr. AlGash River which acts as a physical barrier separating Kr from other populations. Coluzzi (1982) hypothesised that inversions may play an important role in the isolation process among species in the *An. gambiae* complex and between the various forms of *An. gambiae* s.s. He postulated that inversions can group co-adapted gene complexes that confer adaptation in temporarily isolated peripheral populations with marginal ecological conditions. When secondary contact with the source population occur, these inversions protect the co-adapted gene complexes from recombination, resulting in stable inversion polymorphisms and/or expansions of the population into new habitat, finally resulting in a more permanent isolation. Therefore in the present study the populations are highly differentiated.

There is evidence that R_{ST} may not be the best estimator of population substructure for microsatellites data. Forbes *et al.* (1995) studied microsatellite evolution in congeneric mammals of the domestic and bighorn sheep and found that F_{ST} was more sensitive to differences between allopatric populations compared to R_{ST}. Perez-Lezaun *et al.* (1997) conducted studies on microsatellite variation and the differentiation of modern humans and observed that genetic distance methods such as F_{ST} which do not take into account mutational relationships among alleles and which are associated with occurrence of differentiation through drift, have been shown to be better estimators of patterns of human evolution than do R_{ST} which are calculated based on distance methods. Schug *et al.* (1997) suggested that mutation rates at

microsatellite loci are lower than had been previously assumed. By that reasoning, Donnelly *et al* (1999) concluded that if the East African *An. arabiensis* populations had experienced a lower mutation rate and only became isolated very recently, R_{ST} estimators may not be sufficiently sensitive to detect any differentiation. Constrains upon accumulation of mutations in each population and a lower rate of mutation at these loci would increase the influence of drift on allele frequencies. The present study suggests that F_{ST} is a more appropriate estimator than R_{ST} for recently diverged populations and therefore low mutations can be detected, using F_{ST}. Kr population was found to be differentiated from the other populations and reproductively isolated but this was not detected using R_{ST}.

High genetic similarity between My and Hj which are geographically close to each other was observed, suggesting that they may represent a single population or gene pool. According to Francois and Nicolas (2002), neighbouring populations are expected to be genetically more related than distant populations and high gene flow generally prevent local adaptation. Consequently human transportation or wind dispersal may be the reason behind the continuous gene flow between these two localities and to a lower degree, two others namely GW and H.sh.

The high gene flow found between these particular populations in this study is interesting with regards to the spread of insecticide resistance in Sudan. It is concordant with Kent *et al.* (2007) who studied spatial and temporal genetic structure of *An. arabiensis* in Southern Zambia. They observed high gene flow between Macha and Namwala populations, in Southern Zambia. This was also in agreement with Muturi *et al.* (2010) who found high gene flow among the three populations of *An. arabiensis* in Central Kenya. Failloux *et al.* (1997) detected a significant

correlation between gene flow and commercial traffic by planes and/or boats between islands on *Aedes polynesiensis* populations from islands in French Polynesia.

The Mantel test showed that no isolation by distance was found in this study which was similar with that reported by Nyanjom *et al*. (2003) in populations of *An. arabiensis* from Ethiopia and Eritrea. Isolation by distance could occur due to the limited flight range of *An. arabiensis* (Adamas, 1940) but this was not the case for this study. It is known that the distributional range of any species is largely shaped by historical events. The species will extend its range until it reaches a physical (mountain ranges, deserts and major geographic feature) or other forms of barriers (example climatic changes). However, there are no variable ecological zones, or great physical barriers which could have led to population structuring. These results points to great impact of transportation in the genetic structure of *An. arabiensis* along the River Nile. Thus, presumably, any differentiation could largely be due to other factors than distance.

A similar observation was seen within *An. atroparvus* (Vicente *et al.*, 2011) where no correlation between geographic or genetic distances was detected in a study conducted in southern Europe. In agreement, Kamau *et al.* (1999) using microsatellite loci revealed that there were no significant relation between geographic and genetic distance in *Anopheles arabiensis* and *An. gambiae* suggesting that levels of genetic differentiation are not related to geographical distance and not associated to the side on which populations were sampled in relation to the Rift Valley. This finding is in contrast with Azrag (2007) who found that there was significant genetic differentiation related to geographic distance in their study on *An.*

arabiensis in northern Sudan. Samples showed increasing differentiation with increasing geographical separation. Chen *et al.*, (2004) who studied population genetic structure of *An. gambiae* mosquitoes on Lake Victoria islands, west Kenya revealed a significant correlation between geographic distance and pairwise distance. On the other hand, Failloux *et al.*, (1997) found no significant effect of geographic distance on the population genetic structure on *Aedes polynesiensis* populations from islands in French Polynesia in contrast to the genetic structuring pattern of *Culex pipiensquinques fasciatus* from the same islands. In the latter species, genetic differentiation increased considerably ($P < 0.01$) with geographic distance (Pasteur *et al*., 1995). These differences may be due to the variable biology of the two species as well as their histories of colonization.

5.4.3 Population size bottleneck

No severe bottleneck or reduction in population size was detected in the *An. arabiensis* populations of Sudan except in AlHajYousif (Hj). This was evident from the significance in Wilcoxon sign-rank test which was also supported by the "shifted mode" allele distribution. In comparison, the other populations had relatively higher rare alleles than common alleles, a sign that these populations are experiencing mutation-drift equilibrium. The situation at AlHajYousif (Hj) is likely due to the effective vector control programme in this area. This finding did not agree with the Muturi *et al*. (2010) who studied the population genetic structure of *An. arabiensis* in central Kenya. They did not find any evidence of genetic bottlenecks in the area under different agricultural practices. Furthermore, there was no evidence of a genetic bottleneck in *An. arabiensis* despite a drastic reduction in mosquito numbers during the drought year in southern Zambia as reported by Kent *et al.* (2007). This is similar to the present study, where there is a reduction in *An. arabiensis* during the

dry season but no occurrence of genetic bottlenecks apart from Hj which is under vector control programmes. Hj; My and Gw in Khartoum state have very strong programmes of malarial control 'Khartoum Malaria Free Initiative' started from 2001 to 2009. The significant achievement in malarial control in Khartoum state is highly evident. For example, the percentage of malaria cases among the followers of health services decreased from 20 percentage in 2001 to just 3.3 percentage in 2008 and the parasitological incidence has gone down from 91 to just 4 per 10,000 population. Another programme was initiated in 2011 and is due to end in 2015. The objective of this initiative is to decrease the number of morbidity and mortality of malaria cases by 90% by 2015 in northern Sudan compared to the number of reported cases in 2009. However, results of this study in My and Gw did not show any reduction. This could be explained due to the resistance to insecticide in this areas (*Seidahmed et al.*, 2012). Therefore, no reduction in these populations means they are expanding or developing up quickly.

CHAPTER 6

SUMMARY AND CONCLUSION

The objectives of the current study was to investigate the factors affecting the population distribution patterns, abundance, dispersal at two sites, as well as the phylogenetics and population genetics at six populations of *An. arabiensis* in Sudan.

This study has established that *An. arabiensis* is the most important vector of malaria in Sudan. *Anopheles* populations are naturally subject to environmental factors in which they inhabit. These factors play a major role in controlling their population dynamics, population genetic structure and their effectiveness as vectors. The climatic changes in Sudan would predictably cause fluctuations in the *An. arabiensis* populations. This would ultimately affect the population dynamics and population genetics of these vectors. In the ecological study in the two villages, high density was observed in the Abu Algoni village permanent breeding sites in the whole area. In Algerif West the density was low presumably due to effective control programme of malaria vector conducted in this area.

Mosquito age composition plays an essential position in malaria transmission. Parous females that have previously blood fed; have a higher opportunity of being infected with malaria parasites and then transmitting the disease. The temperature is an importance factor that has an effect on the sporogonic cycle of *An. arabiensis*. When its high, the sporozoites will take a shorter time to develop into infective stage in the

Anopheles. It such circumstances it would be broadcast more quickly from the vector to the host.

The ND5 genetic analysis revealed a general trend of high levels of haplotype diversity but coupled with low nucleotide diversity. All populations showed positive but insignificant value for Tajima's D neutrality tests from neutral expectation, excluding Hj which indicates balancing selection or population substructuring or recent bottleneck for this population. Insignificant Tajima's values indicated that the patterns of molecular diversity among populations were not likely due to selection and rapid range expansion but rather of pure demographic expansion due to malaria controlling programmes applied in the country. No genetic divergence was found between My and Se. Low genetic differentiation was observed between Kr and H.sh. Moderate values were observed between My and Hj; Gw and Se; and Gw and Kr. High genetic differentiation were observed between pairwise Kr and Hj, H.sh and Hj and unexpectedly also between Gw and Hj which are geographically closely related populations. Therefore, overall the F_{ST} phylogenetic tree analysis divided the six populations in two groups; 1. Kr, H.sh (Kassala State) and Gw (Khartoum State) 2. Se (Sennar State), My and Hj (Khartoum State). High gene flow was found between My and Hj; Gw and My which are geographically close populations and surprisingly between My and Se; and Gw and Se which are distantly positioned. The latter observation was possibly a result of human transportation and continuous movement from Se towards Khartoum state to My and Gw for selling their crops, vegetables and animals. As expected the lowest gene flow was found between Gw and H.sh; and Se and

H.sh, which are distant populations. However, unexpected low gene flow was observed between Kr and H.sh which are geographically close and this may be to AlGash River played as a physical barrier between them.. The haplotypes phylogenetic tree divided the populations into two major clades with considerable overlapping among haplotypes of populations even those involving Kr and Se which were generally the most distant populations based on other analyses. Phylogenetic divergence among *Anopheles gambiae* complex inferred that *An. arabiensis* and *An. gambiae* are the most closely related taxa based on the evolutionary sequence divergence of the two species.

The ITS analysis genetic diversity revealed generally high levels of haplotype diversity but low nucleotide diversity similar to ND5. Hj and Se populations showed negative and significant value for Tajima's D neutrality test indicating recent directional selection (selection sweep) or recent population growth with excess of rare alleles. My, Gw, H.sh and Kr were negative but insignificant. No genetic differentiation was found among the six populations. Phylogenetic trees showed homogeneity of 99% for the Sudan and most of the GenBank sequences of African populations, in considerable contrast to the ND5 gene, presumably due to the lower mutational rate in the former.

All microsatellite loci tested were polymorphic excluding AG2H29. The number of alleles at locus AG2H143 was the highest among other loci. This locus is inside the 2La fixed chromosomal inversion (Zheng *et al.,* 1996; Petrarca *et al.,* 2000) and for that reason selection pressure may be influencing variation at this locus.

Microsatellite markers analysis showed that high linkage disequilibrium detected between loci and high genetic differentiation was observed between *An. arabiensis* populations from Kr and other populations F_{ST}= 0.17-0.24. Therefore Kr populations were more genetically isolated from the rest and from each other. Additionally Kr may be reproductively isolated due to non random mating or movement of people from Ethiopia to Sudan. It could also be ecologically isolated as a consequence of the Al Gash River which acts a as a physical barrier separating Kr from other populations. Therefore Kr is easier to control compared to other populations. High gene flow was detected among Se (Sennar State) with My and Gw (both Khartoum State); Hj and My (both Khartoum State). Very little gene flow was observed between Kr (Kassala State) and other populations. All populations had not experienced population bottleneck. However, population Hj with shifted mode suggest recent population size reduction. The phylogenetic tree showed that populations Hj, GW and H.sh, My and Se are genetically closely related, while Kr appeared to be comparatively differentiated from other populations.

The ND5 genes showed high levels of haplotype diversity in all populations but lowest nucleotide diversity. While, ITS gene demonstrated low levels of both haplotype diversity and nucleotide diversity in most populations excluding Se, Hj and My. High genetic differentiation were observed between Kr and all other population using microsatellite markers, while with ND5 gene these were observed between pairwise Kr and Hj, H.sh and Hj and unexpectedly also between Gw and Hj which are geographically closely related populations. In contrast ITS gene showed no genetic differentiation among all populations possibly due to the repetitive structure of rDNA

which is known to spread new alleles much faster through a population than other markers. In the case of Hj population based on ND5, Tajima's value indicated recent bottleneck which was confirmed by microsatellite markers, which suggest recent population size reduction in Hj unlike in the other populations. The phylogenetic tree using ND5 positioned Kr with H.sh and Gw, while microsatellite markers isolated Kr. The population subdivision suggested by the ND5 gene and microsatellite markers explained that *An. arabiensis* populations in Sudan are not homogeneous. The differences in the observed level of genetic differentiation among ND5, ITS genes and microsatellite markers suggest that the mutational rates varies significantly among different regions within the genome. More extensive studies on population structure and genetics of *Anopheles arabiensis* in the other regions in Sudan using additional microsatellite loci and obtaining more information on factors that may affect gene flow are recommended for a comprehensive control programme of malaria in Sudan.

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Appendix 1.a: Meteorological data of Algerif West (Khartoum State**)** and Abu Algoni **(**Sennar State) during the study period from June 2010 to May 2011.

Algerif West (Khartoum State)				**Abu Algoni (Sennar State)**		
Month	Mean Temp °C	Mean Hum %	Rainfall mm	Mean Temp °C	Mean Humid %	Rainfall mm
June 2010	36.3	24	7	32.2	44.7	14
July	32	42.9	84	29.4	57.6	113
Aug	31.4	49.3	72	27	78.8	143
Sept	32.4	39.5	27	27.7	74.2	75
Oct	34.2	25	4	29.4	62.2	25
Nov	31.6	20.8	0	27.7	47.8	0
Dec	26.5	24.2	0	23.9	46.6	0
Jan2011	22.9	23	0	23.3	41.2	0
Feb	27.7	14	0	26.2	31.3	0
March	28.6	11.5	0	27.7	26.6	0
April	32.7	8.1	0	32.8	20	0
May	37.5	18.1	0	34.8	26	0

Appendix 1.b: Abu Algoni correlations output

Correlations

		female	meantemp	meanhumid	meanrainfall
female	Pearson Correlation	1	-.090	.805**	.656*
	Sig. (2-tailed)		.780	.002	.020
	N	12	12	12	12
meantemp	Pearson Correlation	-.090	1	-.304	-.053
	Sig. (2-tailed)	.780		.336	.871
	N	12	12	12	12
meanhumid	Pearson Correlation	.805**	-.304	1	.789**
	Sig. (2-tailed)	.002	.336		.002
	N	12	12	12	12
meanrainfall	Pearson Correlation	.656*	-.053	.789**	1
	Sig. (2-tailed)	.020	.871	.002	
	N	12	12	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Correlations

		meantemp	meanhumid	meanrainfall	larvae
meantemp	Pearson Correlation	1	-.304	-.053	-.105
	Sig. (2-tailed)		.336	.871	.744
	N	12	12	12	12
meanhumid	Pearson Correlation	-.304	1	.789**	.798**
	Sig. (2-tailed)	.336		.002	.002
	N	12	12	12	12
meanrainfall	Pearson Correlation	-.053	.789**	1	.813**
	Sig. (2-tailed)	.871	.002		.001
	N	12	12	12	12
larvae	Pearson Correlation	-.105	.798**	.813**	1
	Sig. (2-tailed)	.744	.002	.001	
	N	12	12	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		parity	meantemp	meanhumid	meanrainfall
parity	Pearson Correlation	1	-.399	.327	.363
	Sig. (2-tailed)		.198	.299	.246
	N	12	12	12	12
meantemp	Pearson Correlation	-.399	1	-.304	-.053
	Sig. (2-tailed)	.198		.336	.871
	N	12	12	12	12
meanhumid	Pearson Correlation	.327	-.304	1	.789**
	Sig. (2-tailed)	.299	.336		.002
	N	12	12	12	12
meanrainfall	Pearson Correlation	.363	-.053	.789**	1
	Sig. (2-tailed)	.246	.871	.002	
	N	12	12	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 1.c: Algerif West correlations ouputs

Correlations

		female	meantemp	meanhumid	meanrainfall
female	Pearson Correlation	1	.034	.776**	.550
	Sig. (2-tailed)		.917	.003	.064
	N	12	12	12	12
meantemp	Pearson Correlation	.034	1	.095	.125
	Sig. (2-tailed)	.917		.770	.698
	N	12	12	12	12
meanhumid	Pearson Correlation	.776**	.095	1	.865**
	Sig. (2-tailed)	.003	.770		.000
	N	12	12	12	12
meanrainfall	Pearson Correlation	.550	.125	.865**	1
	Sig. (2-tailed)	.064	.698	.000	
	N	12	12	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		meanhumid	meantemp	meanrainfall	larvae
meanhumid	Pearson Correlation	1	.095	.865**	.542
	Sig. (2-tailed)		.770	.000	.069
	N	12	12	12	12
meantemp	Pearson Correlation	.095	1	.125	.182
	Sig. (2-tailed)	.770		.698	.571
	N	12	12	12	12
meanrainfall	Pearson Correlation	.865**	.125	1	.256
	Sig. (2-tailed)	.000	.698		.421
	N	12	12	12	12
larvae	Pearson Correlation	.542	.182	.256	1
	Sig. (2-tailed)	.069	.571	.421	
	N	12	12	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		parity	meanhumid	meantemp	meanrainfall
parity	Pearson Correlation	1	.613*	-.389	.382
	Sig. (2-tailed)		.034	.211	.220
	N	12	12	12	12
meanhumid	Pearson Correlation	.613*	1	.095	.865**
	Sig. (2-tailed)	.034		.770	.000
	N	12	12	12	12
meantemp	Pearson Correlation	-.389	.095	1	.125
	Sig. (2-tailed)	.211	.770		.698
	N	12	12	12	12
meanrainfall	Pearson Correlation	.382	.865**	.125	1
	Sig. (2-tailed)	.220	.000	.698	
	N	12	12	12	12

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

LIST OF PUBLICATIONS & SEMINARS

1. Mashair, S. M., Abukashawa, S., Zairi, J. & Siti Azizah, M.N. Polytene chromosome of the malaria vector *Anopheles arabiensis* Patton in Sudan**.** The MSPTM 46th Conference Kuala Lumpur 24-26 March 2010. ORAL PRESENTATION.

2. Mashair, S. M., Zairi, J., Siti Azizah, M. N. & Abu Kashawa, S. Studies on the ecology of anopheline mosquitoe in Sennar State, Sudan. The ICWSET Dhaka 2 – 4 Feb 2012. ORAL PRESENTATION.

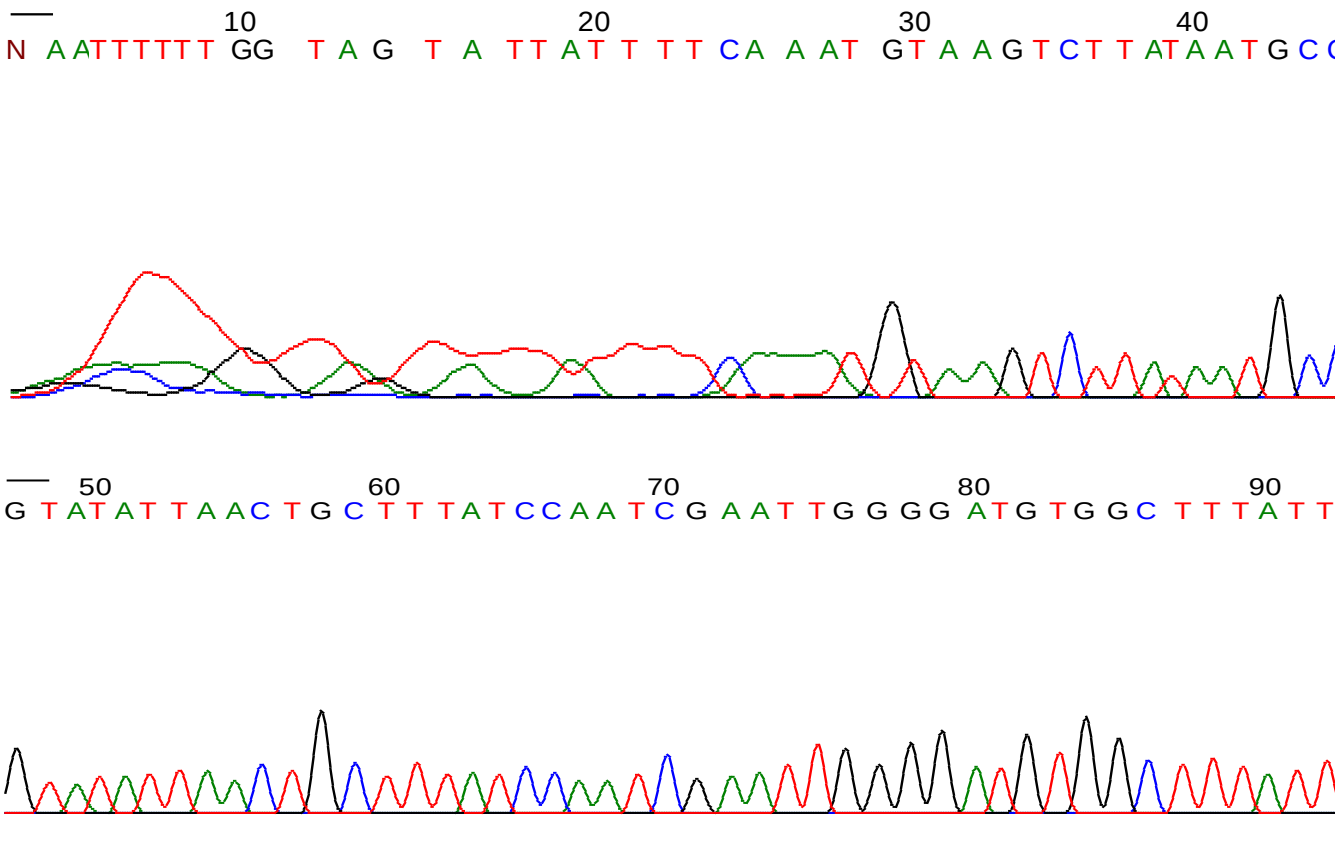
3. Mashair, S. M., Zairi, J., Siti Azizah, M. N. & Abu Kashawa, S. Entomological indices of *Anopheles arabiensis* at Algerif West Khartoum state, Sudan. The MSPTM 48th Conference Kuala Lumpur 27-28 march 2012. ORAL PRESENTATION.

4. Seidahmed, O. M. E., Abdelmajed, M. A., Mustafa, M. S. & Mnzava, A. P. 2012. Insecticide susceptibility status of the malaria vector *Anopheles arabiensis* in Khartoum city, Sudan: differences between urban and periurban areas. *Eastern Mediterranean Health Journal*, 8**(**7**):** 769-776.

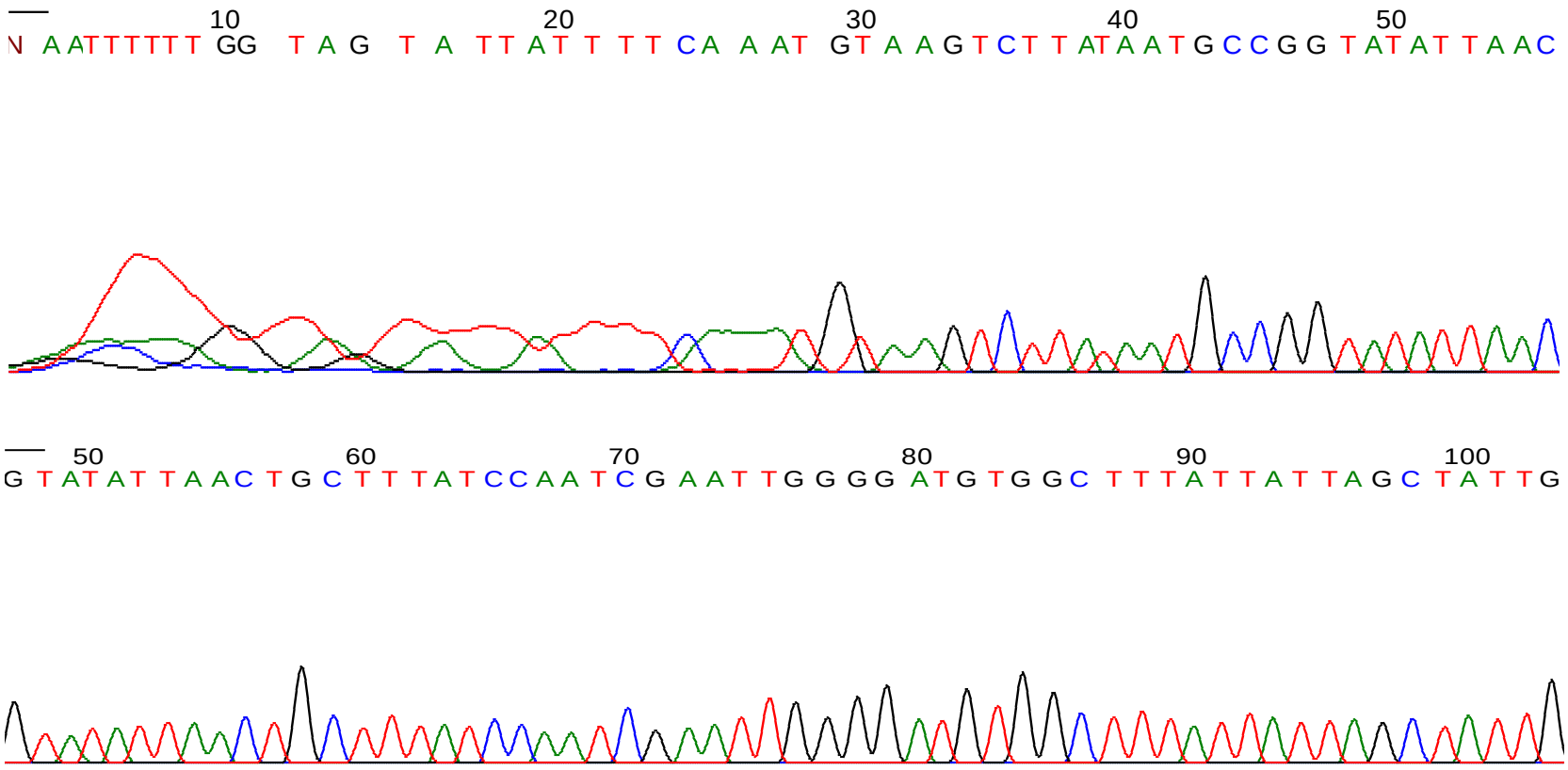
Mashair, S. M., Zairi, J., Siti Azizah, M. N. & Abu Kashawa, S. 2013. Population genetics analysis of the Sudanese malaria vector *Anopheles arabiensis* Patton (Diptera: Culicidae). *Plos one*,(under review).

6- Mashair, S. M., Zairi, J., Siti Azizah, M. N., Hamady Dieng & Abu Kashawa Species composition, abundance and population structure of malaria vectors in two villages of Sudan. *Parasites and Vectors*, (under review).

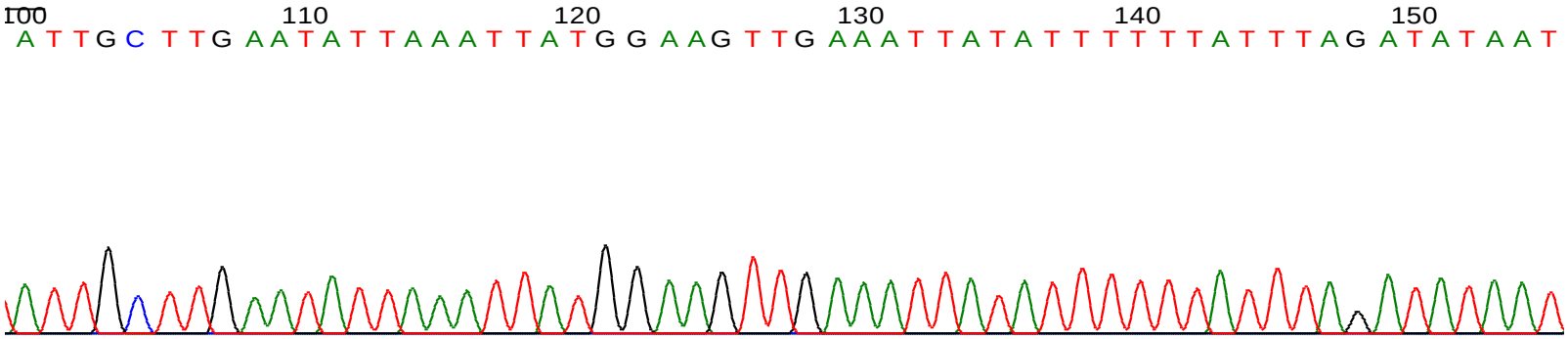
7- Mashair, S. M., Zairi, J., Siti Azizah, M. N. & Abu Kashawa, S Population structure of the Sudanese malaria vector *Anopheles arabiensis* Patton (Diptera: Culicidae) based on mtDNA ND5 gene. Writing.



Appendix 2.a: Chromatography of ND5 sequences.



Appendix 2.a: Chromatography of ND5 sequences.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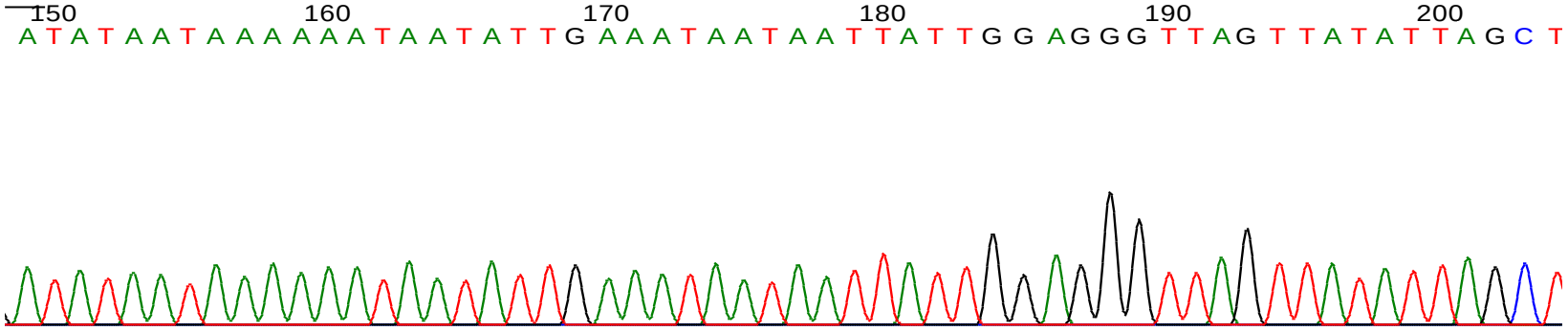
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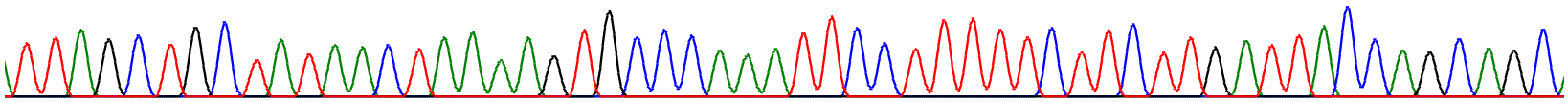

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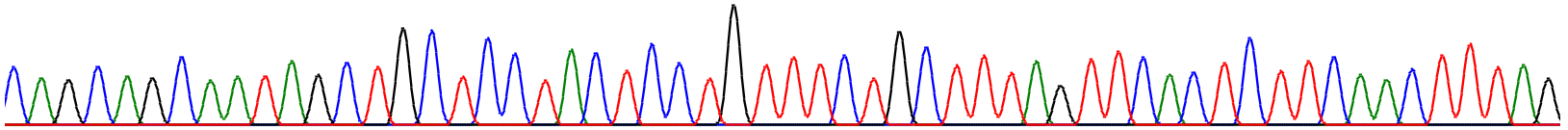

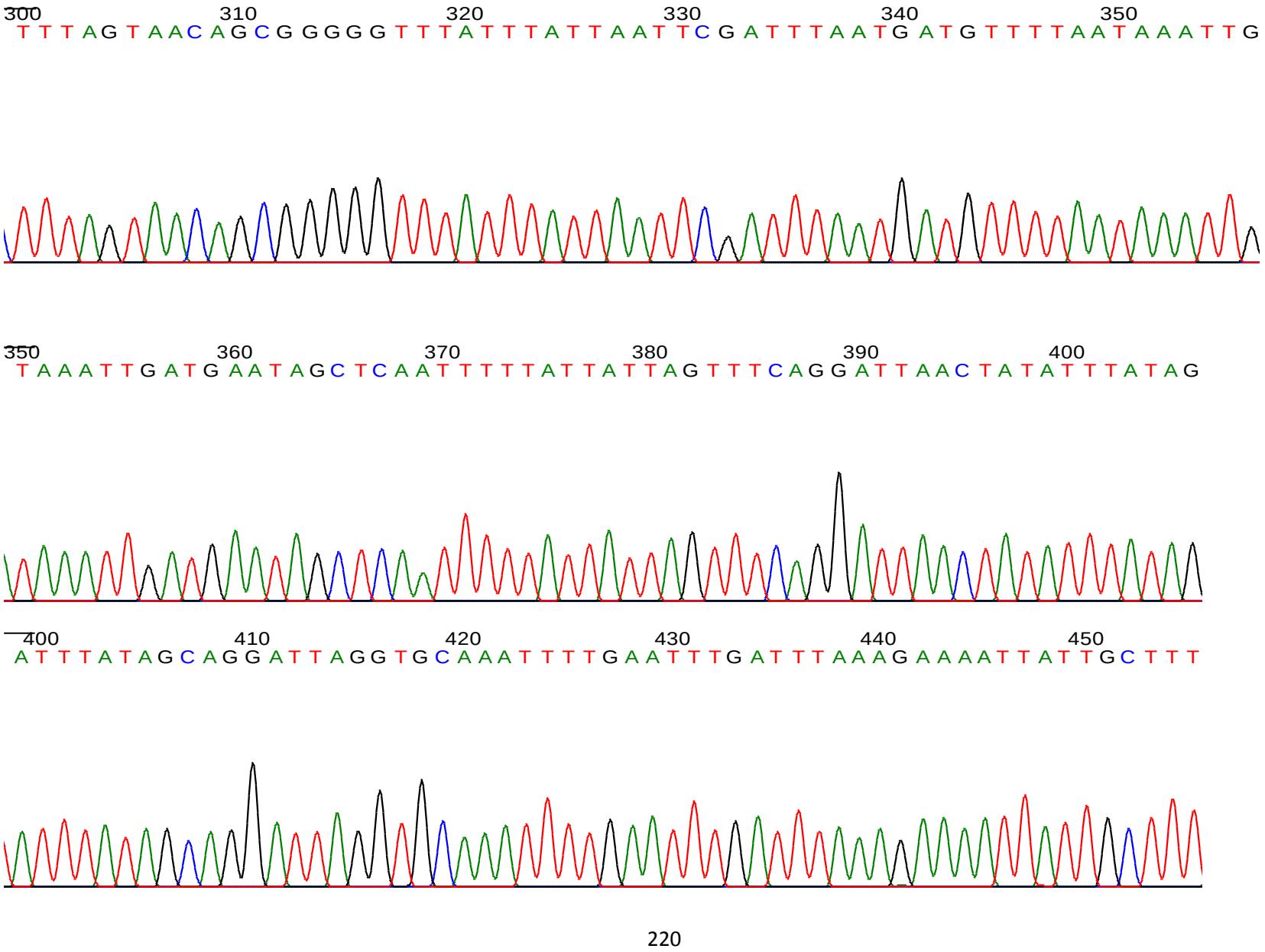
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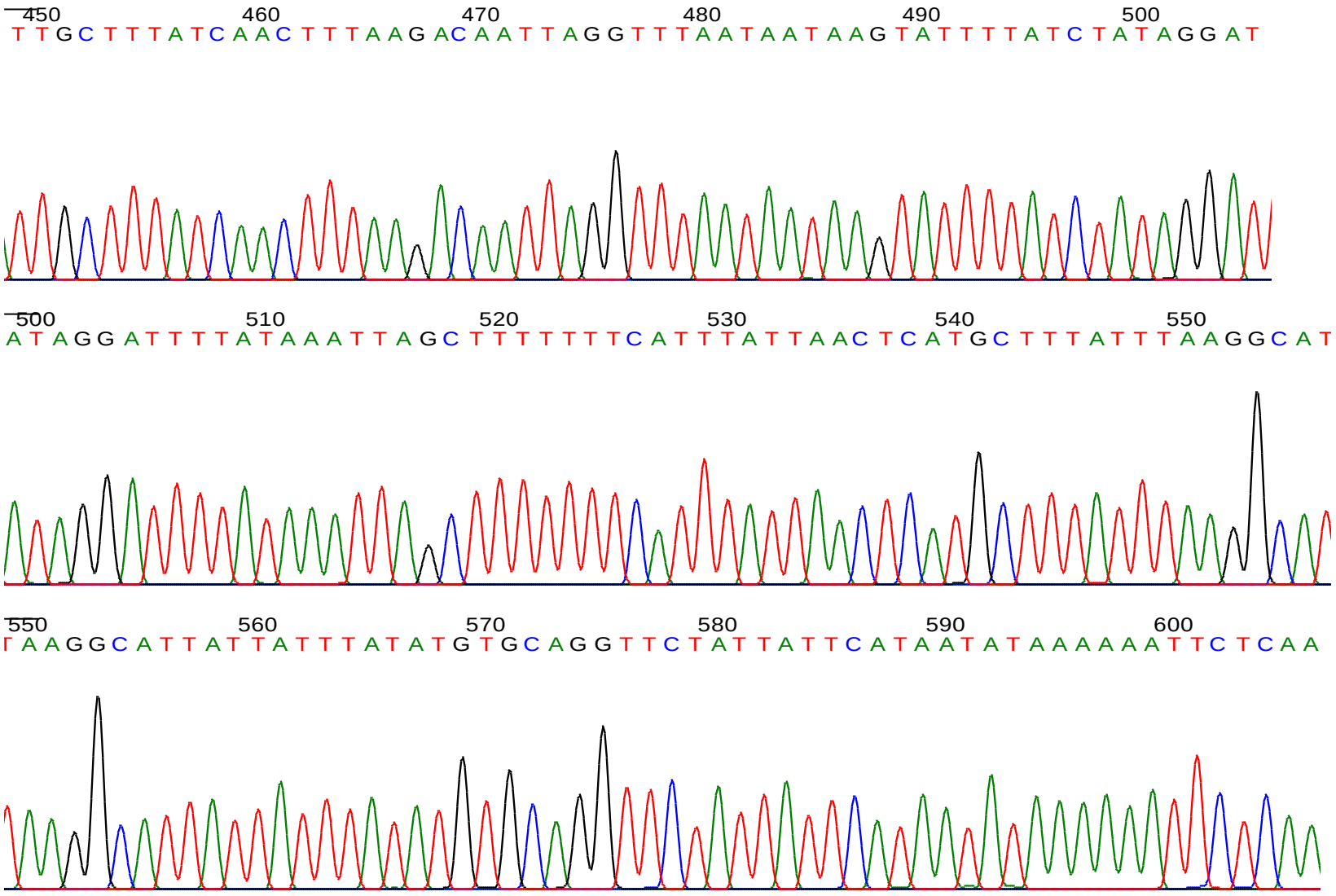
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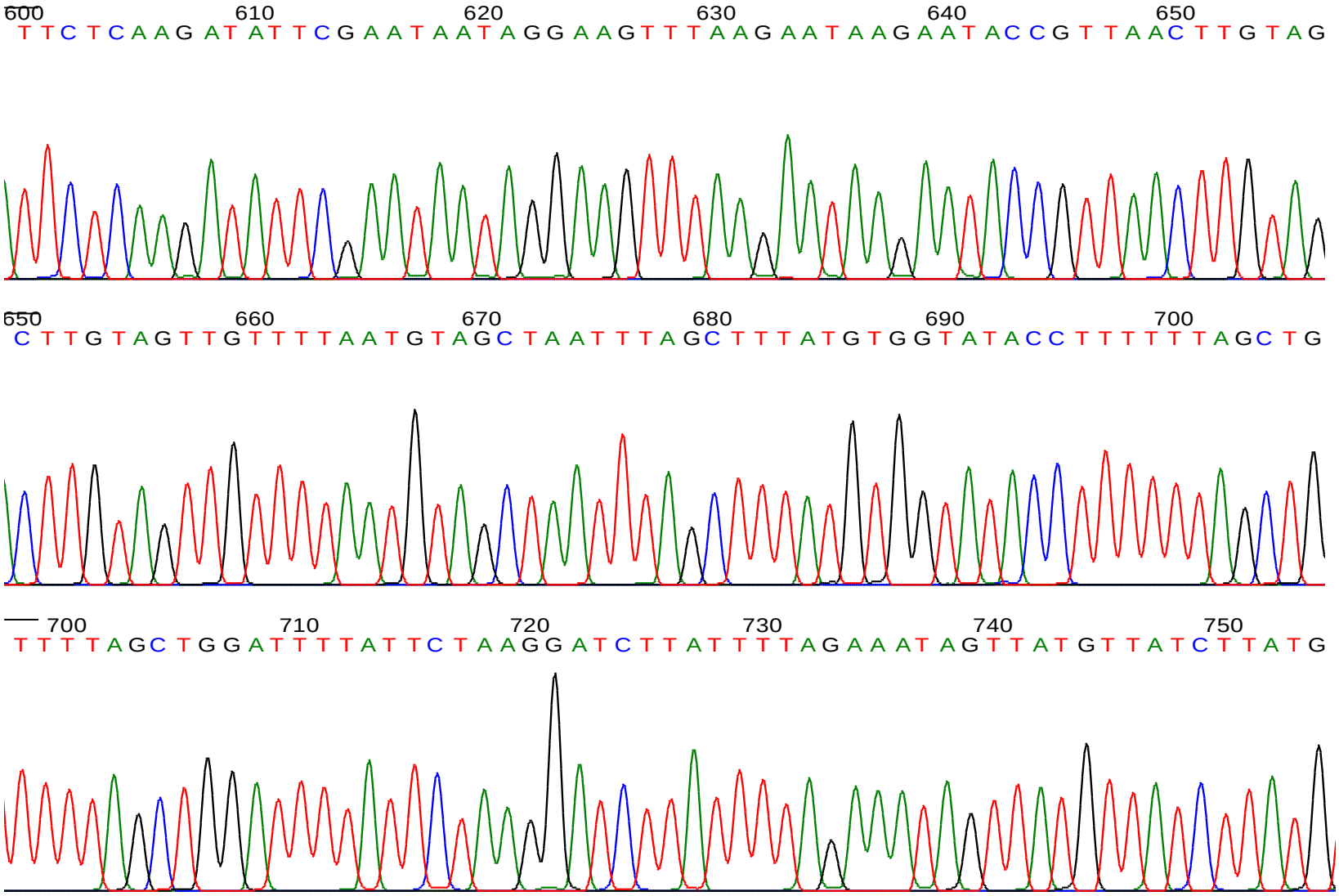
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C T T G T A G T T G T T T T A A T G T A G C T A A T T T A G C T T T A T G T G G T A T A C C T T T T T T A G C T G
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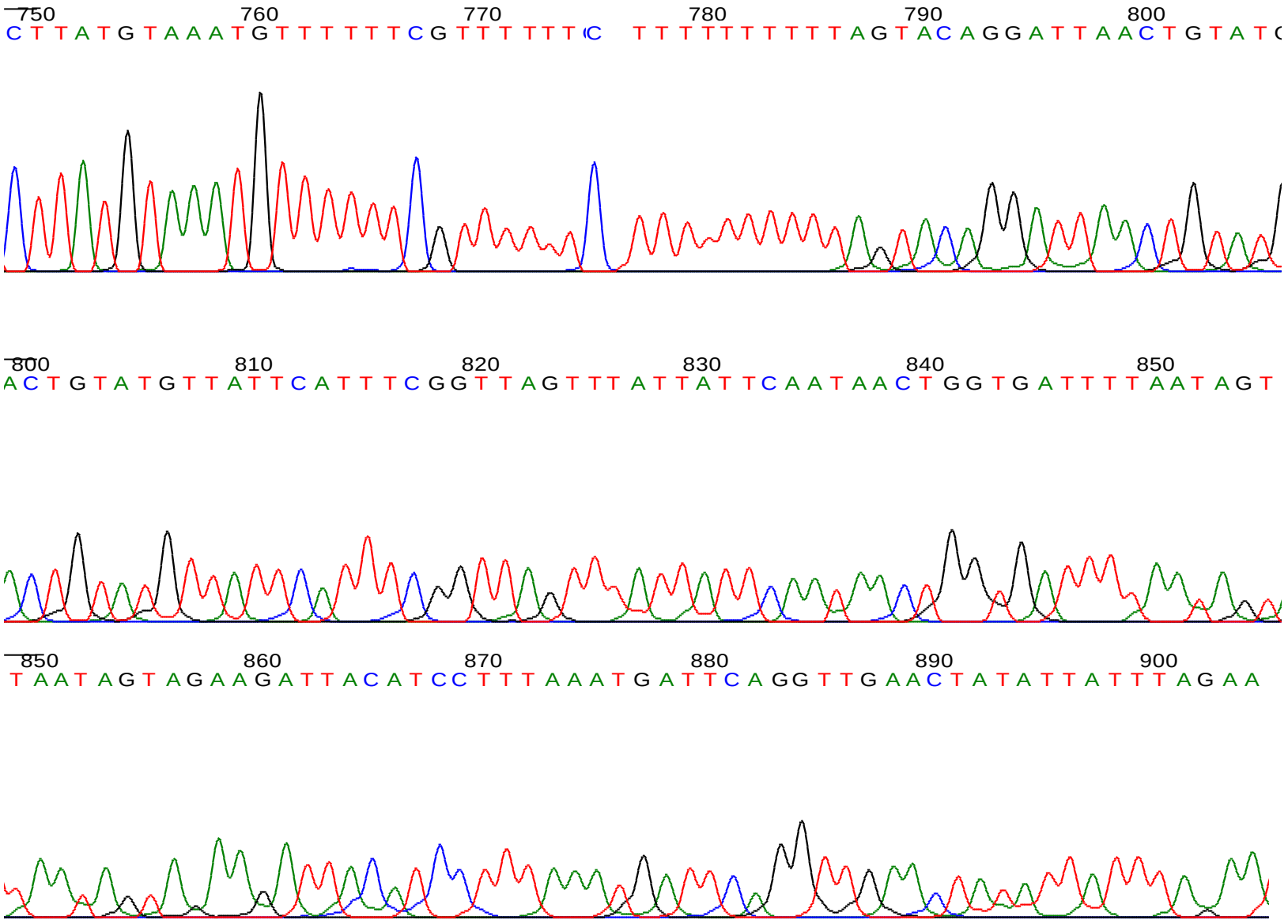
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800 810 820 830 840 850
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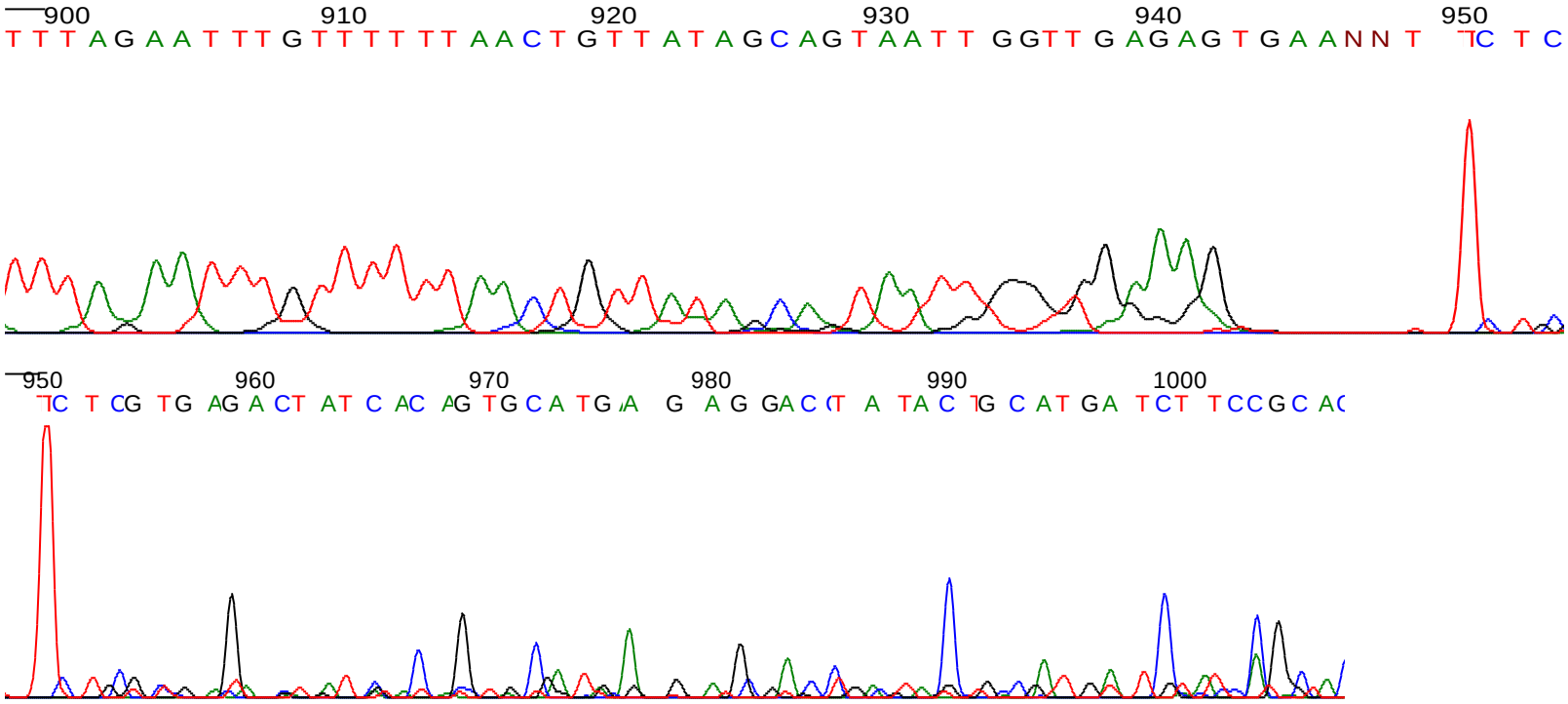
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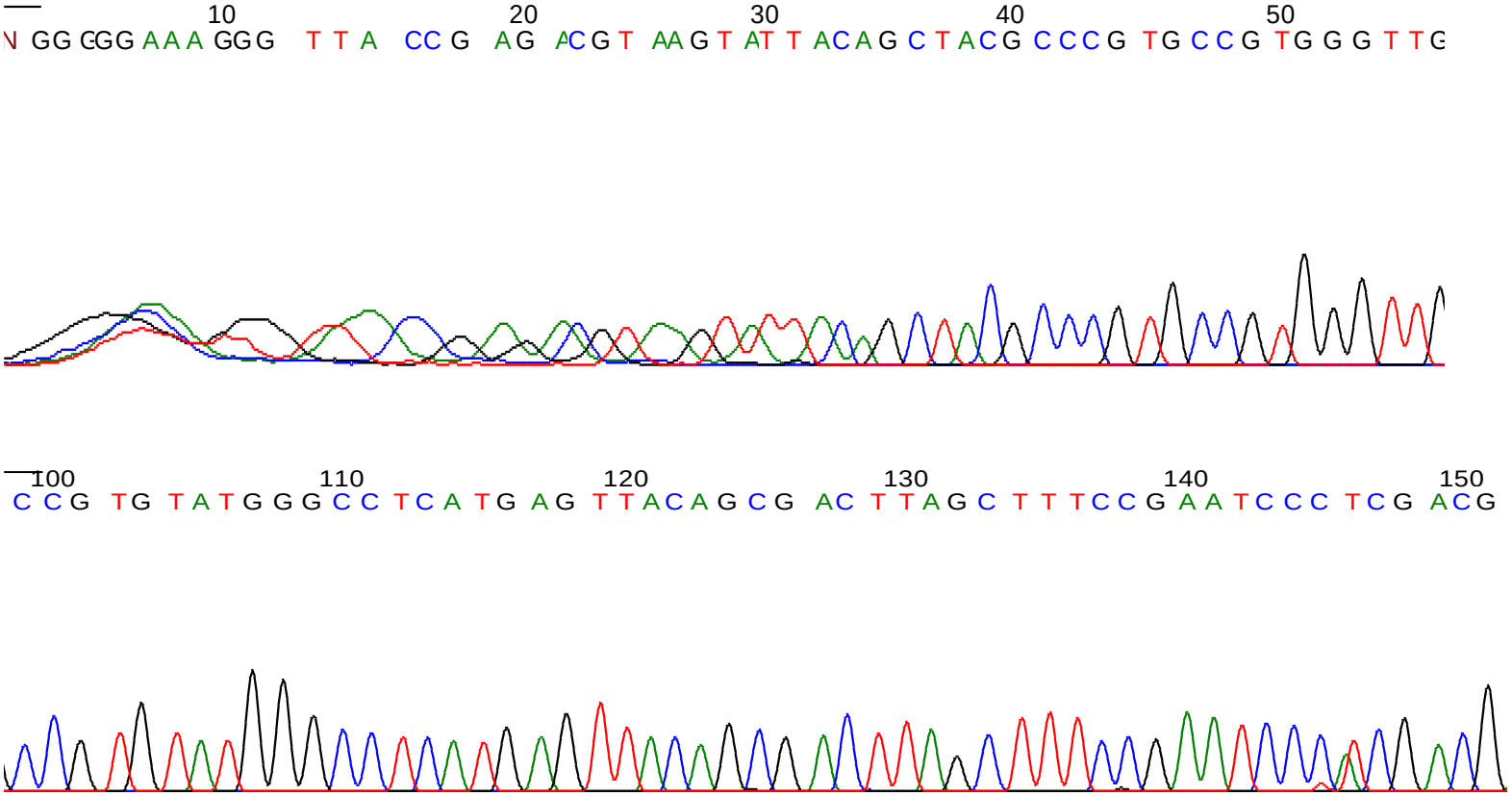
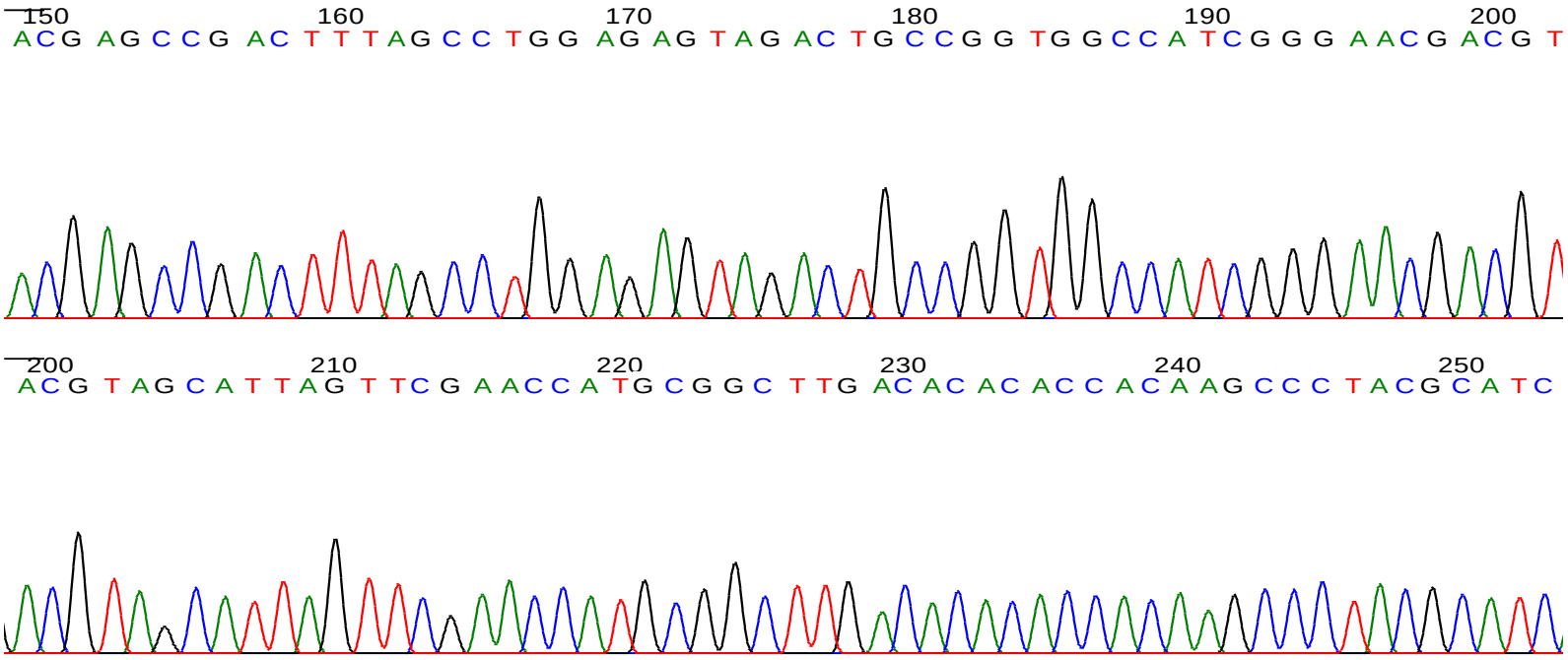


Appendix 2.b: Chromatography of ITS sequences.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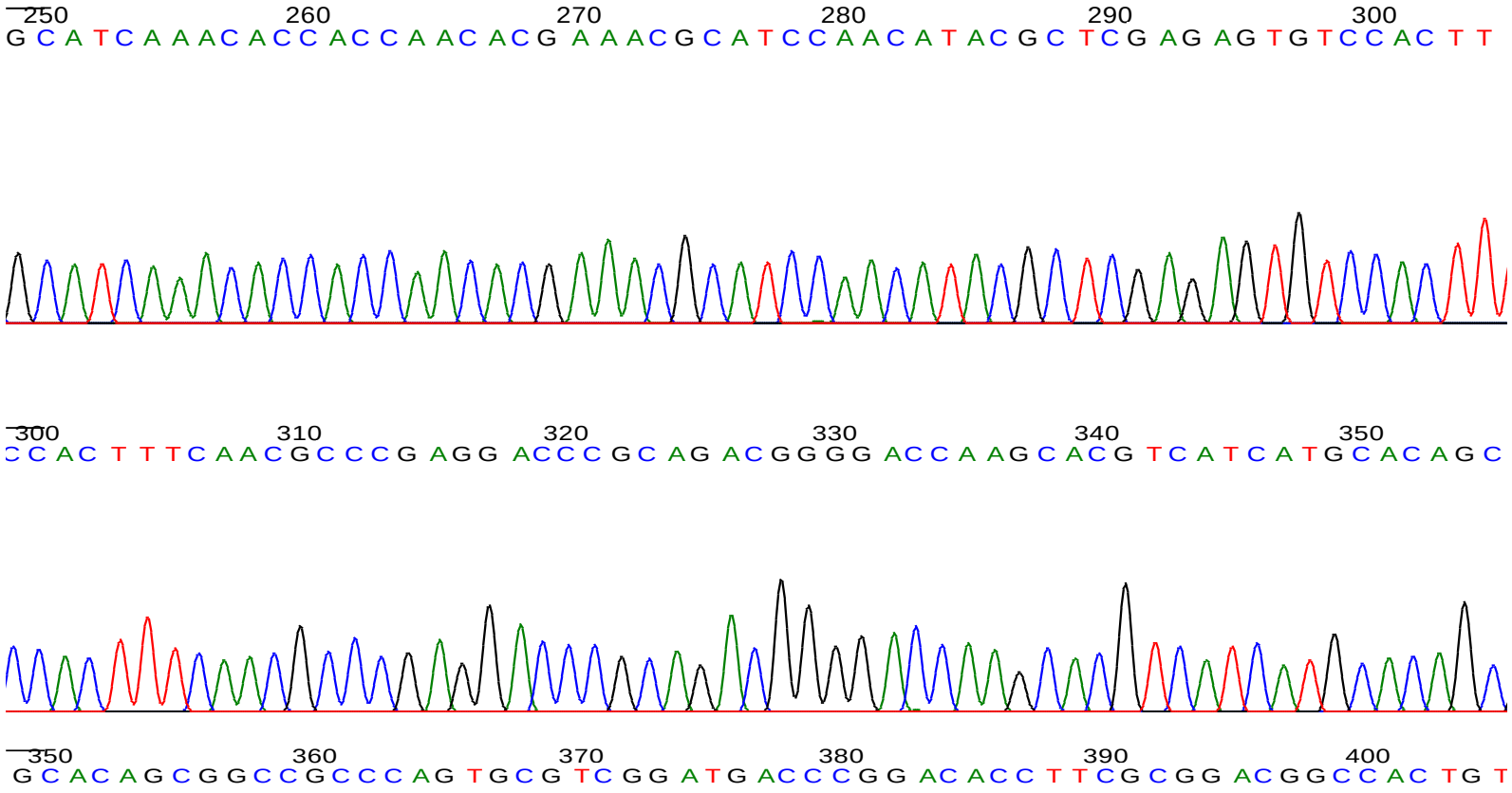
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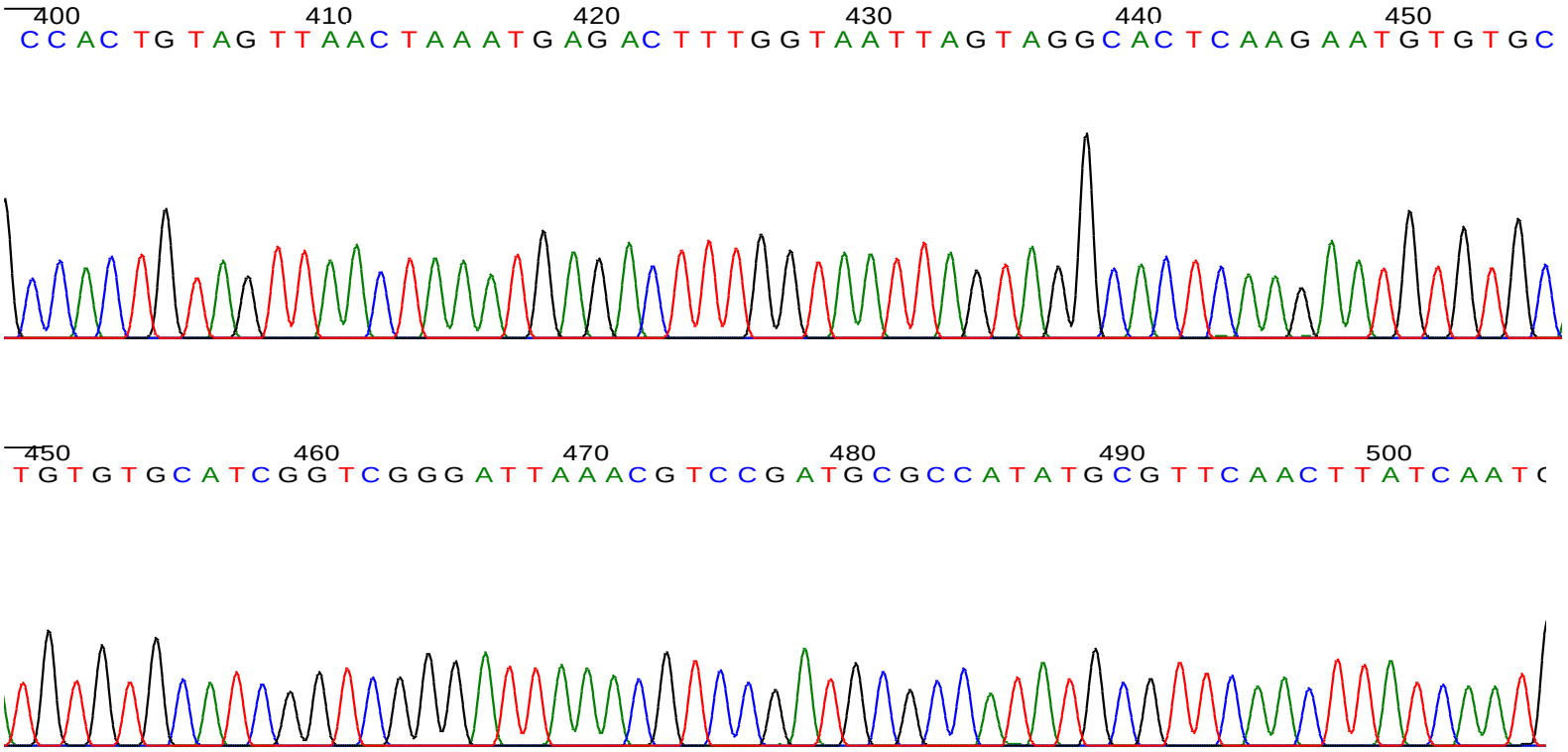
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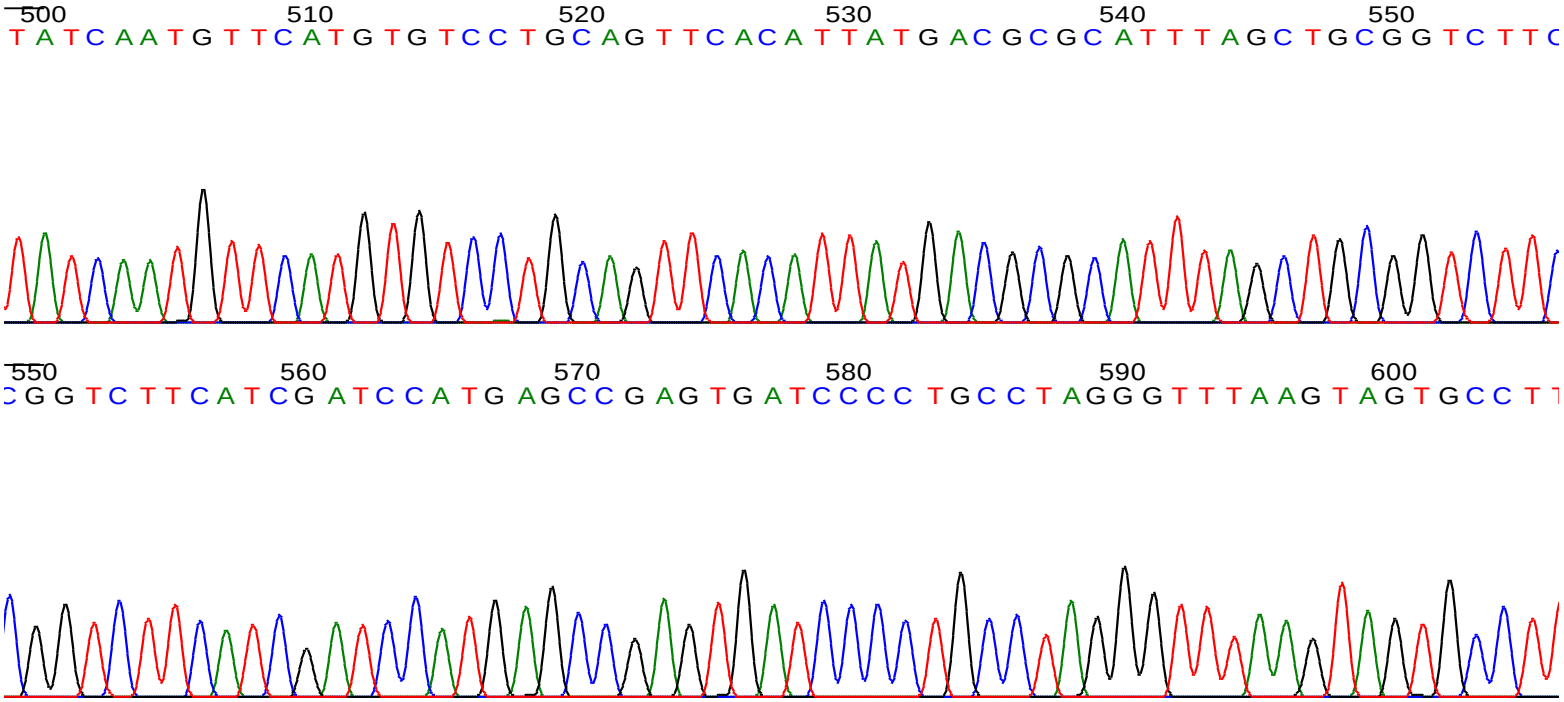
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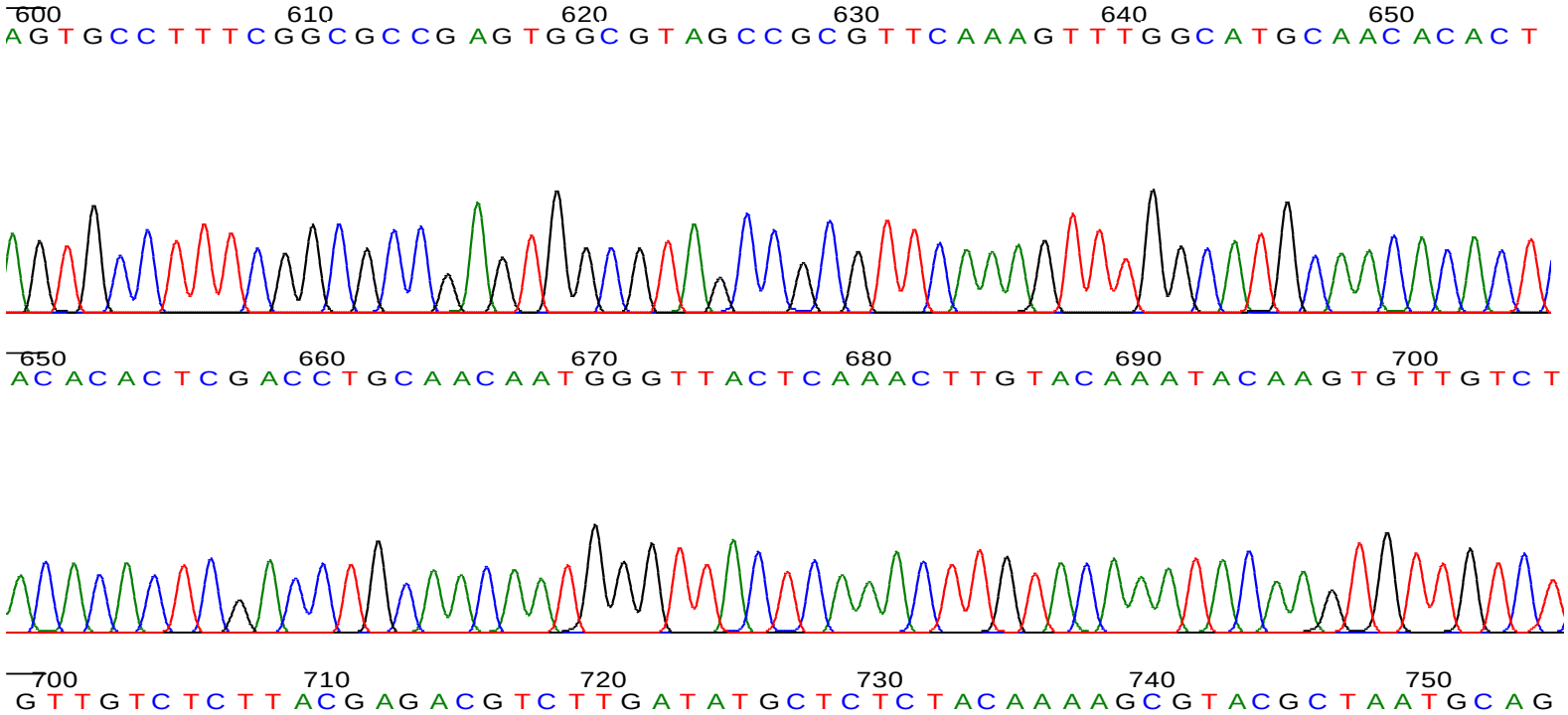
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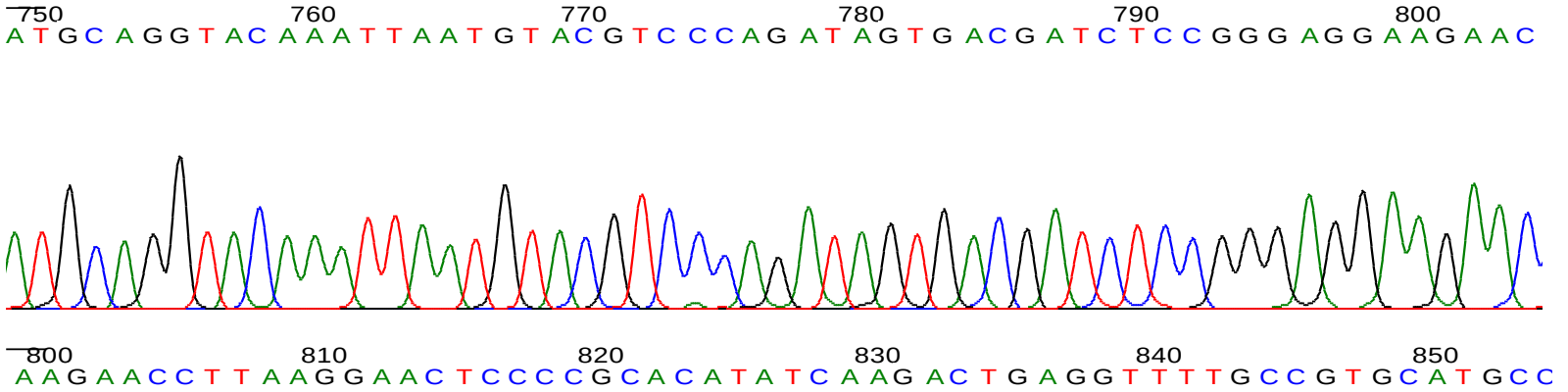
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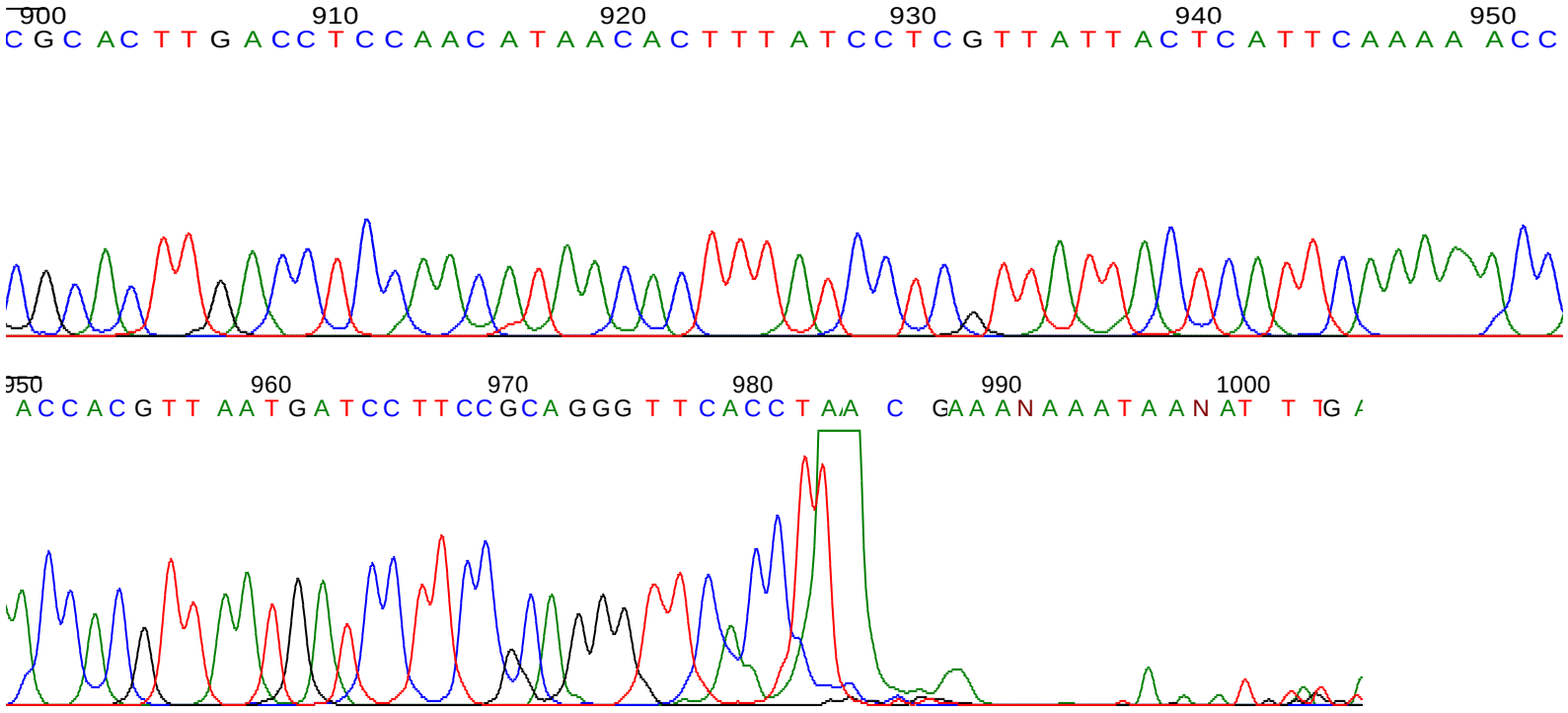
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