FR 1622

Final Report

Contract Number: **RG/2005/HS/09**

Title of the Project: Breeding structure of dengue vector, *Aedes albopictus* in areas at risk of epidemics using RAPD markers.

Internal Memo

From: Dr. Sachie Panawala
To: PIO
Date: 10/09/2012
Subject: Final Report / ~~Thesis~~ of: RG/2005/HS/09

Please find enclosed herewith the

☑ Final Report of the Research Grant: (02 copies)

☐ MD Thesis ☐ MPhil Thesis ☐ PhD Thesis

☐ Soft Copy of the Final Report

Title of the Project: Breeding structure of dengue vector, Aedes albopictus in areas ~~of~~ risk of epidemics using RAPD markers.

Grantee/s: Dr. AA Rasnatilleke

Title of the Thesis: —

Author: -

Thank you.

Section 1

Information regarding Project/Project Personnel

i) **Contract Number:** RG/2005/HS/09

ii) **Title of the Project:** Breeding structure of dengue vector, *Aedes albopictus* in areas at risk of epidemics using RAPD markers.

iii) **Principal Investigator:** Dr. A.A.L. Ratnatilleke

iv) **Co-Investigators:** Nil.

v) **Institute where Research is being carried out:** Department of Chemistry, University of Kelaniya

vi) **Date of award:** ...01.../...06... / ...2005.....

vii) **Date of completion of project:** ...26.../ 11 /...2009.....

viii) **Total allocation of funds:** Rs...2,103,950.00.....

ix) **Total spent:** Rs...2,081,030.52.....

x) **Number of Research Students employed:** One

xi) **Post graduate degree completed with dates:** 23 / 08 / 2010

xii) **Number of Technical Assistants and/or laborers employed and period of service:** Nil.

Section 2

Executive Summary of the project

Dengue fever is currently considered to be the most serious vector-born disease in Sri Lanka. *Aedes aegypti*, and *Aedes albopictus* have been implicated to be the most important vectors of dengue transmission. To understand the transmission of the disease, knowledge of the population (breeding) structure, magnitude of dispersal (gene flow) among *Aedes* mosquito populations is highly important, as it directly influences dengue virus transmission. Random amplified polymorphic DNA (RAPD) analysis was undertaken to examine breeding structure in 19 *A. albopictus* populations from three highly threatened districts in Sri Lanka, namely Colombo, Gampaha and Kandy, with a distance range extending ~100 km. The data were used to determine magnitude of gene flow, genetic diversity, genetic differentiation among *A. albopictus* populations at the macro (among districts) and micro (among cities within district) geographical levels. Allele and genotype frequencies were measured on 62 RAPD loci, assuming that RAPD alleles segregate as dominant markers and the genotype frequencies at those loci are in Hardy–Weinberg equilibrium. The investigation showed that *A. albopictus* appears to be the dominant mosquito species of the sampled areas of each district. The level of gene flow among *A. albopictus* populations within Colombo district is moderate (Nm = 1.6711 mosquitoes per generation). Highest values of gene flow were observed in Gampaha district (Nm = 2.7096) with lowest values from Kandy district (Nm = 0.9955). High levels of population differentiation (structuring) was found in both among districts (Gst = 0.2969; Nm = 1.1841; θ = 0.1915) and among cities of each district (Gst =0.1558 -3343). The intra population mean gene diversity, Hs, in each district is also high and ranged from 0.2009-0.2637. Nei's mean gene diversity, He, in each district ranged from 0.2722 - 0.3173. Cluster analysis based on Nei's genetic distance, indicate that populations from neighboring areas in each district are clustered according to their geographical origin. The result indicate a significant population differentiation and structuring of *A. albopictus* mosquitoes in Sri Lanka, which may be attribute to dynamic equilibrium between factors that favor differentiation and homogenizing factors in recently established populations.

Section 3

Report in Detail

i. INTRODUCTION

1. 1 General introduction

Dengue fever is currently considered to be the most serious mosquito borne arboviral disease, in several tropical and subtropical countries in Asia, Africa and America. It causes more illness and deaths than any other arboviral infection (Malavige et al., 2004).

Dengue fever was first reported in Sri Lanka in 1965, but has become a regular epidemic since 1989. Dengue infections are now well-established in Sri Lanka. The prevailing climatic conditions, environmental pollution, rapid urbanization, over-crowding of cities and careless human practices are proving conducive for the rapid breeding of the mosquito vector and the spread of this infection (Kularatne et al., 2006). Dengue is prevalent mostly in the urban areas like Colombo as well as Gampaha, Kandy and Kurunegala districts. The peak incidence of the disease generally comes after monsoon season, when the density of the two mosquito carrier species- *Aedes aegypti* and *Aedes albopictus* is especially high.

Aedes albopictus known as the Asian 'tiger' is a mosquito that originates from the tropical forest of Southeast Asia. The area is also considered to be the origin of the dengue virus. Presence of *A. albopictus* poses a serious public health problem. Firstly, it has an important role in the transmission of several arboviruses and its susceptibility for the viruses is even greater than that of *A. aegypti* (Mitchell, 1995). Secondly, the species could have naturally transmitted the serotypes 2 and 3 of dengue virus vertically (Ibanez – Bernal et al., 1997). Therefore, the increased risk of human infection via this mosquito species has stimulated a great deal of research on all aspects of the biology, mosquito population structure, behavior and vector competence in the past 15 years. However, despite its epidemiological importance, no studies of the population structure of Sri Lankan *A. albopictus* have been published.

The DNA polymorphism detected by random amplified polymorphic DNA (RAPD) has been used successfully to characterize the genetic structure in several mosquito species (West and Black, 1998). Therefore, during this work, an attempt has been made to demonstrate the existence of high levels of population structuring of *A. albopictus* in Sri Lanka.

1.2 Dengue disease

Dengue viruses are mosquito-borne flaviviruses that have plagued people for centuries (Gubler, 1997). Most people infected with dengue viruses are asymptomatic or develop dengue fever (DF) and a minority of people develops its severe form, dengue hemorrhagic fever (DHF). The disease is caused by one of four closely related viral

serotypes, DEN-1, DEN-2, DEN-3 and DEN-4, which are antigenically distinct. Dengue fever and DHF are primarily diseases of tropical and subtropical areas. Starting in the middle of the 20th century, large-scale urbanization and increasing human populations in tropical parts of the world created conditions especially favorable for dengue transmission. These changes led to the current global dengue pandemic, which is characterized by a dramatic increase in dengue infections and an expanding geographic distribution of the viruses and the mosquito vectors, *A. albopictus* and *A. aegypti*. (Gubler, 1997). The first reported epidemics of dengue fever occurred in 1779-1780 in Asia, Africa and North America. Epidemics of DHF first appeared in the 1950s in Southeast Asia, but it had become a frequent cause of hospitalization and death among children in many countries after 1975.

Dengue, caused by all four serotypes is becoming a prominent health problem all over the world (Figure 1.1). Nearly 90% of infections occur in children.

Figure 1.1. World wide distribution of Dengue, 2008 (WHO, 2008)

1.2.1 Dengue in Sri Lanka

Dengue was first reported in Sri Lanka in 1965 (Kulatilaka and Jayakuru., 1998). Since 1989 hundreds of cases of dengue have been reported and dozens have died annually (Malavige et al., 2004). According to the National Dengue Prevention Unit, since January, 2009, 16,714 dengue cases have been reported all over the country including 240 deaths. Therefore, dengue is becoming an increasingly important public health hazard in Sri Lanka. The central hill district of Kandy is the worst affected, with about 2,200 reported cases. Colombo, Gampaha, and Kalutara districts also have reported high rates of infections and deaths.

The epidemics in Sri Lanka and India were associated with multiple dengue virus serotypes. The relative proportions of the DENV-1, DENV-2, DENV-3 and DENV-4

serotypes in Sri Lanka between 1989 and 2004 are shown in figure 1.2. From 1989 to 2002, DENV-2 was the main circulating dengue serotype in Sri Lanka, followed closely by DENV-3 (Messer et al., 2002). However, during the 2004 epidemic, the DENV-3 virus appeared to be the predominant serotype (Kularatna et al., 2005).

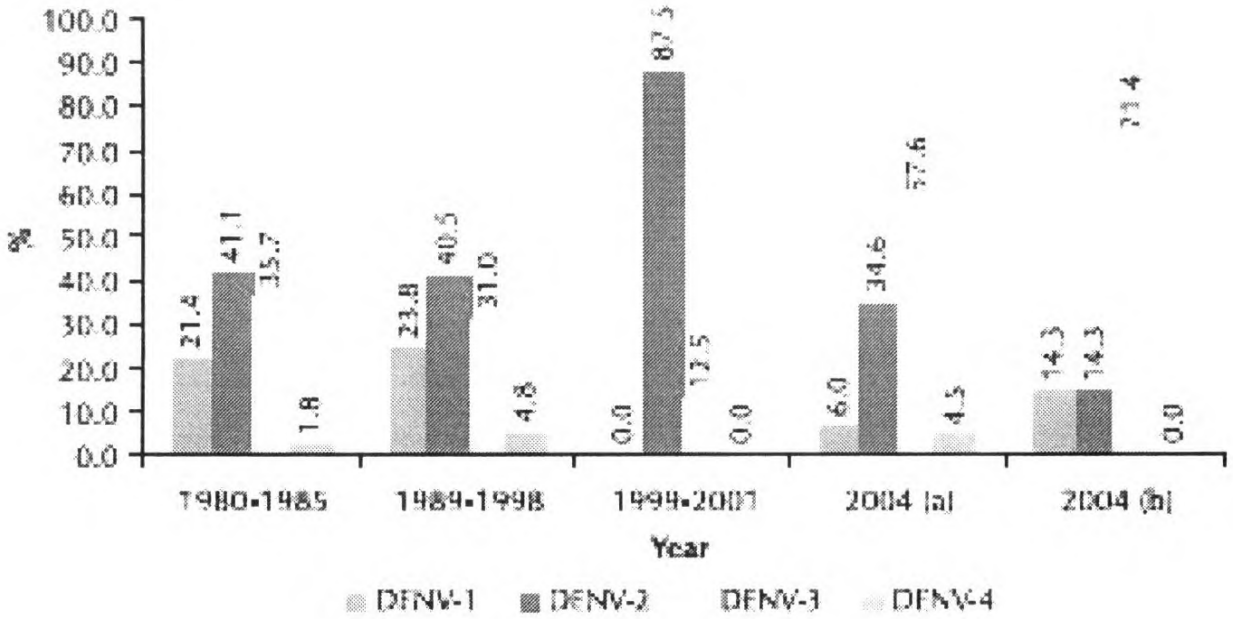

Figure 1.2. Relative percentages of dengue serotypes in Sri Lanka between 1989 and 2004

The four different dengue serotypes are maintained in a cycle that involves humans and the *Aedes* mosquito. Once infected by the virus the mosquitoes remain viruliferous for the rest of their life. No vaccines have proven effective against dengue viruses, and no specific chemotherapeutic drugs are available for dengue treatment. Consequently, the susceptible population has little protection against the disease. It is estimated that the disease kills about 22, 000 people annually in the world. The only viable way to decrease the incidence of dengue is with integrated vector control measures (Gulber, 1989; Rose, 2001). Therefore, attempts to control dengue have been mainly aimed mostly at controlling the *Aedes* mosquito vectors.

1.3 *Aedes* mosquitoes

Aedes is a genus of mosquito originally found in Asian countries, but has spreaded by human activity such as through aircrafts and merchant ships to all continents excluding Antarctica. The genus was named by Johann Wilhelm Meigen in 1818. The name comes from the Greek aédés, meaning "unpleasant" or "odious", so called because of the diseases this mosquito transmits, including dengue fever and yellow fever. The genus contains over 700 species. *Aedes albopictus, Aedes aegypti* and *Aedes polynesiensis* are some of the important species in the *Aedes* genus that transmit diseases to humans (Table 1.1).

Table 1.1. *Aedes* mosquito species and diseases caused

***Aedes* mosquito species**	**Disease caused**
A. albopictus	Dengue fever, Chikungunya
A. aegypti	Dengue fever, Yellow fever
A. polynesiensis	Filariasis

Aedes mosquito species are typically small in size (2.00 mm to 15.00 mm in length). They usually have black with conspicuous white markings and banded legs, hence often called as "Tiger Mosquitoes" (Figure 1.3). Only the female mosquitoes bite humans between a few hours after dawn till an hour or so after sunset, with peaks of activity at mid-morning and late afternoons. These mosquitoes seek human skin from underneath desks or chairs and mainly at the feet and ankles, without much humming sounds. These mosquitoes live close to human dwelling, usually within ninety meters. The females fly for a distance of 50 m, but are capable of going up to 100 – 400 m and reach up to 3 km looking for sites for oviposition. The *Aedes* mosquitoes are commonly found breeding in clear stagnant water collected containers with hard walls such as in flower vases, roof gutters, watering cans, bamboo pole holders, etc. The mosquito can lay eggs about three times in its lifetime and about 100 eggs are produced each time. The development into adult takes about 7 – 14 days. Although they can live for months, the average life span of an *Aedes* mosquito in nature is two weeks.

1.3.1 Species description, biology, geographic distribution and vector state of *Aedes albopictus*

1.3.1.1 Description

A. albopictus known as the Asian tiger mosquito due to the fact that it originates from the tropical forests of south-east Asia and the conspicuous black and white stripes on its body (Figure 1.3). Forest day mosquito and tiger mosquito are also common names for *A. albopictus*. *A. albopictus* is an aggressive outdoor, day biter that attacks humans, livestock, amphibians, reptiles and birds (Eritga et al., 2005). In one survey of biting rates a level of 30 to 48 bites per hour was recorded (Cancrini et al., 2003). *A. albopictus* has four distinct life stages, which consist of egg, larva, pupa and adult. The first three stages occur in water. The adult is the free flying insect.

Asian tiger mosquitoes are small, fragile insects with slender bodies, one pair of narrow wings and three pairs of long, slender legs (Figure 1.3). There is also a distinctive narrow median-longitudinal single median white stripe on the dorsal surface of the thorax and head (Rueda, 2004) (Figure 1.4). It's a most striking identification character for *A. albopictus*.

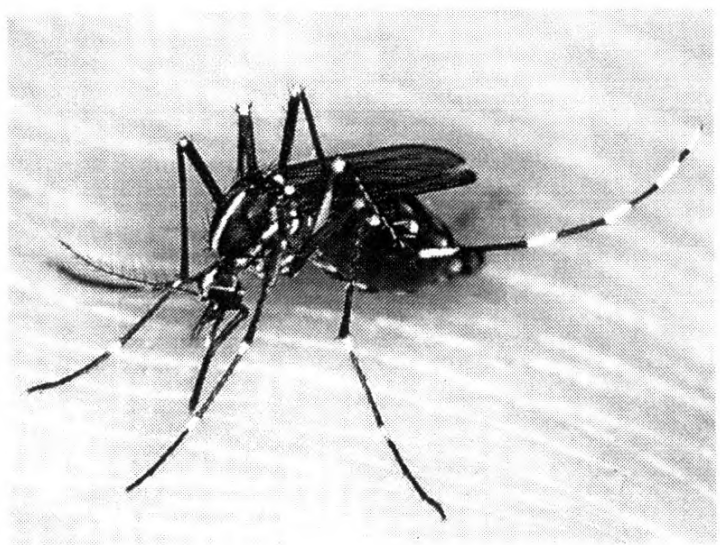

Figure 1.3. *Aedes albopictus*

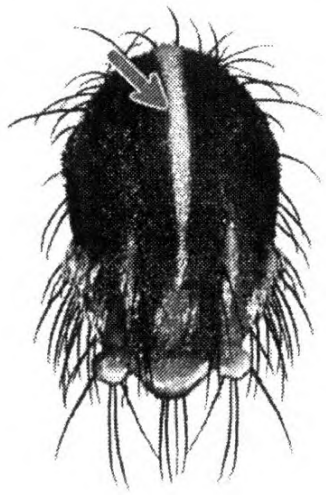

Figure 1.4. A median white stripe on the thorax and head of *A. albopictus*

The typical body length is about 4.75 mm long. The males are roughly 20% smaller than the females, but they are morphologically very similar. They have an elongated proboscis with which the female bites and feeds on blood (Savage et al., 1993; Niebylski et al., 1994). Blood is required for producing eggs in females (Briegel and Horler, 1993; Foster WA, 1995; Xue et al., 2008). Male *A. albopictus* obtains energy by feeding on plant nectar. Typically they fly and feed in the day time rather than at night or at dust and dawn. So they exhibit diurnal activity with two distinct peak periods of activity, in the early morning and late afternoon. *A. albopictus* mosquitoes have a short flight range (200 to 600 m) and they have not been able to fly in strong winds. They are readily transported in containers like waste tires and vehicles (Moore and Mitchell., 1997).

1.3.1.2 Oviposition

The Asian tiger mosquito is a container inhabiting species which lays its eggs in any water containing receptacle in urban, suburban, rural and forested areas. This mosquito prefers to lay its eggs above the water surface on dark rounded vertical surfaces (Juliano, 1998). But not directly laying eggs into water as other mosquitoes do. Field studies show that they have a preference for black, red or woody substrates. Eggs can be collected effectively with black oviposition cans (Focks, 2003). *A. albopictus* need no more than one blood meal for oviposition activity (Mori and Wada, 1977; Neto and Navarro-Silva, 2004). Also the females are able to oviposit more than once, usually one to four times

(Hawley, 1988). This behavior suggests that female seek more than one breeder for oviposition. Eggs are black and oval with a length of 0.5 mm. Eggs can withstand desiccation up to one year. 150 to 250 eggs are laid per oviposition. The eggs may require several water submersions before hatching (Hawley, 1988). Additionally, O_2 tension greatly affects egg hatching (Hawley, 1988). A number of studies have shown that low O_2 tension stimulates the hatching of *A. albopictus* eggs and is a more important factor than flooding or temperature on inducing egg hatching (Hawley, 1988). But higher temperatures allow *A. albopictus* to grow faster and reach the adult stage earlier. The combination of higher temperature and water renewal increased egg viability and shortened the incubation period (Neto and Navarro-Silva, 2004).

1.3.1.3 Laval behavior

The primary immature habitats of *A. albopictus* mosquitoes are in artificial containers such as tires, flower pots, cemetery urns vases, buckets, tin cans, rain gutters, ornamental ponds and drums (Hawley, 1988; Pena et al., 2003). Larvae are also found in natural containers such as tree holes, bamboo pots and leaf axils (Chow, 1949; Joyce, 1961). Depending on the temperature and the availability of food, *A. albopictus* can complete larval development between 5 and 10 days (Hawley, 1988). Larvae also called wigglers are active filter feeders that move within S shaped motion. They feed on fine particulate organic matter in the water. Increased larval density or a decrease in food can cause increased mortality and a decrease in adult size (Mori, 1979). The larvae use a breathing siphon to acquire O_2 and must periodically come to the surface to do so.

There are several characteristics that are unique to the *A. albopictus* larvae. The air tube of *A. albopictus* has a slightly inflated appearance and is much lighter in color. The anal gills of *A. albopictus* are much longer than the saddle and are equal in size. The lateral hairs are doubled in *A. albopictus* on the saddle and four long caudal hairs of the dorsal brush.

The larvae develop through four instars prior to pupation. Pupae (tumblers) are comma shaped, appearing to tumble through the water when disturbed. The pupae of mosquitoes are active and short lived. They do not feed but can move. After 2 -3 days in the pupal stage, the adult mosquito emerges.

1.3.1.4 Geographic distribution

A. albopictus, a mosquito native to Asia, has been one of the fastest spreading animal species over the past two decades. A mosquito has a short flight range (about 10 – 800 m) during its entire life-time (McDonald, 1977; Reiter et al., 1995) but it has ability to disperse by passive migration. Modern transportation of people and goods by aircraft (Gratz et al., 2000), boats (Chadee, 1984) as well as vehicles (Durrheim, 1995) has recently increased. Therefore, it has facilitated the diffusion of both mosquitoes and the

viruses carried in infected people. This contributes to increased dengue incidence and a spread of the dengue world wide.

A. albopictus has spreaded from its native range to at least 28 other countries around the globe, largely through the international used tire trade (Figure 1.4; Reiter and Sprenger 1987; Benedict et al., 2008). Therefore *A. albopictus* occurs throughout the oriental region from the tropics of South Asia, the Pacific and Indian Ocean Islands, north through China and Japan and west to Madagascar. Although *A. albopictus* is native to tropical and subtropical regions, they are successfully adapting themselves to cold regions too. Specific biological characteristics of *A. albopictus* that are relevant to the invasiveness and ability to displace other species have been reviewed (Juliano and Lounibos, 2005).

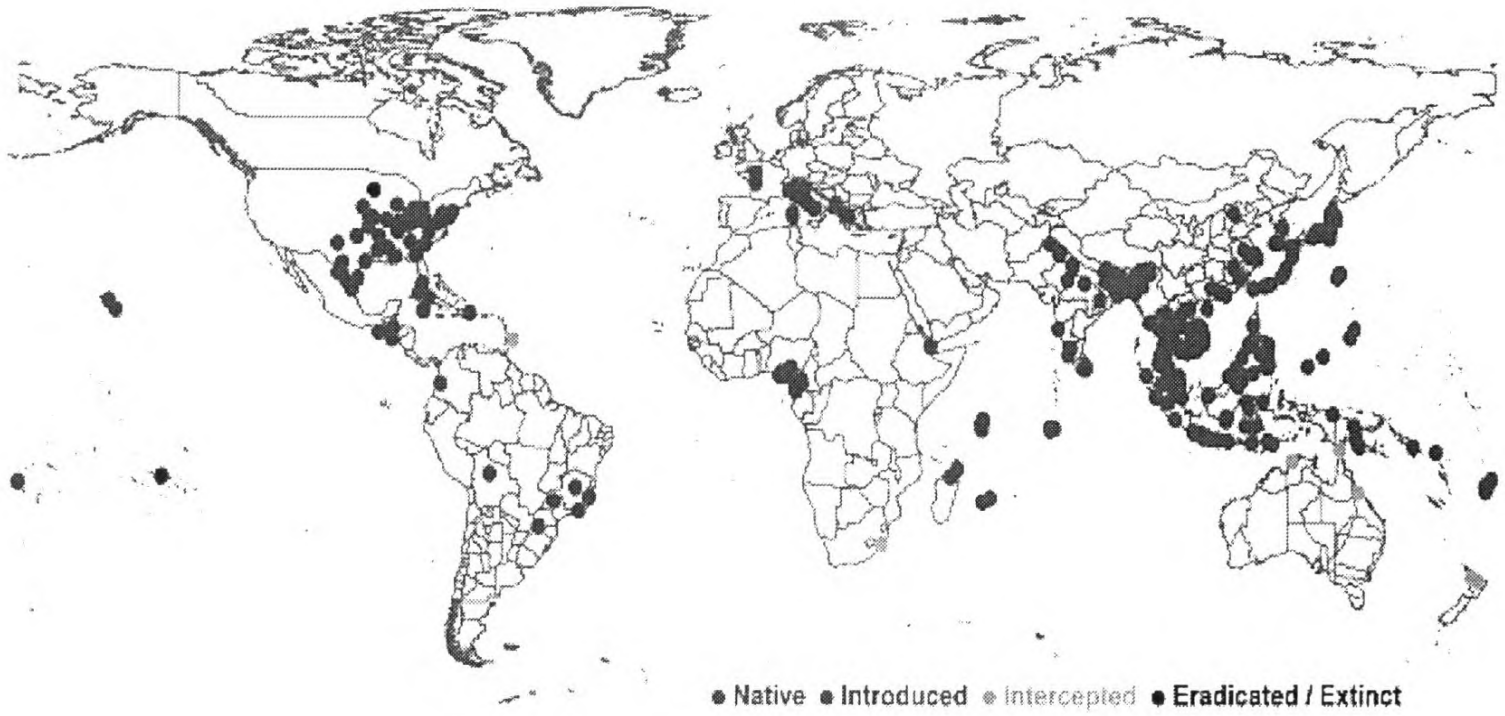

Figure 1.5. Global distribution of *A. albopictus* in 2008

1.3.1.5 *Aedes albopictus* as a disease vector

A. albopictus transmit varieties of pathogenic viruses. It is a competent laboratory vector of more than 30 viruses. However only a few are known to affect humans. They are Eastern Equine Encephalitis (EEE), Cache Valley Virus, dengue, St. Louis and LaCrosse encephalitis viruses, chikungunya fever virus, yellow fever virus, west nile virus (Hawley, 1988; Mitchell, 1991). The species has the ability to colonize different environments, and could also sustain urban cycles of the yellow fever by connecting the sylvatic and urban environments. *A. albopictus* along with *A. aegypti* is considered to be one of the most important dengue vectors. *A. albopictus* is also a competent experimental vector for dengue (DEN) serotypes 1, 2, 3 and 4. *A. albopictus* has been involved in the transmission of DEN viruses in Southeast Asia, Southern China, Japan and the Seychelles (Hawley, 1988). In addition to the dengue virus *A. albopictus* is a vector for seven Alpha viruses including Chikungunya, EEE, Mayoro, Ross River, Western equine encephalitis, Venezuelan equine encephalitis and Sindbis viruses.

Overall, mosquito borne diseases have re-emerged as a significant human health problem due to a number of factors, including the lack of progress in vaccine development, the emergence of drug resistance in pathogens and insecticide resistance in mosquitoes, and the decline in socioeconomic conditions in many countries that limit disease monitoring and mosquito control efforts. At present, mosquito control remains the only viable strategy for preventing dengue and other mosquito borne disease. In a control program it is important to understand the population structure and the molecular basis of the genomic flexibility of the vector species and hence the mechanisms ensuring the diversity of its populations.

At present, mosquito populations are being investigated at the molecular and behavioral levels, with the goal of developing innovative control strategies. *A. albopictus* has been the subject of numerous studies conducted in laboratories throughout the world (Ayeres et al., 2002; Cancrini et al., 2003; Mitchell, 1991).

1.4 Markers used in population genetics

Genetic factors are responsible for most of the characteristics that contribute to the success of the insect vectors, such as susceptibility, vector competence, biting behavior, insecticide resistance and other traits of epidemiological importance. Biotypes or geographic races may be identified using genetic markers (Tabachnick, 1991). *A. albopictus* has been one of the best studied species in this respect. Assessment of the population structure, genetic relatedness among different geographic populations also provides important evidence to infer vector movement or from a site of initial colonization (Ballinger – Crabtree et al., 1992). Demographic and genetic processes can help to understand several events, such as gene flow, migration, selection and extinction of populations among others.

Therefore, knowledge of the mosquito population structure may lead to novel ways of controlling disease transmission (James, 1992). Early population genetic studies defined genetic relationships throughout the world's range; where as more recent studies have focused on local patterns of dispersal (Apostol et al., 1996; Urbanelli et al., 2000; Ayres et al., 2002).

Efficient techniques are now available to study population genetic structure. They depend mainly on three types of markers.

1.4.1 Morphological markers

Traditional morphological markers analyze mutant traits in a population which can be physically identified. Yellow larva (Craig and Gilham, 1959), sex ratio distorter (Craig and VandeHey, 1960) and white eye (Bhalla, 1968) of *A. aegypti* are some of the earliest morphological mutant traits described by morphological markers. More than 80 morphological mutants affecting all parts of the *A. aegypti* body was characterized

within few years. 28 of these were assigned to the three linkage groups. Over the years, much progress was made in genetic mapping of *A. albopictus* (Bat-Miriam and Craig, 1966). There are several undesirable factors that are associated with morphological markers. The first is their high dependency on environmental factors. Second, these mutant traits often have undesirable features such as dwarfism or albinism.

1.4.2 Biochemical markers

Biochemical markers are superior to morphological markers because they are generally independent of environmental conditions. Isozymes / allozymes are the most widely used biochemical markers. Allozymes can be assayed and surveyed from nearly any species. The enzymes are extracted, and run on denaturing electrophoresis gels. They consist of starch, polyacrylamide or cellulose media and stained using specific enzyme reaction mixtures. The denaturing component in the gels (usually Sodium Dodecayl Sulphate) unravels the secondary and tertiary structure of the enzymes and they are separated on the basis of their molecular weight.

Allozymes marker detects both homo-and heterozygous banding patterns (Loxdale and Lushai, 1998). The rates of mutations that produce detectable differences in allozyme alleles are in the order of 10^{-6} (Voelker *et al.*, 1980). Allozymes have been used for various purposes, e.g. species identification (Miless, 1978; 1979), and population genetics. Investigations using isozyme analysis have demonstrated genetic variation at the protein level between geographic populations of *Aedes aegypti* (De Sousa et al., 2000).

Allozyme analysis is cheap, often much quicker to isolate and develop and is easy to use. The drawbacks of this method include low detectable variability (Tabachnick, 1991) and a need for fresh or frozen material (-20 °C). Because allozymes do not represent a random sample of the genome they may bias some population genetic interferences (Loxdale and Lushai, 1998).

1.4.3 Molecular markers

Molecular markers are very popular, powerful tools as a complement to morphological, biochemical information. They are found at specific locations of the genome and used to 'flag' the position of a particular gene or the inheritance of a particular trait. Molecular markers have greatly facilitated research in a variety of disciplines such as taxonomy, phylogeny, ecology, etc. DNA based markers are unlimited, less affected by age, physiological condition of samples and environmental factors. They are not tissue specific and thus can be detected at any phase of organism development. Only a small amount of sample is sufficient for analysis and the physical form of the sample does not restrict detection. Molecular marker technologies are low labor required, easy to score and easy to share data between laboratories.

For quite a long period of time, allozymes (proteins) have been the molecular markers of choice. In recent years, however attention has increasingly focused on the DNA molecule as a source of informative polymorphisms, because each individual's DNA sequence is unique. This sequence information can be exploited for any study of genetic diversity and relatedness between organisms. Molecular makers have several properties.

1. Highly polymorphic behavior
2. Co dominant / dominant inheritance
3. Frequent occurrence in the genome
4. Even distribution throughout the genome
5. Selectively neutral behavior (i.e., no pleiotropic effects)
6. Easy access
7. Easy and fast assay (e.g., by procedures amenable to automation)
8. High reproducibility
9. Easy exchange of data between laboratories

However, no molecular markers are available yet that fulfill all of these criteria.

1.5 DNA based genetic studies

In recent years, different marker systems such as Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNAs (RAPDs), Sequence Tagged Sites (STS), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) or microsatellites, Single Nucleotide Polymorphisms (SNPs) etc. have been developed and applied to a range of genetic studies. They can be categorized as dominant or co-dominant. Dominant markers allow for analyzing many loci at once, e.g. RAPD. However, inability to detect heterozygotes in diploids is one of the major disadvantages of these markers. In contrast, co-dominant markers analyze one locus at a time. A primer amplifying a co-dominant marker would yield one targeted product. Ability to detect heterozygotes in diploids is a major advantage of this marker system. The relative advantages and disadvantages of these markers are summarized in Table 1.2.

However, according to the kind of study to be undertaken, one marker can be chosen from among varieties of marker systems.

Any DNA marker based genetic study, typically involve few main steps. At the initial step, samples are collected and DNA is extracted. Then extracted DNA is analyzed for molecular markers. Allelic bands are then visualized by gel electrophoresis. Polymorphic or / and monomorphic data matrix is constructed using gel bands. Data are fed in to suitable genetic data analysis programmes to generate descriptive statistical data.

Table 1.2. Comparison of the most commonly used marker systems

Feature	**RFLPs**	**RAPDs**	**AFLPs**	**SSRs**	**SNPs**
DNA required (µg)	10	0.02	0.5-1.0	0.05	0.05
DNA quality	High	High	Moderate	Moderate	High
PCR based	No	Yes	Yes	Yes	Yes
No. of polymorphic loci analyzed	1.0-3.0	1.5-50	20-100	1.0-3.0	1.0
Nature	Co dominant	Dominant	Dominant	Co dominant	Co dominant
Ease of use	Not easy	Easy	Easy	Easy	Easy
Amenable to automation	Low	Moderate	Moderate	High	High
Reproducibility	High	Low	High	High	High
Development cost	Low	Low	Moderate	High	High
Cost per analysis	High	Low	Moderate	Low	Low

1.5.1 DNA extraction

The extraction of DNA from specimen is the starting point for any DNA marker analysis. The analysis depends on the ability to prepare pure, high molecular weight DNA. Initially this technique should be followed to lyse the cells gently and solubilize the DNA. Next, basic enzymatic or chemical methods must be used to remove contaminating protein, RNA and other macromolecules. Co-isolation of contaminants such as polysaccharides, polyphenols and other compounds cause damage to DNA. Therefore, to obtain good quality DNA, it is essential to remove these contaminants before precipitation of DNA in the final step. In a typical extraction procedure, after cell disruption, it is incubated in hot lysis buffer. Proteins are separated by phenol-chloroform- isoamyl alcohol extraction, and the DNA is precipitated using ethyl alcohol (Weising et al., 1995). However, DNA degradation could still occur due to the presence of contaminating endogenous nucleases. In order to reduce nuclease activity fresh samples must be extracted immediately or stored for hours or days at appropriate low temperature before extraction. Frozen samples must be thawed only in the presence of lysis buffer containing a high concentration of EDTA (5 mM). EDTA can chelate bivalent cations and there by inhibit metal dependent DNase activity (Weising et al., 1995).

DNA pellet resulting after centrifugation is washed, dried and re-dissolved in TE buffer. Depending on the species, additional purification steps may or may not be necessary in

order to remove RNA, polysaccharides, polyphenols and other contaminating substances. Inclusion of RNase treatment and ammonium acetate precipitation as an additional step, removes RNA and some polysaccharides (Weising et al., 1995). Purification can be performed by fractionated precipitation using solvents such as phenol, chloroform, ethanol, isopropanol, and /or by adsorption on solid matrices (anion exchange resins, silica or glass gel, etc.). Sometimes several DNA purification principles may be combined, so that extraction and purification can be performed within same step.

1.5.2 Quantification of isolated DNA

DNA absorbs light in the UV range. The absorption spectra of DNA and RNA have maxima at 260 nm. As described by the Beer-Lambert law, the amount of absorbance at a particular wavelength is a function of the concentration of the absorbing material. DNA concentration is measured using the spectrophotometer as a function of its absorbance (A) or optical density (OD). Absorbance data for nucleic acids are expressed in A_{260} or OD_{260} units.

For double stranded DNA, an OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml of DNA. The concentration of DNA sample is measured at 260 nm (OD_{260}) against a blank. Although it is possible to estimate the concentration of DNA solution by measuring their absorption at a single wavelength (260 nm), OD values can also be taken at other wavelengths i.e. at 230 nm, 280 nm, and 340 nm. The ratio of absorbance at 260 nm to absorbance at other wavelength (230 nm or 280 nm) is a good indicator of the purity of the preparation. Significant absorption at 230 nm, indicates contamination by phenolic ion, thiocyanates and other organic compounds, where as absorption at higher wavelength (330 nm and higher) is usually caused by light scattering and indicates the presence of particular matter. Absorption at 280 nm indicates the presence of protein, because of aromatic amino acids absorb strongly at 280 nm. Ideally, a good preparation should have 260 nm / 280 nm ratio between 1.8 and 2.0 contaminated with (protein) result in lower values (Weising et al., 1995).

There are other methods to quantify extracted DNA. DNA is run with known amount of standard DNA marker (Ladder) in an agarose gel. Band intensities were compared visually with a bands of ladder, each of which has known DNA amount. Then amount of DNA present in bands are estimated.

1.5.3 Analysis for molecular markers - DNA amplification using Polymerase Chain Reaction (PCR)

The PCR is based on the enzymatic *in vitro* amplification of DNA. Starting from a very low amount of template DNA (mostly in the nanogram range), millions of copies of one or more particular target DNA fragments are produced in a very short period of time. This amplicons are electrophoresed and visualized by staining or autoradiography. PCR is characterized by its high speed, selectivity, and sensitivity.

In a typical PCR, three temperature controlled steps can be discerned, which are repeated in a series of 25 to 50 cycles. A typical reaction mix consists of,

1. A buffer, usually containing Tris-HCl, KCl and $MgCl_2$
   The standard buffer for Taq polymerase mediated PCR contains 50 mM KCl, 10 mM Tris. HCl (pH 8.3 at room temperature) and 1.5 mM $MgCl_2$ (Sambrook et al., 1989).

2. A thermo stable DNA polymerase
   This enzyme catalyzes the addition of nucleotides to the 3' end of a single stranded DNA being synthesized.

3. All four deoxynucleotides (dNTPs: dATP, dCTP, dGTP, dTTP)
   The amount of dNTPs depends on the number of target sequence and size of the target sequences to be amplified. Therefore, for amplification of a longer target sequence a high concentration of dNTP is recommended.

4. Forward and reverse oligonucleotide primers
   Primers are typically 15-30 bases long and can be designed to be exactly complementary to the template. Primers can be purchased from several manufactures or synthesized by using a DNA synthesizer (Weising et al., 1995). The GC content has a high prediction value. A high GC content is positively correlated with primer strength.

   The ratio between the primers and template is important for the specificity of PCR. Normally each primer should be at least 10-fold in excess of the target sequence to avoid unspecific amplifications and primer - dimers taking place when the ratio is too high and if the ratio is too low, the amplification efficiency is reduced (Sambrook et al., 1989).

5. Template DNA
   Purified extracted DNA sample is used as the template DNA. It must be in appropriate concentration for the best amplification.

1.5.3.1 PCR cycle

In the first step, the template DNA is made single stranded by raising the temperature to 94 °C (denaturing step). In the second step, lowering of the temperature to about 25 - 65 °C (depending on primer sequence and experimental strategy) results in primer annealing to their target sequences on the template DNA (annealing step). For the third step, a temperature is chosen so as the activity of the thermo stable polymerase is optimal, i.e., usually 72 °C (elongation step). The polymerase now extends the 3' ends of the DNA primer hybrids toward the other primer binding site. Since this happens at both primer annealing sites on both DNA strands, the target fragment is completely replicated (cycle 1).

In the next cycle, the two resulting double stranded DNAs are again denatured, and both the original strand as well as the product strand now acts as a template. Repeating these three step cycles 25 to 50 times results in the exponential amplification of the target (amplicon) between the 5' ends of the two primer binding sites.

1.5.4 Randomly amplified polymorphic DNA (RAPD)

RAPD is used as a DNA based molecular marker to detect polymorphism. This method was first described by Williams et al. (1991) and uses a single nucleotide primer from an 8-10 base pairs long sequence to amplify random sections of nuclear DNA. The method exhibits dominant banding patterns whereby both homozygotes and heterozygotes produce a band.

Therefore using several different primers, polymorphisms at many RAPD loci can be detected among individuals. So RAPD - PCR can potentially increase the resolution of genetic differences among individuals in population genetic studies. However, the genotype of heterozygous individuals with amplification products arising from a single chromosome can not be distinguished from homozygous with amplification products arising from both chromosomes (Williams et al., 1991).

RAPDs have been used to analyze genetic relationships among populations of insects with high ability to invade and colonize new habitats. As well as used in a variety of studies for species diagnosis, population differentiation, genetic fingerprinting and genetic mapping (Williams et al., 1991; Black et al., 1992; Kambhampati et al., 1992; Ballinger – Crabtree et al., 1992; Puterka et al., 1993; Clark and Lanigan, 1993; Apostol et al., 1996 and Sharma et al., 2009).

These markers successfully have identified the origin of the infestation (Hamer et al., 1997). Ballinger – Crabtree et al. (1992) used RAPDs to estimate polymorphism in *A. aegypti* populations from Africa, Australia and America. Discrimination of frequencies of fragments produced with three different RAPD primers permitted unequivocal identification of the geographical origin in 89% of the individuals. Apostol et al. (1996) used the same technique to analyze the reproductive structure in 16 *A. aegypti* populations from Puerto Rico. Despite the high rate of dispersal estimated for the whole area, their results showed a significant genetic differentiation among different sites within cities.

Gorrochotegui – Escalante et al. (2000) studied 10 *A. aegypti* populations from seven cities along the north eastern coast of Mexico. Their results with RAPDs markers indicated free gene flow among populations within a 90 to 250 km radius, but isolation at greater distances.

De Sousa et al. (2001) applied RAPDs as genetic markers to estimate levels of intra-specific polymorphism and genetic relatedness of *A. aegypti* populations from Argentina. The genetic variability of *A. albopictus* Brazilian populations was analyzed using RADP for the first time by Ayres et al. (2002). RAPD markers were used to demonstrate the existence of high levels of population structuring of *A. aegypti* in Brazil,

both at the macro (up to 2 600 km) and micro geographic (within several kilometers) levels by Ayres et al., (2003). Therefore DNA polymorphism detected by RAPD has been used successfully to characterize the genetic structure in several mosquito species.

The RAPD - PCR methodology has many advantages. It avoids the use of radioactive materials and uses a minimum amount of DNA. This methodology is accomplished without the knowledge of a target sequence, cost efficient, short processing time, relatively easy to perform and reveals large numbers of polymorphisms (Williams et al., 1991 and Welsh et al., 1991).

The disadvantage of RAPD polymorphisms in population genetics is that the majority of alleles (> 90 %; Williams et al., 1991) segregate as dominant markers. RAPD – PCR produces a fragment with template DNA from individuals that are either homologous dominant or heterozygous for an amplifiable allele. No fragment is produced in homologous recessive individuals because amplification is disrupted in both alleles. As well as this method requires stringent standardization before the markers can be used reliably and consistently. Also, results are not easily reproduced making them difficult to interpret.

1.5.5 Visualization of amplified product using Agarose gel electrophoresis

Electrophoresis through agarose gel is the standard method to separate, identify and purify DNA fragments. A gel is a complex network of polymeric molecules and DNA molecules are negatively charged. Under an electric field, small DNA molecules migrate faster than the larger molecules (Sambrook et al., 1989). The migration rates of the DNA molecules are inversely proportion to the logarithm of their molecular weights. Agarose gel is convenient for separating DNA fragments ranging from 200 bp to about 50 kb in length but have low resolving power. Agarose gels are usually run in horizontal configuration with a constant electrical power supply (Sambrook et al., 1989).

Agarose gels are casted by melting the agarose in the presence of desired electrophoresis buffer. The melted solution is poured into a mold and allowed to harden. The density of gel matrix is determined by the concentration of the agarose.

The rate of migration of DNA bands is determined by several factors.

1) Molecular size of the DNA.
   Larger molecules migrate more slowly than smaller molecules.

2) Agarose concentration
   There is a linear relationship between the concentration of the agarose and logarithm of the electrophoretic mobility of DNA fragments. Table 1.3 shows the separation ranges in gels containing different amount of agarose (Sambrook et al., 1989).

Table 1.3. Separation ranges of linear DNA molecules (kb)

Amount of agarose in gels (%w/v)	Efficient range of separation of linear DNA molecules (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

3) Concentration of the DNA.
   Super helical circular, nicked circular and linear DNA of the same molecular weight migrates through agarose gels at different rates.

4) Applied voltage.
   At low voltage, migration of linear fragments is proportional to the voltage applied with increasing electric field. The pattern of migration of large fragments is different. For DNA fragments greater than 2 kb in size, agarose gels should be run at no more than 5 v/cm.

5) Composition of the electrophoretic buffer.
   Composition and ionic strength affect the DNA mobility. In the absence of the ions and minimal electrical conductance, DNA migrates very slowly. Several different buffers are used and available for electrophoresis of dsDNA.

Direction of the electric field, base composition, temperature and presence of intercalating dyes also affect the migration rate.

1.5.6 Detection of PCR products

Staining of agarose gels with fluorescent dye Ethidium Bromide (EtBr) has become a convenient method to detect DNA on a gel (Sharp et al., 1973). EtBr intercalates between bases of DNA. Ultraviolet radiation at 254 nm is absorbed by the DNA and transmitted to the dye. Affinity of the dye for single stranded nucleic acid (RNA and DNA) is much lower than that of double stranded DNA and fluorescence yield is comparatively low (Sambrook et al., 1989).

Stock solution of EtBr can be prepared to a concentration of 10 mg/ml and stored in a dark bottle or bottle wrapped in an aluminum foil. The dye can be incorporated into the gel (0.5 μg/ml), which enables one to observe DNA bands in the gel under UV during electrophoresis. However, due to the dye, electrophoresis mobility is reduced by 15%. Therefore, staining also can be done after the electrophoresis. In this case the gel is

immersed in electrophoretic buffer or water containing EtBr (0.5 μg/ml) for 30-40 minutes at room temperature. De-staining is usually not needed. However, for detection of very small quantities (<10 ng) of DNA, staining for 20 minutes at room temperature is sufficient (Sambrook et al., 1989).

1.5.7 Band evaluation

The preciseness and accuracy of band scoring is critically dependent on several parameters, including DNA quality, completeness of PCR, electrophoretic conditions, and means of signal detection. Mobility artifacts may be caused by irregularities in the electric field as well as by residual impurities in the DNA preparations (proteins) and the resulting band shifts can lead to several misinterpretations of band matching. This can be checked by replicating individual samples and by the inclusion of molecular weight standards (Weising et al., 1995). Markers are particularly important in large gels where the smiling effect is especially pronounced (Sulston et al., 1989). Even if highly efficient standardization procedures are followed, the decision to regard two closely spaced bands as different or identical is always subjective and prone to error (Weising et al., 1995). Due to the need for pruning loci with low frequency null alleles, more loci are needed to be sampled with RAPDs and some problems of bias cannot be completely eliminated. To minimize errors, the following precautions are suggested (Weising et al., 1995).

1. Only the easily scorable part of the fingerprinting should be considered for analysis.
2. Bands that cannot be accurately scored throughout all lanes to be compared should be executed from the analysis.
3. Co-migrating fragments of different intensities should not be treated as identical if the intense band is more than twice as strong as the faint band.

Banding pattern can be either evaluated by the eye of the investigator or by automated methods. An elegant technique to visualize fragments is the use of fluorescence labeled PCR primers. With this technique, sizing of the PCR products can be done by real time scanning with an automated sequencing device (Ziegle et al., 1992).

1.6 Statistical analysis

Statistics is a mathematical science pertaining to the collection, analysis, interpretation or explanation and presentation of experimented data. This descriptive statistics is useful in research, when communicating the results of experiments. Therefore, descriptive statistics summarize the population data obtained from band evaluation by describing what was observed in the sample numerically or graphically.

Early statistical models were almost always from the class of linear models. But rapid and sustained increases in computing power starting from the second half of the 20^{th} century have had a substantial impact on the practices of statistical science. Powerful computers coupled with suitable numerical algorithms and developed an open source statistical packages (Statistical software).

Many software programs for molecular population genetic studies have been developed for personal computers. Their easy access, implementation of sophisticated and powerful statistical techniques, and user-friendliness make them an attractive alternative to performing calculations on spreadsheets or by writing simpler programs for oneself. TFPGA (Miller, 1997), Arlequin, (Schneider et al., 1997), GDA (Lewis and Zaykin, 1999), GENEPOP (Raymond and Rousset, 1995), GeneStrut (Constantine et al., 1994), and POPGENE (Yeh and Boyle, 1997) are some of the software which can be used to analyze band patterns, polymorphisms generate from intraspecific genetic variation within the framework of Hardy-Weinberg equilibrium. These programs are broad in their application, i.e., they were designed for a variety of marker types and analyses. All are available free on the Internet and can be run on Windows PCs and Apple Macintosh computers. These particular programs were chosen because each can accommodate a variety of molecular marker types and perform many different types of analyses. Although there is much overlap in their functionality, each program has unique features to offer potential users.

1.6.1 POPGENE (version 1.32)

POPGENE (version 1.32) program can be used to analyze the haploid or diploid, dominant or co-dominant data. Input details are gained from bands in the gels. They are imported to POPGENE-formatted text files or files created by the program's text editor. Data analysis is done through menus and dialog boxes.

The program can estimate allele frequencies for dominant markers (Chong et al., 1994). The user can optionally specify an inbreeding coefficient in a dominant marker data set for a population if it is not in Hardy-Weinberg equilibrium, when estimating allele frequencies.

Population structure is estimated by *G*-statistics for haploid data and *F*-statistics for diploid data. Gene flow (*Nm*) is estimated from G_{ST} or F_{ST} (Slatkin and Barton, 1989). Nei's genetic distances and identities (Nei, 1972, 1978) can be estimated between groups or populations, and a dendrogram can be generated based on an unweighted pair group method algorithm (UPGMA) analysis (Sokal and Michener, 1958) of the distance matrix. Those results appear in an output window which can be saved as a text file or cut and pasted into another text editor. Therefore, POPGENE program is used in a wide range of dominant data analysis but it has some disadvantages too. Its manual is sketchy and methods are not explained in detail

1.6.2 Tools for Population Genetic Analyses (TFPGA)

Haploid or diploid, dominant or co-dominant data extracted from run gels can be imported as TFPGA formatted text files. The *Analyze* menu lists seven items: Descriptive Statistics, *F*-statistics, Genetic Distance, Hardy-Weinberg, UPGMA, Exact Tests, and Mantel Test. The program will estimate allele frequencies for diploid

dominant markers by one of two methods: as the square root of the frequency of the recessive genotype or by Taylor expansion (Lynch and Milligan, 1994).

Descriptive statistics include allele and heterozygote (but not genotype) frequencies, observed and expected heterozygosities (Nei, 1978), and percent of polymorphic loci, with the option to obtain these estimates for any level of the user-defined hierarchy. Various genetic distances and identities can be calculated for any level of the hierarchy—Nei's and Nei's minimum (original and unbiased, Nei, 1972, 1978), Rogers' (1972) and modified Rogers' (Wright, 1978) and co ancestry (Reynolds et al., 1983). Cluster analysis is by UPGMA (Sokal and Michener, 1958); bootstrap values and consistency indices (the number of loci that support each node) can be generated. TFPGA manual includes well documented descriptions of methods. Therefore, instructions could be followed easily. As well as explanations of minor flaws, common error codes and references with suggested reading are included in the TFPGA software. Therefore, it is widely used by the researchers to analysis their data.

ii. Scientific scope of the project

2.1 Aims and objectives of the study

Our study is aimed to determine the breeding structure of *A. albopictus* in the areas where high rates of dengue infections and deaths occurred recently.

The population (breeding) genetic structure of *A. albopictus* would provide valuable information about the dispersal patterns of these mosquito vectors within and among mosquito populations, transmission patterns of the dengue virus, and existence of possible epidemiological units for development of effective vector control strategies (Crampton et al., 1994). Further, such knowledge could also be useful to understand the spread of genes such as those involved in vector competence, parasite susceptibility, insecticide resistance etc. (Hargreaves et al., 2003) and have a great impact on the existing dengue control programs.

During this work, *A. albopictus* eggs are sampled from breeding sites located in each population under study, and reared to adults. Genomic DNA from adult mosquitoes are extracted and used to amplify polymorphic DNA (RAPD) alleles by PCR. These allele data are then used to calculate population genetic indices, such as gene flow (vector dispersal patterns) , genetic diversity (variability), genetic differentiation (population sub division), genetic distance etc. within and among populations to uncover the population (breeding) structure of *A. albopictus*.
]

2.1.1 Overall Objective of the study

To determine the dispersal patterns of *Aedes albopictus* populations (genes) within and among districts at risk of epidemics, for improving the future vector control strategies.

2.1.2 Specific objectives:

1) To determine gene flow among *A. albopictus* mosquito populations within and among districts at risk of epidemics, to explore exchange of alleles among populations

2) To determine genetic distance D, among mosquito populations, the effective migration rate, *Nm* and the variance effective population size *Ne*, in each city under investigation.

iii. MATERIALS AND METHODS

3.1 Specimen collection

3.1.1 Study sites

Mosquitoes were collected from three different districts in Sri Lanka, Colombo (6° 55'N, 79° 51'E), Gampaha (7° 05'N, 79° 59'E) (Western province) and Kandy (7° 17'N, 80° 38'E) (Central province). Samples were taken from 6 – 7 neighborhoods in each district. Sampling sites in Colombo district include Narahenpita, Kirulapana, Maharagama, Nugegoda, Maligawatta and Kolonnawa; Sampling sites in Gampaha district include Mahara, Ragama, Wattala, Gampaha, Weliweriya and Biyagama. Whereas in Kandy district they are Peradeniya, Galaha, MC Kandy, Kundasale, Katugasthota, Wattegama and Muruthalawa. Altogether a total of 19 *A. albopictus* populations were analyzed. The geographic locations of all sampling sites are shown in figure 4.1.

Sampling sites were in the expanded circular design to allow the analysis of gene flow at two levels, among collections within a district (at micro geographic levels) and among districts (at macro geographic levels).

3.1.2 Mosquito collection

A. albopictus eggs were collected using ovitraps in March 2006 to May 2008. The ovitrap consisted of a plastic cup (painted black on the inner side filled with 150 ml well water). A thin paddle (3 x 12 cm) made of A4 white paper was placed in the container for use as an oviposition substrate. Total of 209 ovitraps were installed in both the interior and exterior of houses in all sampling sites. Indoors, the ovitraps were placed in low lying (< 2 m) dark corners, such as areas underneath beds. Outdoor ovitraps were placed in low lying area areas (< 2 m), protected from direct sun light and rainfall. After 1-2 week, the ovitraps were collected and transported to the laboratory. Paddles were allowed to air dry for 24 hours at room temperature and eggs were identified (section 3.1.3) and counted using magnifying lense. Eggs from each positive ovitrap were separately pooled and transferred in to 250 ml beaker, containing 150 ml of well water for egg hatching. The larvae were fed with fish food according to Arrivillaga and Barrera, (2004) until they became adult mosquitoes.

3.1.3 Morphological species identification

Each emerged *A. albopictus* a mosquito was identified using a pictorial key (Rueda, 2004). Adult *A. albopictus* were then stored at -20 °C awaiting analysis. A random sampling was used in this work, in order to minimize relationship between mosquitoes.

3.2 Genomic DNA extraction

Three different protocols were tested for genomic DNA extraction from mosquitoes. Two of them were based on phenol/ chloroform extraction (Apostol et al., 1993), (Rivero et al., 2004) and the other on high salt extraction protocol (Sunnucks and Hales, 1996; Aljanabi and Martinez, 1997). The Apostol et al. procedure was found to be superior to other methods because it gave high quality DNA in reasonable yield (45.9 µg/ml). Therefore Apostol et al. procedure was used to extract genomic DNA.

Briefly, individual mosquito was homogenized in 300 µl of lysis buffer containing 10 mM Tris-HCl (pH 8.0), 5 mM NaCl, 5 mM EDTA, 0.1 % sodium dodecyl sulphate (SDS), 0.015 mM Spermine, 0.05 mM Spermidine and 0.33 µl/ml Proteinase K. The homogenate was incubated at 50 °C overnight. After extraction with an equal volume of phenol, followed by chloroform: isoamyl alcohol (24:1) 0.2 volumes of 10 M ammonium acetate was added to the aqueous phase. Genomic DNA was recovered by precipitation with 2.5 volumes of ethanol at room temperature and centrifugation for 5 min. at 15,800 g. DNA pellet was air dried and resuspened in 50 µl of sterile water. The samples were stored at 4 °C for until further analysis.

3.3 Quantification of genomic DNA

The concentration of each extracted DNA sample was estimated by gel electrophoresis using *λEco*RI/*Hind*III size marker. 500ng of size marker was loaded onto the gel and the band intensity of size markers were compared with that of sample DNA to estimate the amount of genomic DNA.

3.4 RAPD-PCR

RAPD –PCR amplification was performed using extracted genomic DNA in a Techne® 312 thermocycler (Techne Limited, Cambridge, UK). Amplification proceeded through 35 cycles at 94 °C for 1 min., 35 °C for 1 min., 72 °C for 2 min. followed by a final extension step at 72 °C for 10 min. Finally the samples were held at 4 °C. Each PCR was carried out in a final volume of 25 µl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001 % gelatin, 0.2 mM of each dNTPs (Promega Corporation, USA), 25 pmol of RAPD primer (Operon technologies GmbH, Germany), 0.5 Units of Taq. DNA polymerase (Sigma-Aldrich, Inc, USA) and 10 ng of mosquito genomic DNA. The parameters for RAPDs such as the concentration of primer, $MgCl_2$, template DNA, Taq. DNA polymerase and the PCR program were optimized to obtain intense, reproducible, polymorphic bands. 30 arbitrary primers (A – C, Operon Biotechnologies GmbH, Germany) were tested and five were selected on the basis of their reproducibility, intensity and polymorphism during the PCR amplification. The reproducibility of the RAPD amplifications was tested by performing the same PCR in duplicate using the same genomic DNA. The selected oligonucleotide primers and their sequences are given in table 3.1.

Table 3.1. Primers used, number and size range of fragments obtained after RAPD amplification

Primer	**Nucleotide sequence**	**Number of amplified fragments**	**Fragment size range (bp)**
A8	5'-GTGACGTAAGG-3'	15	200-2250
B7	5'-GGTGACGCAG-3'	14	300-1000
B8	5'-GTCCACACGG-3'	10	290-990
C18	5'-TGAGTGGGTG-3'	12	200-1375
C19	5'-GTTGCCAGCC-3'	11	450-3000

3.5 Agarose gel analysis of RAPD bands

Amplified RAPD bands were analyzed on agarose gel electrophoresis system (SCIA-PLAS, Harvard Bioscience Inc, United States).

A 1.2 % agarose gel was prepared as described by Sambrook et al. (Sambrook et al.,1989). 10 µl of each PCR product was mixed with 2 µl of 6x loading buffer, containing 0.25 % bromophenol blue, 40 % sucrose in water (Sambrook et al., 1989) and loaded onto the agarose gel.

The electrophoresis was performed at a constant voltage of 75 V for 3 hours in 0.5X Tris-Borate-EDTA (TBE) buffer with ethidium bromide (5 mg/ml) (Sambrook et al. 1989). The allelic RAPD bands were visualized on an UV transilluminator (Hoefer Inc., San Fancisco, USA) at 254 nm and photographed prior to scoring of bands. The sizes of DNA fragments were estimated by comparing against the DNA size marker, *λEco*RI/*Hind*III and 100 bp ladder (Sigma-Aldrich, Inc, USA).

3.6 Statistical analysis

Allelic bands on agarose gel were scored visually for each primer and data were used to prepare a matrix suitable for statistical analysis using POPGENE (version 1.32) and TFPGA population genetic packages. Only strong bands were scored while omitting faint bands. Co-migrating and incompletely stained bands were also scored. Presence of a band was scored as "1", absence of a band was scored as "0". The faint bands were scored as ".". For TFPGA population genetics software, presence of a band was scored as "1" and absence of a band was scored as "2".

The RAPD markers were analyzed using the following assumptions:

1. RAPD alleles segregate in a Mendelian fashion,
2. bands that co-migrate are homologous,
3. different loci segregate independently and
4. populations are in Hardy-Weinberg equilibrium.

Based on these assumptions, allele frequencies were estimated using square root method (Nei, 1987). The allele frequencies were used to compute total gene diversity, H_T, intra population mean gene diversty, H_S, genetic indices (genetic differentiation), Gst and θ (McDermott and McDonald, 1993), effective migration rates (gene flow) Nm, Nei's original genetic distances, D, and Nei's gene diversity, He (Nei's 1972). A dendrogram, summarizing the genetic relationships among all populations was constructed, using the unweighted pair group mean analysis (UPGMA) algorithm for which bootstrap values based on 1000 replicates were added (Felsenstein 1985).

Pairwise Fst values were calculated with AFLPsurv (Vekemans et al., 2002), transformed into F_{ST} / (1 - F_{ST}), and regressed on geographic distances among populations (Rousset (1997), to determine whether geographic distance among populations serves as a barrier to the gene flow,

iv. RESULTS

4.1 Mosquito collection

2096 eggs were collected using 209 ovitraps fixed in 19 collection sites in three different districts (Figure 4.1). 775 larvae emerged from those eggs, which represented a hatching rate of 36.9%. The numbers of eggs collected in the outdoor traps were significantly higher than the indoor traps. Of the total 376 mosquitoes emerged, 341 were *A. albopictus*. Locations and sample sizes of mosquito collections are presented in figure 4.1 and table 4.1.

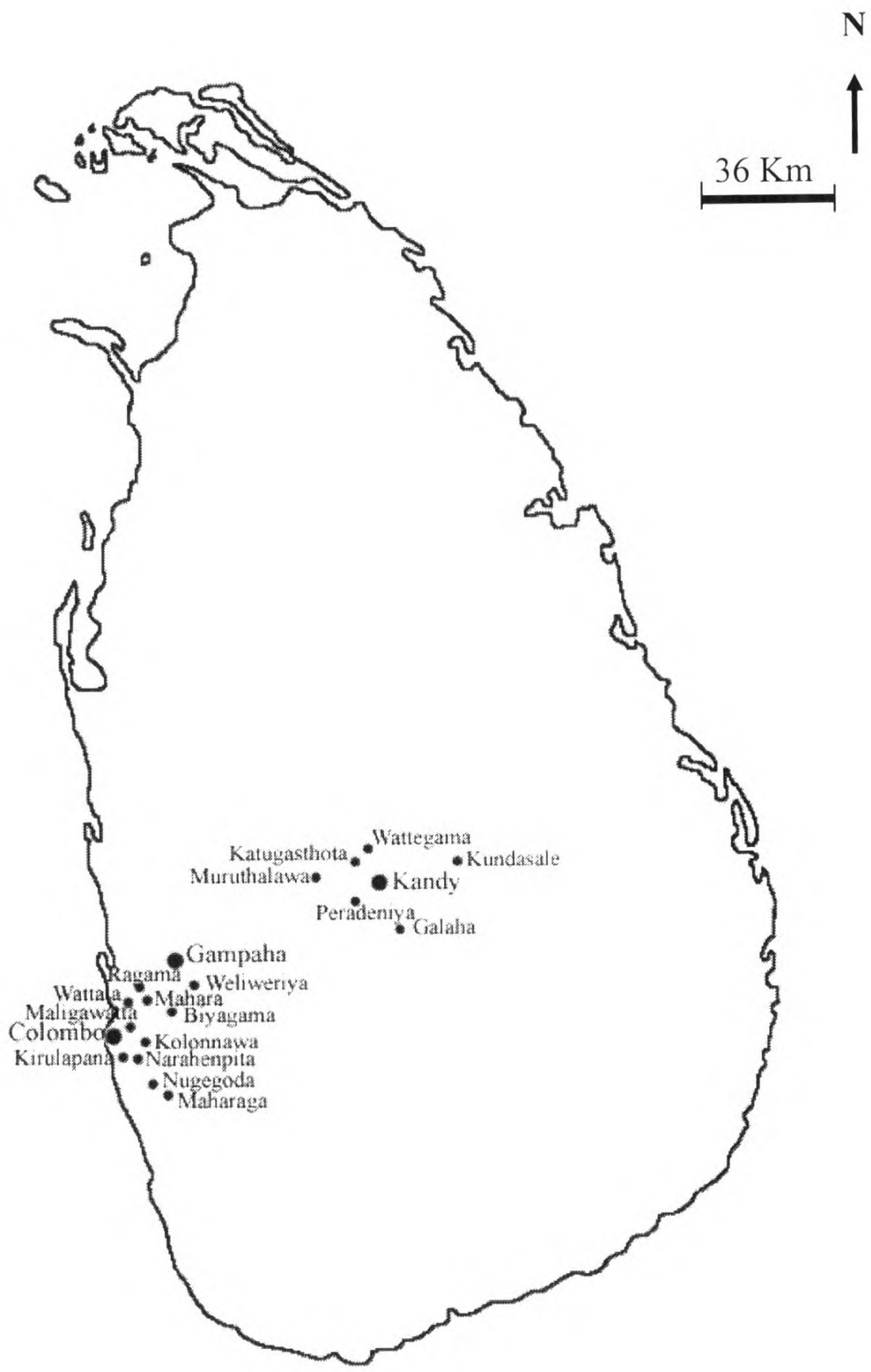

Figure 4.1. The geographic locations of sampling sites

Table 4.1. Locations and sample sizes of mosquitoes emerged.

District	Area	No. of traps fixed	No. of eggs	No. of larvae	Total no. of mosquitoes	No. of *A. albopictus*	No. of *A. aegypti*
Colombo	Narahenpita	20	64	22	14	8	6
	Kirulapana	15	53	23	8	8	-
	Maharagama	11	97	16	9	9	-
	Nugegoda	11	160	37	17	13	4
	Maligawatta	12	29	12	3	3	-
	Kolonnawa	9	39	19	5	5	-
Gampaha	Mahara	10	63	57	30	30	-
	Ragama	11	86	54	26	26	-
	Wattala	11	18	9	7	7	-
	Gampaha	8	97	56	37	37	-
	Weliweriya	7	200	61	37	37	-
	Biyagama	4	108	29	19	19	-
Kandy	Peradeniya	11	154	68	32	30	2
	Galaha	11	54	24	16	16	-
	MC Kandy	11	149	38	10	8	2
	Kundasale	21	259	78	30	22	8
	Katugasthota	10	100	45	27	18	9
	Wattegama	8	184	49	18	18	-
	Muruthalawa	8	182	78	31	27	4
Total		209	2096	775	376	341	35

4.2 DNA extraction

High purity and good yield per *Aedes* mosquito were the main factors expected in the DNA extraction procedure. Therefore A_{260}/A_{280} ratio and yield of extracted DNA from three different procedures (Section 3.2) were determined and compared. In comparison to Rivero et al., (2004) and standard high salt extraction protocol, highest concentration (45.9 µg/ml) of genomic DNA with average A_{260}/A_{280} ratio of 1.73 were obtained with Apostol et al., 1993 procedure.

When analyzed on 0.8% agarose gel extracted DNA gave clear, intense, sharp bands in almost all samples. This indicated the presence of intact high molecular weight mosquito genomic DNA. RNA also appeared as a smear in the gel in the low molecular weight region. The concentration of DNA was calculated by gel assay as described earlier (Section 1.5.2) (Figure 4.2). Amount of DNA per mosquito varied from ~ 40 ng to ~ 240 ng. Average amount of extracted DNA per mosquito was
~ 80 ng.

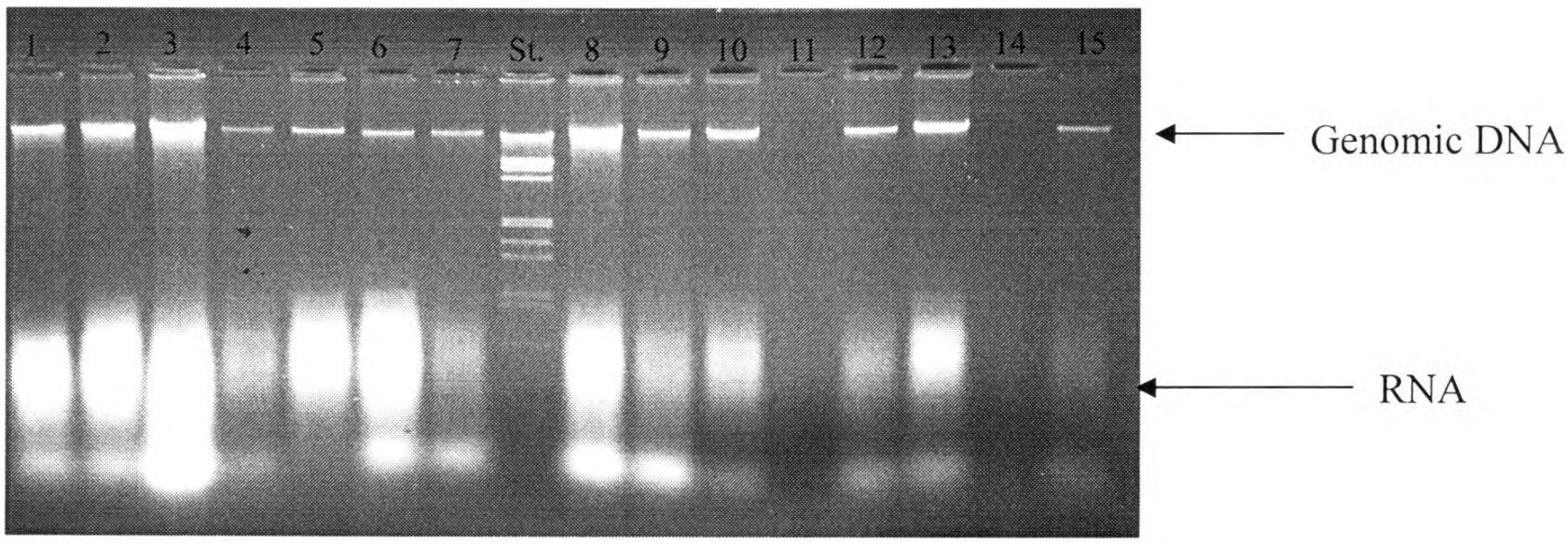

Figure 4.2. High molecular weight genomic DNA extracted from *A. albopictus* mosquitoes visualized in 0.8% agarose gel (Each lane 1 -15 contain ~ 80 ng of DNA)

4.3 RAPD - PCR

Strong and reliable amplification products for RAPD-PCR with random primers were obtained from DNA isolated by Apostol et al. 1993 procedure. The parameters for RAPDs such as the concentration of primer, $MgCl_2$, template DNA, Taq. DNA polymerase, and the temperature of denaturation, annealing were optimized to get clear intense, reproducible, polymorphic bands.

Optimization of primer concentration

Primer concentration was optimized from 0.2 mM to 2.0 mM. Large numbers of non specific bands were observed at high primer concentration. In addition some bands were not amplified with some primers at 0.2 mM concentration. Further optimization yielded clear intense bands at 0.4 mM primer concentration (Figure 4.3).

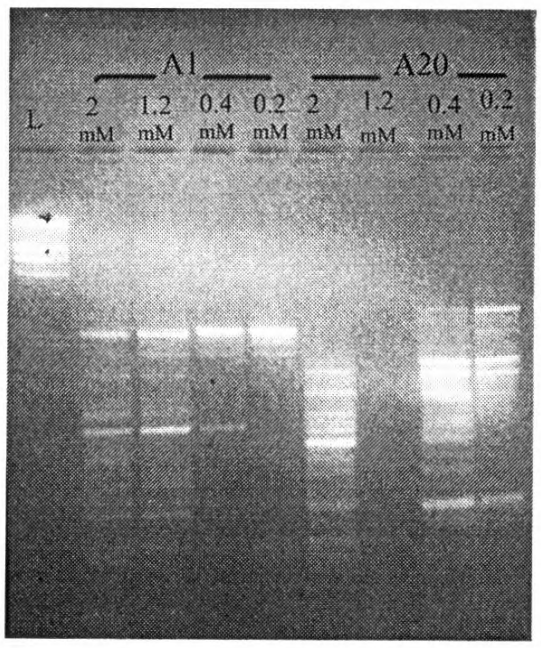

Figure 4.3. Effect of primer concentration on RAPD patterns
(4 ng/ μl of mosquito DNA were amplified with primer A1 and A20 at four different primer concentrations of 0.2, 0.4, 1.2 and 2.0 mM. Lane L, *Lamda DNA/ Eco* R I/ *Hind* III marker).

Optimization of $MgCl_2$ concentration

$MgCl_2$ concentrations tested, ranged from 1 to 2.5 mM. No amplification product was observed at 1 mM $MgCl_2$. The number of detectable products increased when $MgCl_2$ concentration changed from 1.5 mM to 2.5 mM. Similar banding profiles were obtained using 2.0 and 2.5 mM $MgCl_2$. But the intensity of band was higher at 2.5 mM $MgCl_2$ concentration (Figure 4.4).

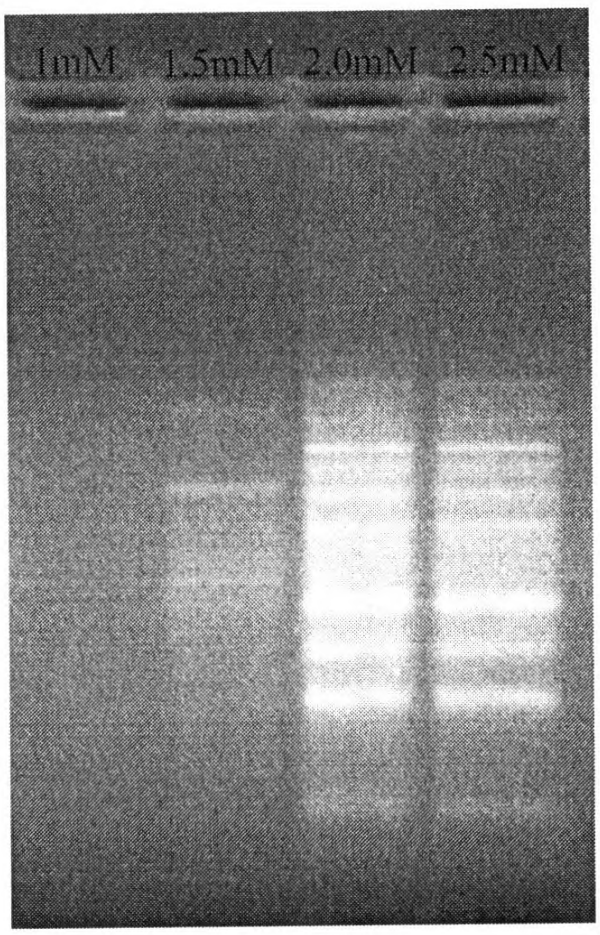

Figure 4.4. Effect of $MgCl_2$ concentration on RAPD patterns
(4 ng/ µl of mosquito DNA were amplified with primer A2 at four different $MgCl_2$ concentrations of 1.0, 1.5, 2.0 and 2.5 mM).

Optimization of template DNA concentration

Template DNA concentrations tested, ranged from 4 to 20 ng in 25 µl reaction mixture. Numbers of amplified bands were increased with the increase of template concentration. Clear, intense band profile was observed with the 12 ng of template DNA per 25 µl reaction (Figure 4.5).

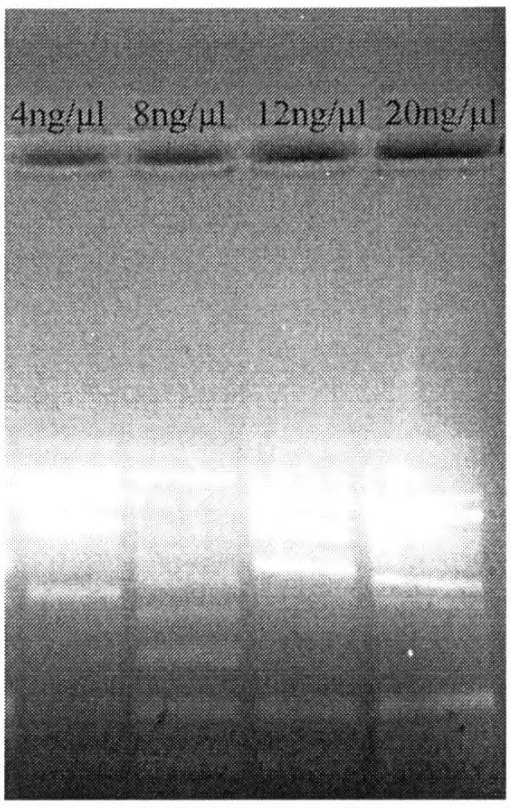

Figure 4.5. Effect of template DNA concentration on RAPD patterns
(Mosquito DNA was amplified with primer A2 at four different template DNA concentration of 4, 8, 12 and 20 ng/ µl).

Primer screening

A set of random primers that differentiate intra genus polymorphic variations within mosquito species was screened from a pool of 30 primers (Operon A-C). Initially 30 RAPD primers (10mer oligonucleotides with arbitrary sequences) were screened with *A. albopictus* randomly selected from three different districts. Then a set of RAPD primers which produce reliable, reproducible and polymorphic fingerprints were selected for the study. These primers gave reproducible fingerprinting pattern when run in triplicate. Out of the 30 primers, five primers, A8, B7, B8, C18 and C19 that revealed clear, consistent and discrete banding pattern were selected for the diversity analysis (Table 3.1) (Figure 4.6 and 4.7).

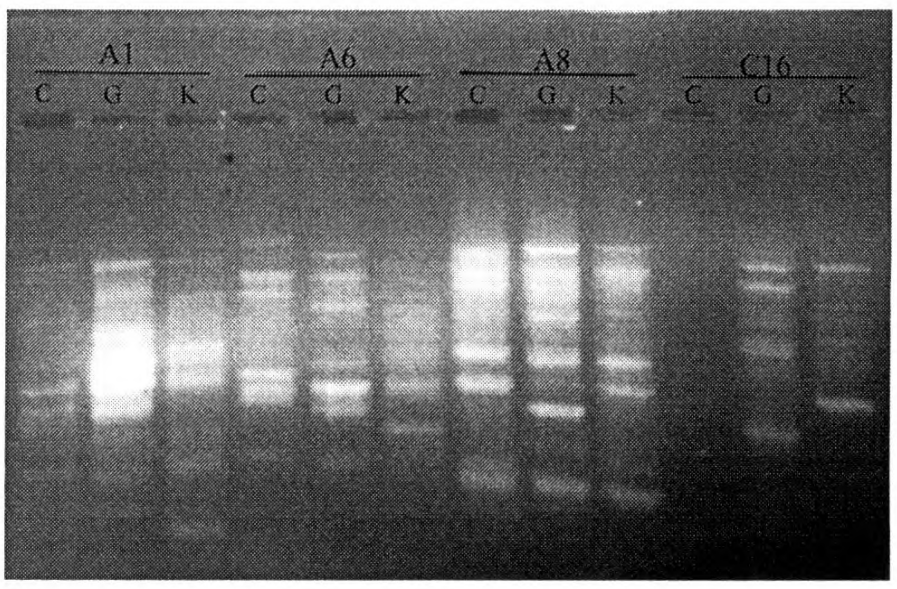

Figure 4.6. RAPD amplification patterns of mosquito samples (4 ng/ µl) from three districts (C-Colombo, G-Gampaha, K-Kandy). lane 1-3 with primer A1; lane 4-6 with A6; lane 7-9 with primer A8; lane 10-12 with primer C16 in 1.2 % agarose gel.

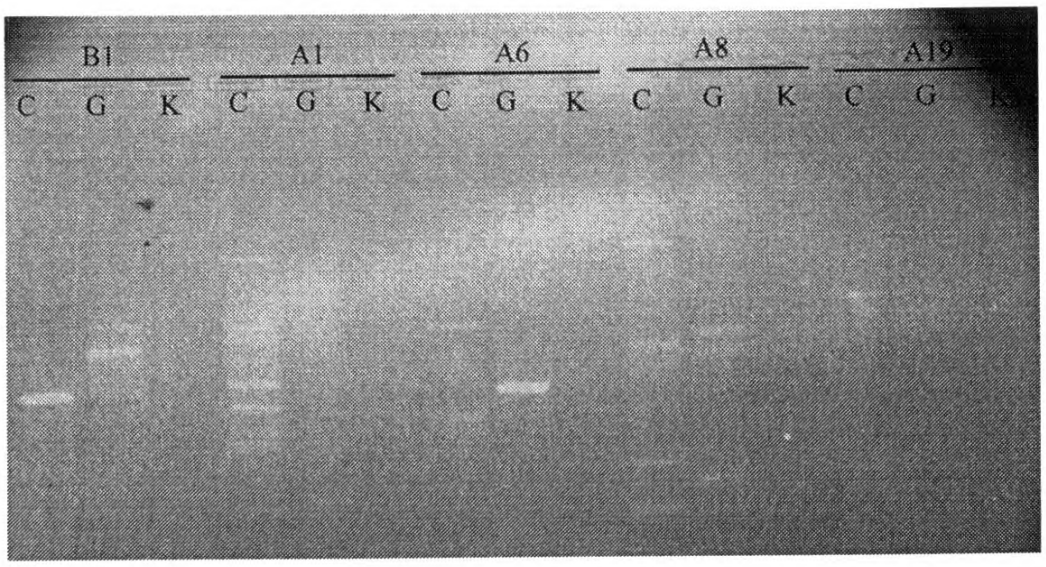

Figure 4.7. RAPD amplification patterns of mosquito samples (4 ng/ μl) from three districts (C-Colombo, G-Gampaha, K-Kandy). lane 1-3 with primer B1; lane 4-6 with A1; lane 7-9 with primer A6; lane 10-12 with primer A8; lane 13-15 with primer A19 in 1.2 % agarose gel.

4.4 RAPD-PCR and gel electrophoresis

All the extracted DNA samples from three different districts were amplified with selected primers. A total of 62 bands were produced and overall amplification consisted of 10 to 15 bands ranged from 200 bp to 3.0 kbp size. The A8 primer was able to generate 15 bands. Primer B7 produced 14 bands and B8 produced 10 bands. While C18 produced 12 bands followed by C19 with 11 bands. Therefore, altogether 62 loci (bands) were amplified. All the amplified bands produced by the 5 primers are polymorphic with no detectable monomorphic bands.

Some of the amplification products were shown in following figures (Figure 4.8 – 4.12)

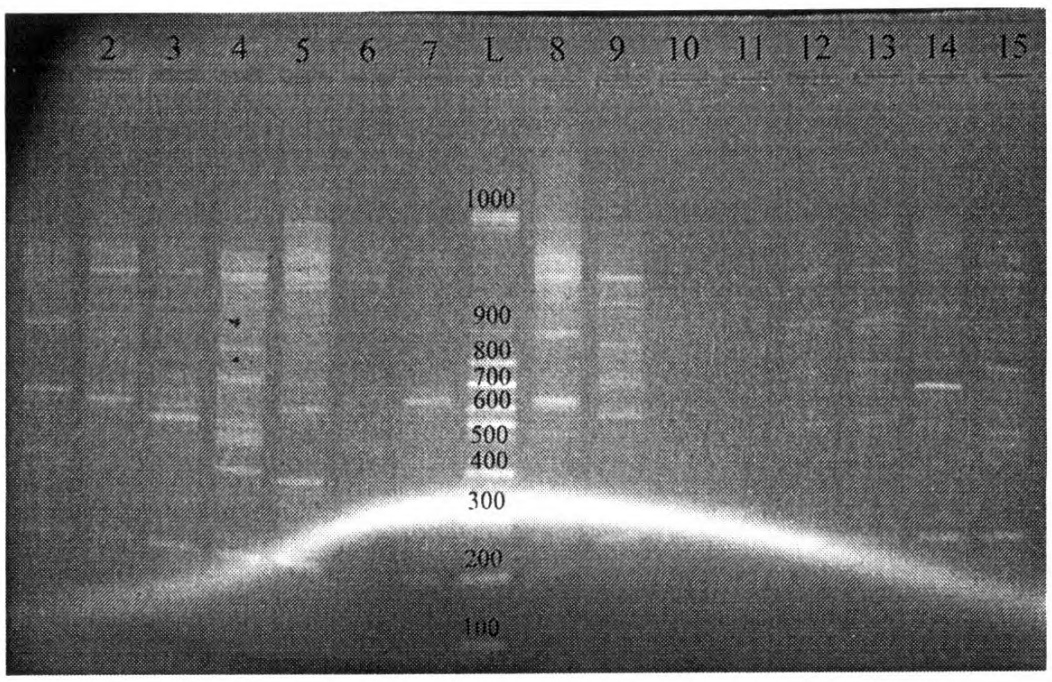

Figure 4.8. RAPD patterns of genomic DNA from *A. albopictus* populations in Colombo district with primer A8, lane L-100 bp ladder.

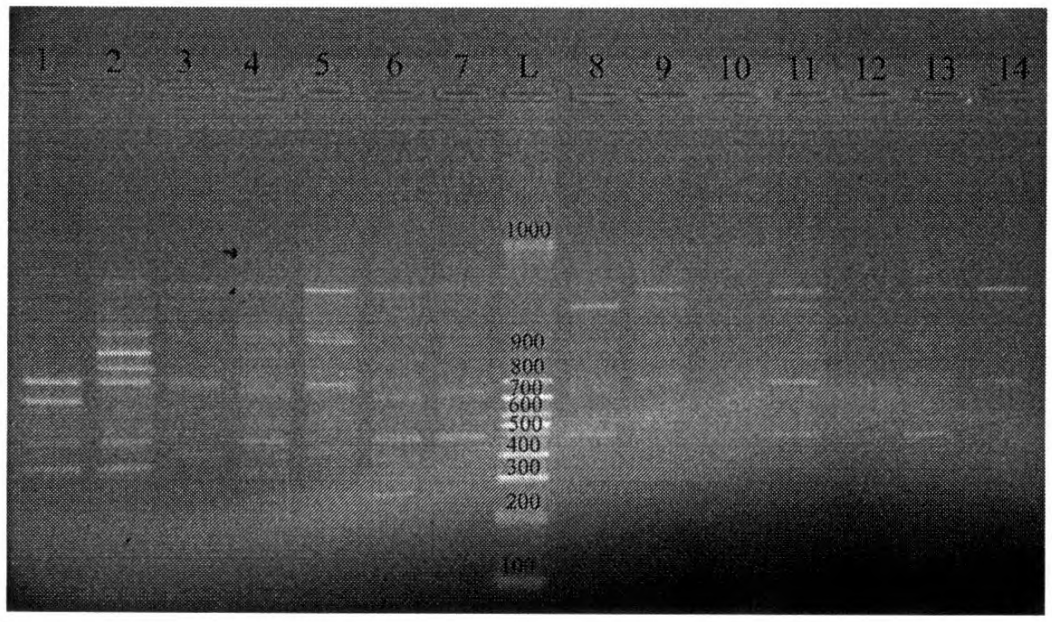

Figure 4.9. RAPD patterns of genomic DNA from *A. albopictus* populations in Colombo district with primer B7, lane L-100 bp ladder.

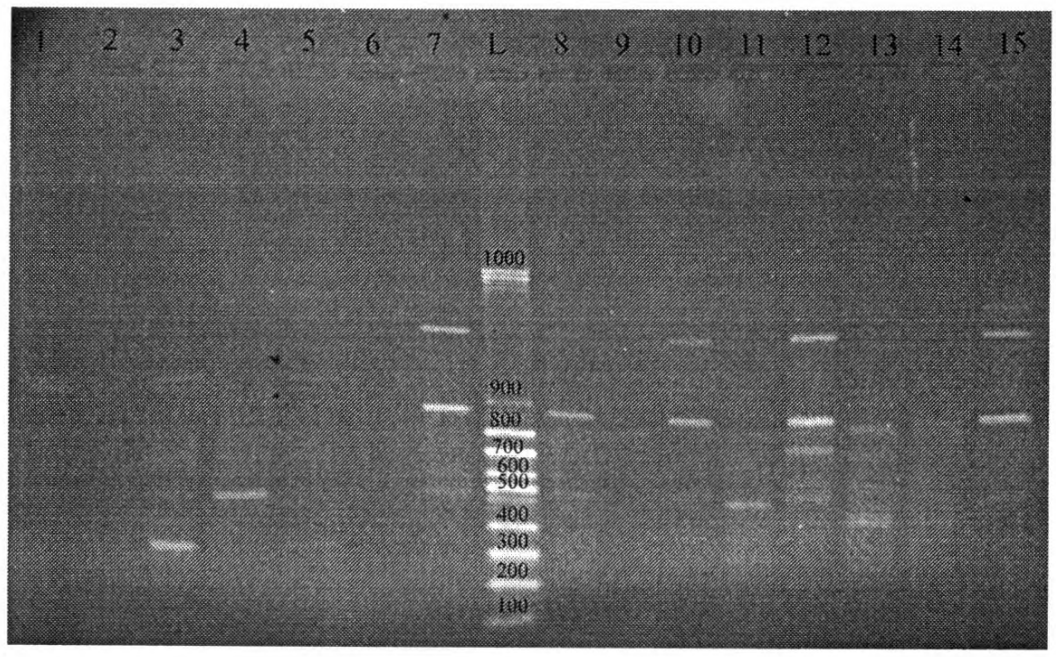

Figure 4.10. RAPD patterns of genomic DNA from *A. albopictus* populations in Gampaha district with primer B8, lane L-100 bp ladder.

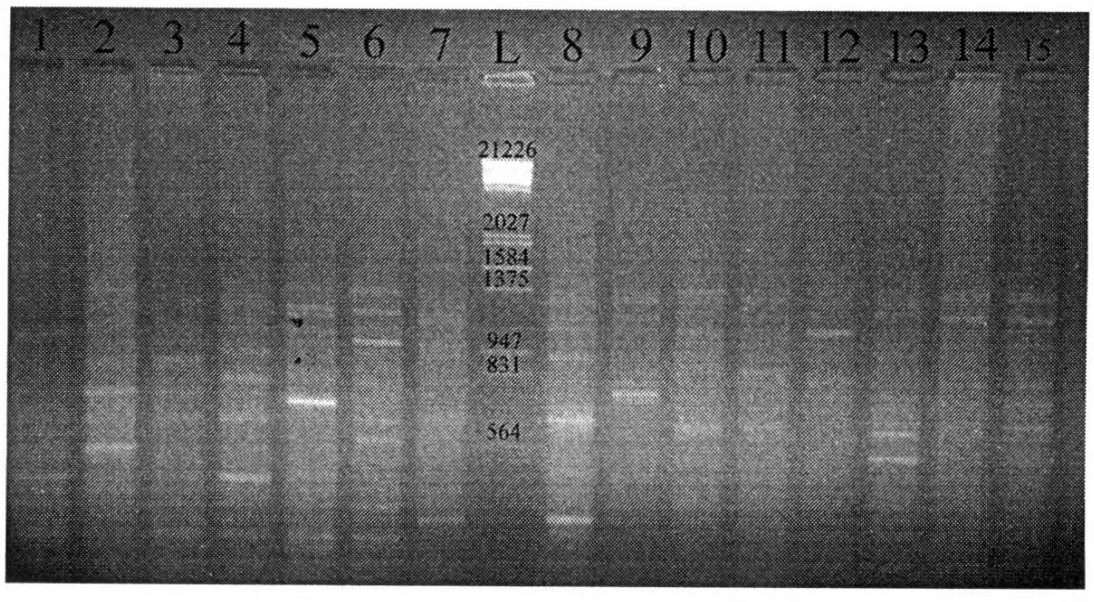

Figure 4.11. RAPD patterns of genomic DNA from *A. albopictus* populations in Kandy district with primer C18, lane L- *Lamda DNA/ Eco*R I*/ Hind* III marker.

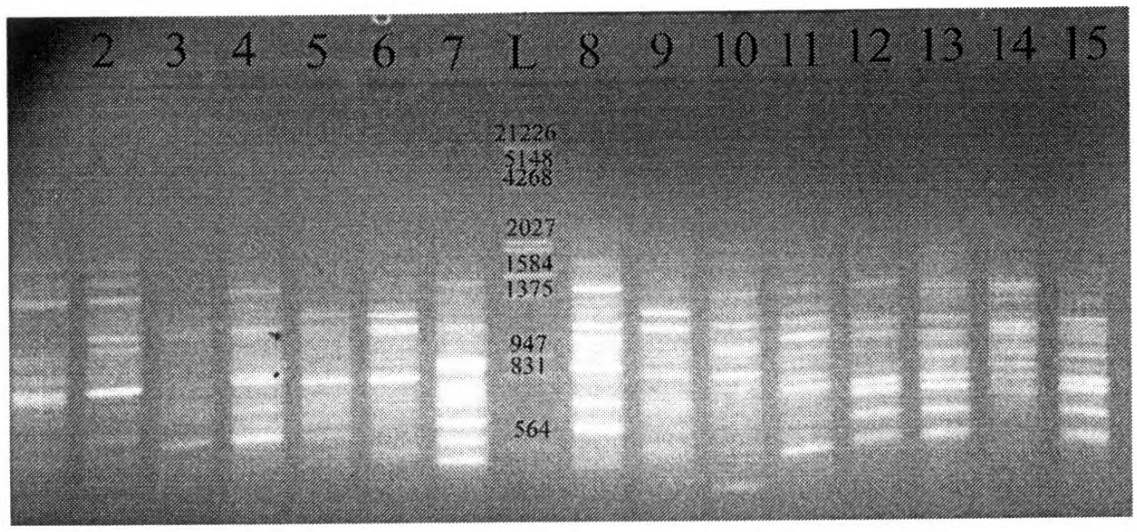

Figure 4.12. RAPD patterns of genomic DNA from *A. albopictus* populations in Kandy district with primer C19, lane L- *Lamda DNA/ Eco*R I/ *Hind* III marker.

4.5 Statistical analysis

Total of 62 loci representing the 19 populations from three districts were used for the statistical analysis. Amplified bands were scored and presented in a data matrix (see appendix). Nei's genetic distance (D), Total gene diversity, H_T; mean gene diversity within population, Hs; genetic differentiation, Gst; gene flow, Nm; and average expected heterozigosity, He values were calculated using POPGENE genetic package. F statistics- θ was calculated using TFPGA genetic package. Both TFPGA and POPGENE (version 1.32) population genetics programs were used to calculate Nei's original genetic distances (Nei's 1972) and to build dendograms.

Following equations were used for the calculations of genetic parameters.

Nei's genetic distance (D)

Consider two randomly mating diploid populations X and Y,

$D_{XY} = -\ln (I_{XY})$

Where, it based on the concept of genetic identity
$I_{XY} = J_{XY} / \sqrt{J_X J_Y}$
J_X = average homozygosity in population X
J_Y= average homozygosity in population Y
J_{XY}= average interpopulation homozygosity

Total gene diversity (Ht)

The gene diversity between the i^{th} and j^{th} populations as

$D_{ij} = H_{ij} - (H_i + H_j)/2$

Where, $H_i = 1 - J_i$ and $H_{ij} = 1 - J_{ij}$. (J = gene identity)
While, the gene identity in the total population J_T is,

$J_T = J_S - D_{ST}$
Where, J_S is the average gene identity within subpopulations, and D_{ST} is the average gene diversity between subpopulations, including the comparisons of subpopulations with themselves. The gene diversity in the total population ($Ht = 1 - J_T$) is

$Ht = Hs + D_{ST}$
Where $Hs = 1 - Js$, within population gene diversity. Thus, the gene diversity in the total population can be analyzed into the gene diversities within and between subpopulations.

Genetic differentiation; Gst

$G_{ST} = D_{ST}/Ht$
$= (H_T - H_S)/Ht$
Where, D_{ST} is the average gene diversity between subpopulations.

Gene flow is estimated from Gst (Nm)

$Nm = 0.5(1-Gst)/ Gst$

Average expected heterozygosity (He) or Nei's genetic diversity

$$He = \sum_j^L hj / L$$

Where, $hj = 1 - \sum Pi$, heterozygosity per locus j with i alleles. Where, P_i is the frequency of the i^{th} allele. L = Total number of loci. The average He over all loci is an estimate of the extent of genetic variability (diversity) of the population.

Table 4.2. Genetic variability indices (polymorphism and Nei's (1973) genetic diversity (He) for each population based on RAPD analysis

Localities	**Geographic distance range among populations (km)**	**Polymorphism (%)**	**Genetic Diversity (He)**
Colombo	2.1 – 11.7	98.39	0.2722
Gampaha	2.3 – 16.2	100.00	0.3173
Kandy	4.6 – 16.5	93.55	0.2924
Gampaha & Colombo	2.1 - 26.0	100.00	0.3129
Gampaha & Kandy	2.3 – 85.0	100.00	0.3198
Colombo,Gampaha & Kandy	2.1 – 96.5	100.00	0.3169

Table 4.3. Genetic indices, (total gene diversity, Ht; gene diversity (within population), Hs; genetic differentiation, Gst and θ) and gene flow (Nm) among Sri Lankan *A. albopictus* populations, pooled by district based on RAPD analysis

Population	**Ht**	**Hs**	**Gst**	**Nm**	**θ**
Colombo	0.2671	0.2056	0.2303	1.6711	0.1377
Gampaha	0.3124	0.2637	0.1558	2.7096	0.1110
Kandy	0.3018	0.2009	0.3343	0.9955	0.2127
Gampaha & Colombo	0.3027	0.2346	0.2248	1.7244	0.1576
Gampaha & Kandy	0.3217	0.2299	0.2853	1.2525	0.1899
Colombo,Gampaha & Kandy	0.3160	0.2222	0.2969	1.1841	0.1915

4.5.1 Intra population analysis

4.5.1.1 Colombo health region

The average expected heterozygosity, He among six populations in Colombo district was 0.2722 and polymorphism value was 98.39% (Table 4.2). The total gene diversity (Ht) was 0.2671 and within population gene diversity, Hs were 0.2056. The overall differentiation among 6 population was high (θ = 0.1377; Gst = 0.2303; F_{ST} = 0.1104; Nm = 1.6711) (Table 4.3). Similarly, the genetic distances varied between 0.0546 and 0.1355; genetic differentiation (F_{ST} value) varied between 0.0000 and 0.1890 (Table 4.4 and 4.5).

Table 4.4. Pair wise genetic identities (diagonal above) and genetic distances (diagonal below) between six *A. albopictus* populations in Colombo district based on RAPD analysis

pop ID	Narahenpita	Kirulapana	Maharagama	Nugegoda	Maligawatta	Kolonnawa
Narahenpita	****	0.9469	0.8852	0.8733	0.8975	0.9438
Kirulapana	0.0546	****	0.8884	0.9017	0.9130	0.9355
Maharagama	0.1220	0.1183	****	0.9318	0.8831	0.9145
Nugegoda	0.1355	0.1035	0.0707	****	0.8912	0.8946
Maligawatta	0.1082	0.0910	0.1243	0.1152	****	0.9223
Kolonnawa	0.0578	0.0667	0.0894	0.1114	0.0809	****

Table 4.5. Pair wise genetic differentiation (F_{ST}) values between six *A. albopictus* populations in Colombo district

pop ID	Narahenpita	Kirulapana	Maharagama	Nugegoda	Maligawatta	Kolonnawa
Narahenpita	****					
Kirulapana	0.0000	****				
Maharagama	0.1546	0.1890	****			
Nugegoda	0.1831	0.1573	0.0526	****		
Maligawatta	0.1108	0.1184	0.1826	0.1626	****	
Kolonnawa	0.0000	0.0567	0.0956	0.1396	0.0667	****

The geographical distance between the populations in Colombo health region was comparatively small (2.1 – 11.7 km). The correlation matrix between F_{ST} / (1- F_{ST}) and the respective geographic distances was positive (R^2 = 0.2428) (Figure 4.13).

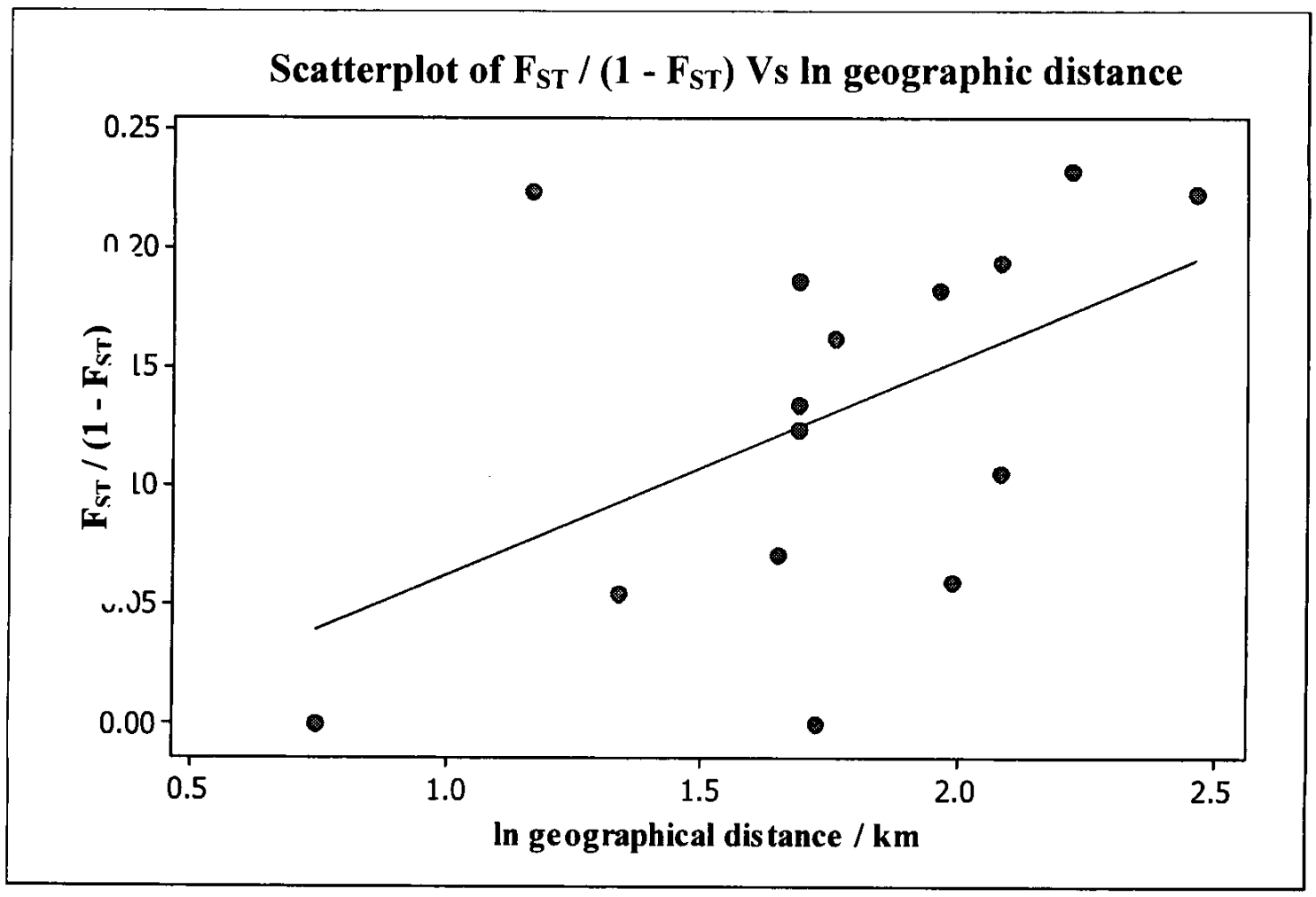

Figure 4.13. Scatter plot of genetic differentiation between the pairs of populations of *A. albopictus* from Colombo health area against the ln geographical distance separating them.

The unweighted pair-group method with arithmetic average dendogram (Figure 4.14) demonstrated two separate clusters. One cluster represented Maharagama and Nugegoda populations. The second cluster further divided into two subgroups, one containing Maligawatte and the other was further divided into Kolonnawa population and small cluster representing Narahenpita and Kirulapana populations.

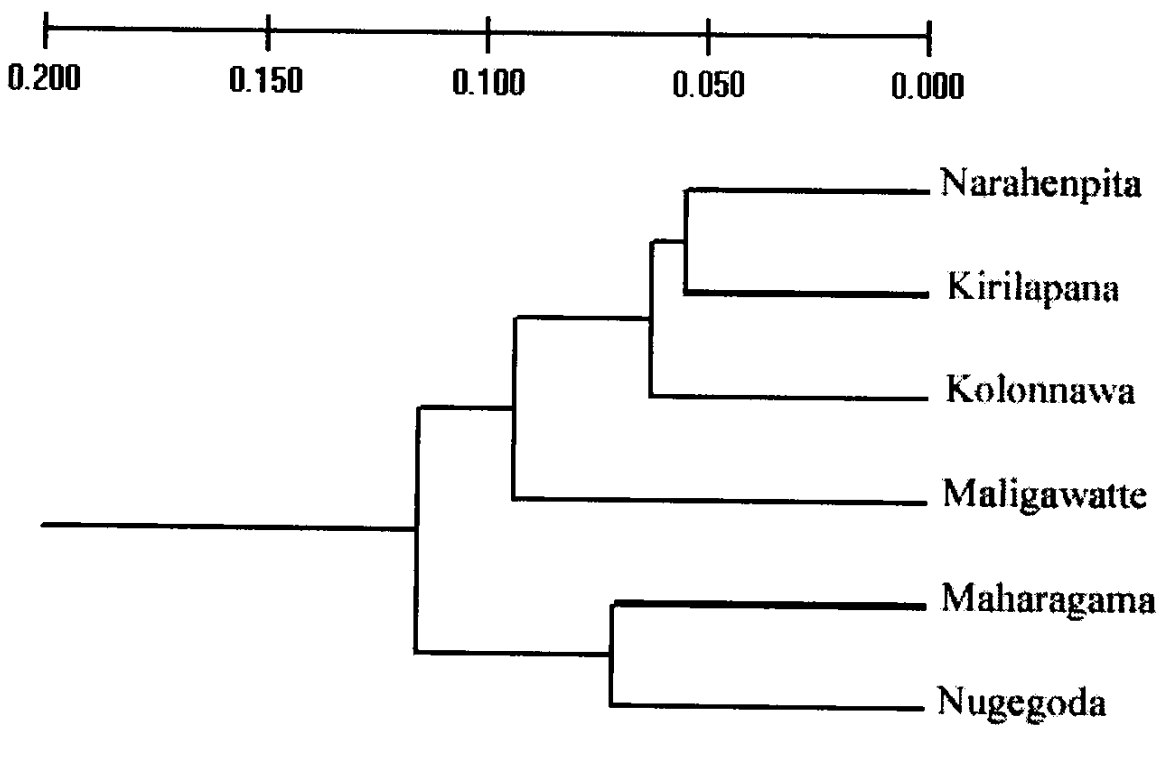

Figure 4.14. Dendrogram based Nei's (1972) genetic distances among *A. albopictus* populations in the Colombo health areas (Table 3.4).

4.5.1.2 Gampaha health region

Analysis of six populations in Gampaha health area revealed polymorphism value of 100.00 and average expected heterozygosity, He of 0.3173 (Table 4.2). The total gene diversity (Ht) was 0.3124 and within population gene diversity (Hs) was 0.2637 (Table 4.3). The overall differentiation (Gst) among six *A. albopictus* populations analyzed was 0.1558 (θ = 0.1110; F_{ST} = 0.0985) (Table 4.3). The number of migrants (Nm) was 2.7096 mosquitoes per generation (Table 4.3). The genetic distances varied from 0.0522 and 0.1216 (Table 4.6). F_{ST} values between pairs of populations ranged from 0.0314 – 0.1518 (Table 4.7).

Table 4.6. Pair wise genetic identities (diagonal above) and genetic distances (diagonal below) between six *A. albopictus* populations in Gampaha district based on RAPD analysis

pop ID	Mahara	Ragama	Wattala	Gampaha	Weliweriya	Biyagama
Mahara	****	0.9351	0.9248	0.8855	0.9295	0.9007
Ragama	0.0671	****	0.9461	0.9012	0.9484	0.9193
Wattala	0.0782	0.0554	****	0.8958	0.9331	0.9140
Gampaha	0.1216	0.1040	0.1101	****	0.9186	0.9118
Weliweri.	0.0732	0.0530	0.0692	0.0849	****	0.9492
Biyagama	0.1046	0.0841	0.0900	0.0923	0.0522	****

Table 4.7. Pair wise genetic differentiation (F_{ST}) values between six *A. albopictus* populations in Gampaha district

pop ID	Mahara	Ragama	Wattala	Gampaha	Weliweriya	Biyagama
Mahara	****					
Ragama	0.0761	****				
Wattala	0.0787	0.0314	****			
Gampaha	0.1518	0.1252	0.1172	****		
Weliweri.	0.1321	0.0812	0.0663	0.1411	****	
Biyagama	0.1333	0.0951	0.0880	0.0955	0.0366	****

F_{ST} / (1- F_{ST}) showed a positive correlation with geographic distance of the populations of *A. albopictus* in the Gampaha health region (R^2 =0.1431) (Figure 4.15).

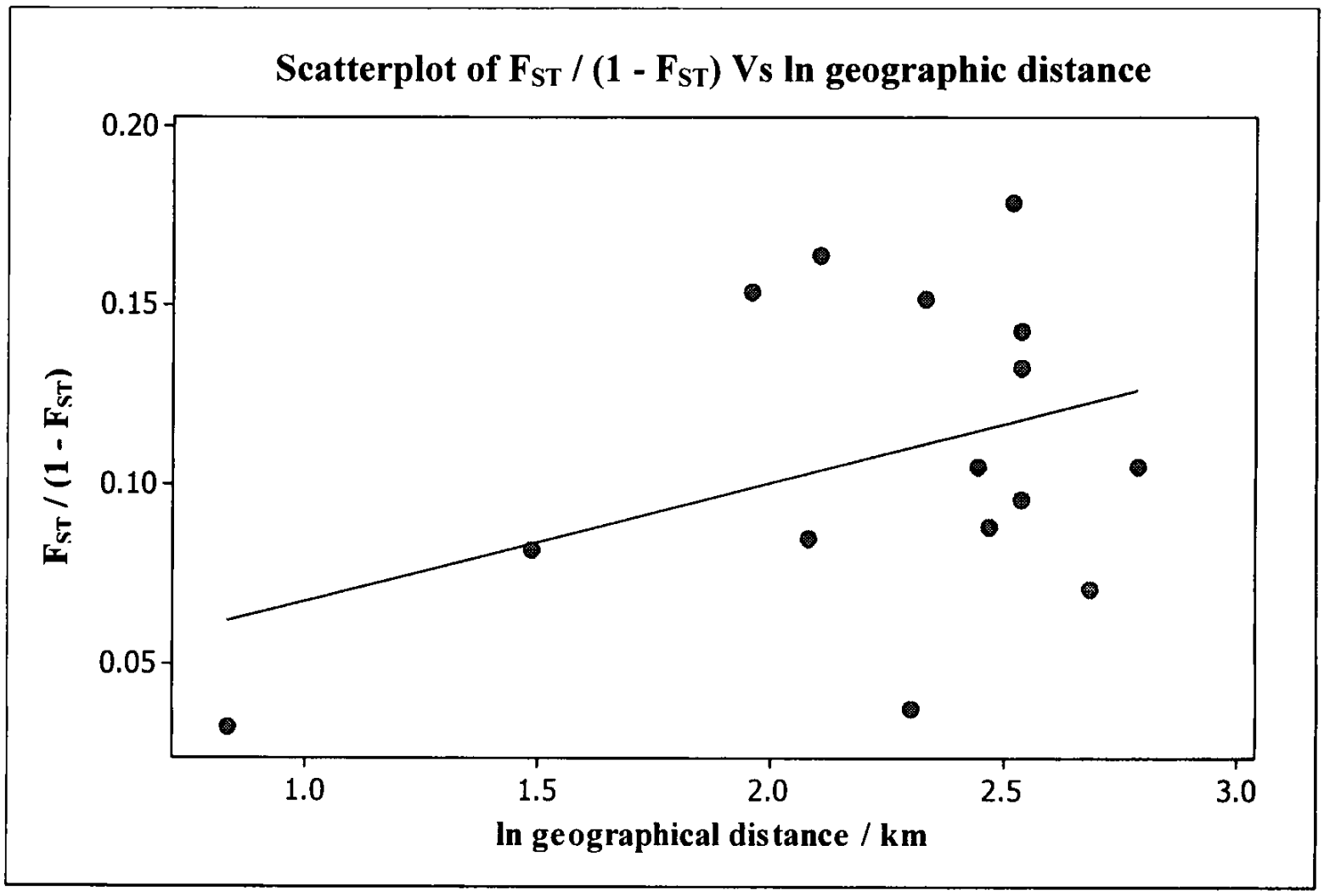

Figure 4.15. Scatter plot of genetic differentiation between the pairs of populations of *A. albopictus* from Gampaha health area against the ln of geographical distance separating them.

The dendogram constructed using the UPGMA method (Figure 4.16) showed that population in Gampaha city area represents a distinct group from the remaining *A. albopictus* populations in Gampaha district. Remaining populations further separated into two clusters, one representing Weliweriya and Biyagama populations, while the other representing Mahara population and a small cluster containing Ragama and Wattala populations.

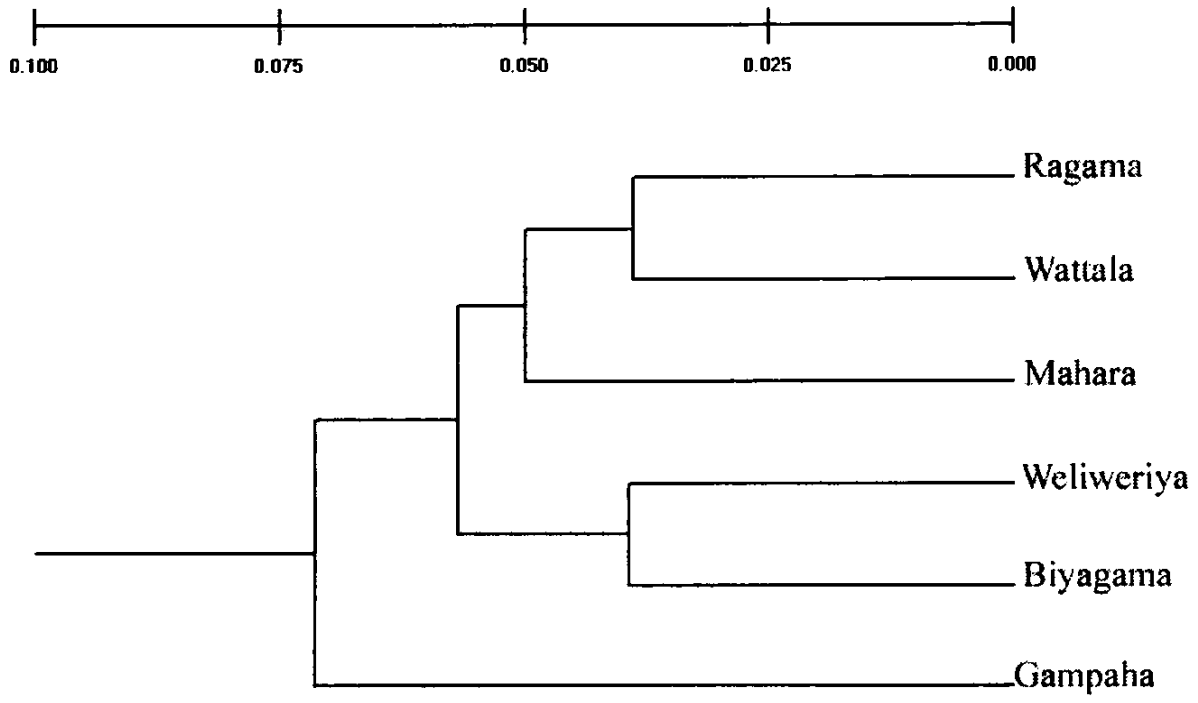

Figure 4.16. Dendrogram based on Nei's (1972) genetic distance among *A. albopictus* populations in the Gampaha health area (Table 4.6).

4.5.1.3 Kandy health region

Average expected heterozygosity, He of seven *A. albopictus* populations in the Kandy district were 0.2924. A comparatively lower polymorphism value of 93.55 was also observed (Table 4.2). The total gene diversity (Ht) was 0.3018 (Table 4.3). The overall differentiation among seven populations was extremely high (Gst = 0.3343; F_{ST} = 0.0625; Nm = 0.9955) (Table 4.3). Pair wise genetic distances were also high, and varied between 0.0373 and 0.2951 (Table 4.8). F_{ST} values between pairs of populations ranged from 0.0000 – 0.1614 (Table 4.9).

Table 4.8. Pair wise genetic identities (diagonal above) and genetic distances (diagonal below) between seven *A. albopictus* populations in Kandy district based on RAPD analysis

pop ID	Peradeni.	Galaha	Kandy	Kundasale	Wattega.	Katugast.	Murutha.
Peradeni.	****	0.8990	0.8864	0.7626	0.9256	0.9094	0.8377
Galaha	0.1065	****	0.8531	0.8626	0.8890	0.8787	0.8529
Kandy	0.1206	0.1588	****	0.7445	0.9201	0.9001	0.8804
Kundasa.	0.2710	0.1478	0.2951	****	0.7716	0.7722	0.7466
Wattega.	0.0773	0.1177	0.0833	0.2593	****	0.9634	0.8623
Katugast.	0.0950	0.1293	0.1052	0.2585	0.0373	****	0.8334
Murutha.	0.1771	0.1591	0.1274	0.2923	0.1481	0.1823	****

Table 4.9. Pair wise genetic differentiation (F_{ST}) values between seven *A. albopictus* populations in Kandy district

pop ID	Peradeni.	Galaha	Kandy	Kundasale	Wattega.	Katugast.	Murutha.
Peradeni.	****						
Galaha	0.0087	****					
Kandy	0.0087	0.0558	****				
Kundasa.	0.1279	0.0229	0.1552	****			
Wattega.	0.0199	0.0599	0.0202	0.1614	****		
Katugast.	0.0388	0.0685	0.0314	0.1608	0.0000	****	
Murutha.	0.0335	0.0192	0.0000	0.1216	0.0424	0.0646	****

Compared to the Colombo and Gampaha health region the geographic distance between the populations in Kandy health region was comparatively high (4.6 – 16.5 km). F_{ST} / (1-F_{ST}) showed no significant correlation with geographic distance of the populations of *A. albopictus* in the Kandy health region (R^2 =0.0017) (Figure 4.17). However it reveled no significant correlation between genetic differentiation and geographic distance in Kandy health region.

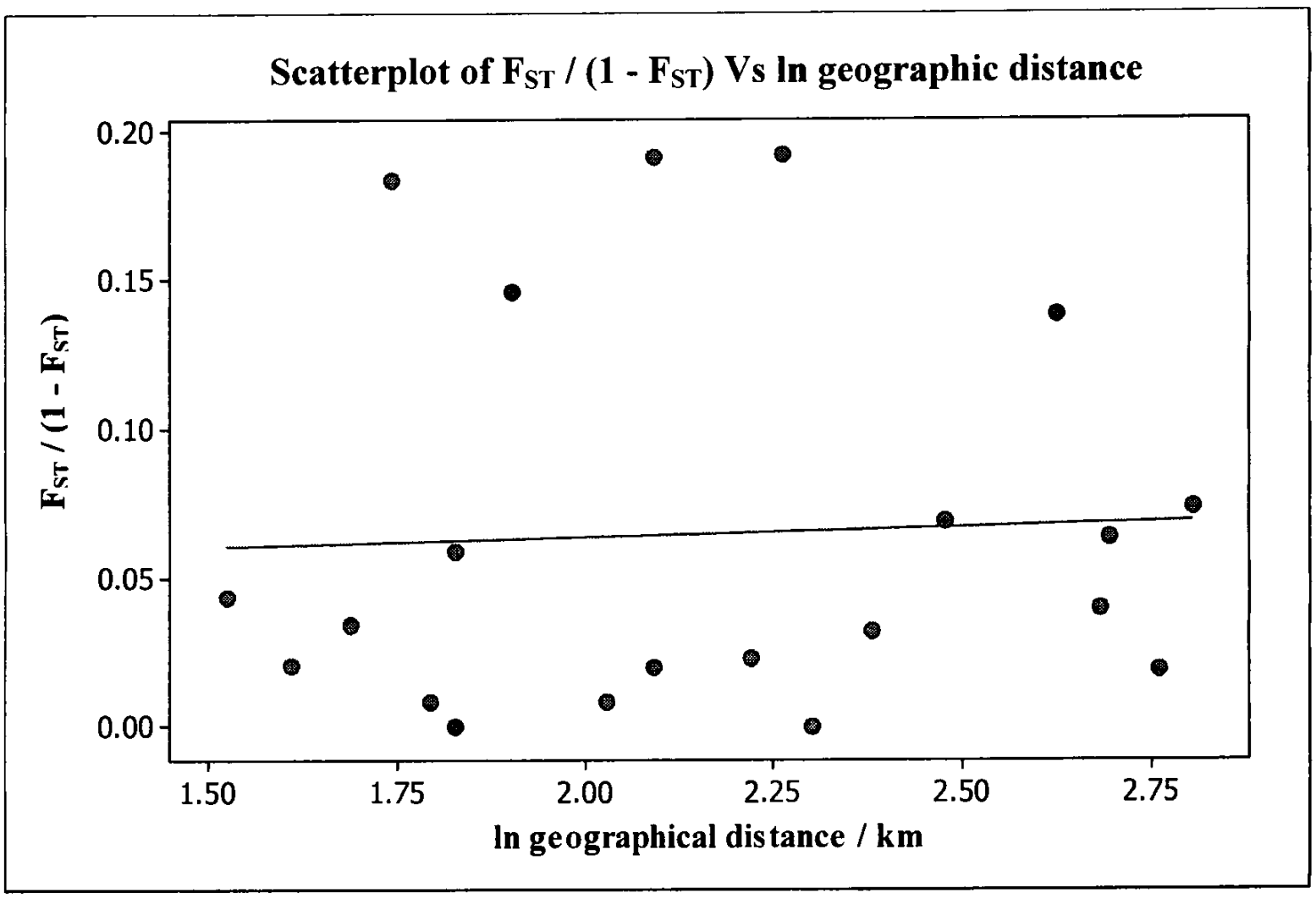

Figure 4.17. Scatter plot of genetic differentiation between the pairs of populations of *A. albopictus* from Kandy health area against the ln of geographical distance separating them.

The dendogram constructed by UPGMA method revealed two separate clusters (Figure 4.18). First cluster consist of highly diverge Kundasale population. The second cluster,

representing the remaining populations, was further divided to Muruthalawa, Galaha and MC Kandy. The remaining populations further grouped to Peradeniya population and to a small cluster containing Katugasthota and Wattegama populations.

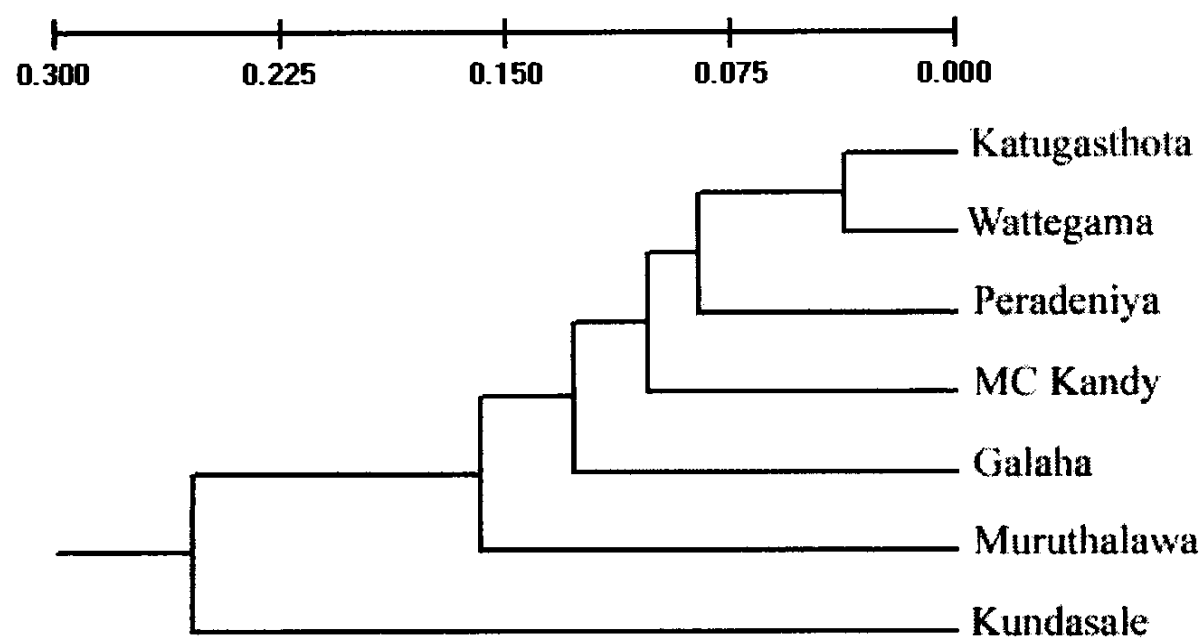

Figure 4.18. Dendrogram based on Nei's (1972) genetic distance among *A. albopictus* population in the Kandy health area (Table 3.8).

4.5.2 Inter population analysis

4.5.2.1 Gampaha and Colombo health regions

Genetic differentiation (Gst) of 12 *A. albopictus* mosquito populations among Gampaha and Colombo district was 0.2248. The number of migrants (Nm) was 1.7244 mosquitoes per generation. The total gene diversity (Ht) among Gampaha and Colombo populations was 0.3027 (Table 4.3). Pair wise genetic distances were varied between 0.0528 and 0.1667 (Table 4.10).

The dendogram constructed using the UPGMA method showed that populations from Colombo and Gampaha districts clustered separately (Figure 4.19). Maharagama and Nugegoda populations represented a distinct, highly diverged group from the remaining *A. albopictus* populations. Colombo and Gampaha populations clustered separately.

Table 4.10. Pair wise genetic identities (diagonal above) and genetic distances (diagonal below) between 12 *A. albopictus* populations in Gampaha and Colombo based on RAPD analysis

pop	1	2	3	4	5	6	7	8	9	10	11	12
1	****	0.9469	0.8852	0.8733	0.8975	0.9438	0.9099	0.9025	0.8768	0.9146	0.9144	0.8837
2	0.0546	****	0.8884	0.9017	0.9130	0.9355	0.9398	0.9267	0.9173	0.9122	0.9486	0.9106
3	0.1220	0.1183	****	0.9318	0.8831	0.9145	0.8714	0.9122	0.8922	0.8793	0.8991	0.8745
4	0.1355	0.1035	0.0707	****	0.8912	0.8946	0.8878	0.8887	0.8917	0.8964	0.9236	0.8831
5	0.1082	0.0910	0.1243	0.1152	****	0.9223	0.8510	0.8481	0.8465	0.8798	0.8678	0.8612
6	0.0578	0.0667	0.0894	0.1114	0.0809	****	0.9146	0.8978	0.8887	0.8902	0.9313	0.8972
7	0.0944	0.0620	0.1377	0.1190	0.1613	0.0893	****	0.9351	0.9248	0.8855	0.9295	0.9007
8	0.1025	0.0761	0.0919	0.1180	0.1648	0.1078	0.0671	****	0.9461	0.9012	0.9484	0.9193
9	0.1315	0.0863	0.1141	0.1147	0.1667	0.1180	0.0782	0.0554	****	0.8958	0.9331	0.9140
10	0.0893	0.0919	0.1286	0.1094	0.1281	0.1163	0.1216	0.1040	0.1101	****	0.9186	0.9118
11	0.0895	0.0528	0.1063	0.0794	0.1418	0.0712	0.0732	0.0530	0.0692	0.0849	****	0.9492
12	0.1237	0.0936	0.1341	0.1244	0.1495	0.1085	0.1046	0.0841	0.0900	0.0923	0.0522	****

1- Narahenpita, 2- Kirulapana, 3- Maharagama, 4- Nugegoda, 5- Maligawatta, 6- Kolonnawa, 7-Mahara, 8-Ragama, 9-Wattala, 10-Gampaha, 11- Weliweriya, 12- Biyagama

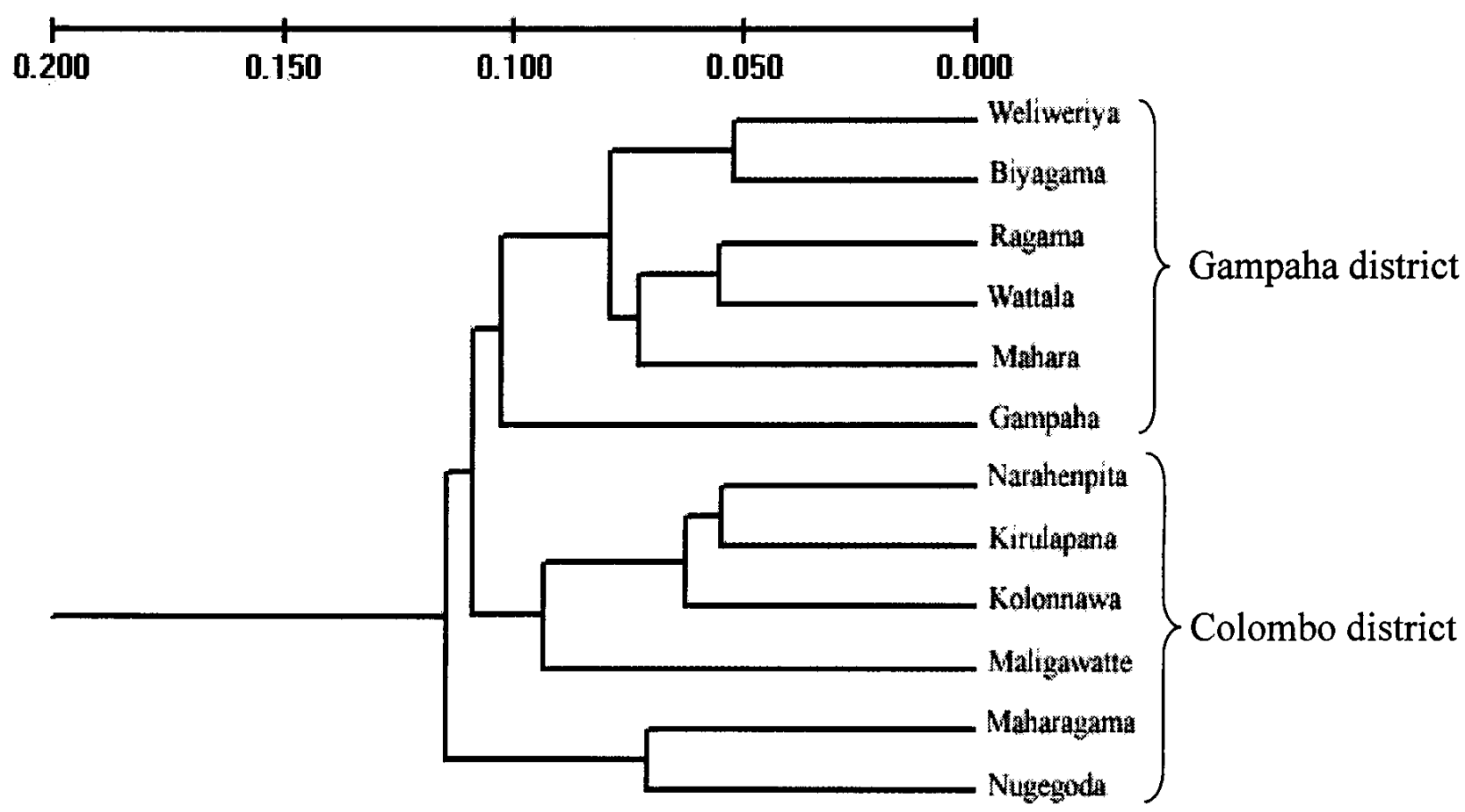

Figure 4.19. Dendrogram based on Nei's (1972) genetic distance among *A. albopictus* populations in the Gampaha and Colombo health area (Table 3.10).

4.5.2.2 Gampaha and Kandy health regions

Genetic differentiation (Gst) of 13 *A. albopictus* mosquito populations in Gampaha and Kandy district was 0.2853 and the number of migrants (Nm) was 1.2525 mosquitoes per generation. The total gene diversity (Ht) among Gampaha and Kandy populations was 0.3217 (Table 4.3). Pair wise genetic distances varied between 0.0245 and 0.2800 (Table 4.11).

Table 4.11. Pair wise genetic identities (diagonal above) and genetic distances (diagonal below) between 13 *A. albopictus* populations in Gampaha and Kandy based on RAPD analysis

ID	1	2	3	4	5	6	7	8	9	10	11	12	13
1	****	0.9577	0.9525	0.9113	0.9547	0.9274	0.9362	0.9264	0.8951	0.7558	0.9424	0.9216	0.8972
2	0.0432	****	0.9758	0.9286	0.9754	0.9478	0.9344	0.9330	0.8947	0.7929	0.9623	0.9191	0.8706
3	0.0487	0.0245	****	0.9283	0.9652	0.9477	0.9178	0.9137	0.8910	0.8174	0.9459	0.9054	0.9080
4	0.0929	0.0741	0.0744	****	0.9493	0.9446	0.8928	0.9047	0.8744	0.8191	0.9220	0.8726	0.8975
5	0.0464	0.0249	0.0355	0.0521	****	0.9815	0.9301	0.9349	0.9126	0.7848	0.9469	0.9093	0.9167
6	0.0753	0.0536	0.0537	0.0570	0.0187	****	0.9177	0.9056	0.9048	0.7749	0.9432	0.9013	0.8983
7	0.0659	0.0679	0.0858	0.1133	0.0725	0.0858	****	0.9361	0.9227	0.8011	0.9568	0.9421	0.8842
8	0.0765	0.0694	0.0903	0.1002	0.0674	0.0992	0.0660	****	0.8836	0.9015	0.9142	0.9057	0.8957
9	0.1108	0.1112	0.1154	0.1343	0.0914	0.1001	0.0804	0.1237	****	0.7778	0.9459	0.9276	0.9242
10	0.2800	0.2321	0.2016	0.1995	0.2423	0.2550	0.2218	0.1037	0.2513	****	0.8004	0.8029	0.7908
11	0.0593	0.0384	0.0556	0.0812	0.0546	0.0585	0.0442	0.0897	0.0556	0.2226	****	0.9857	0.8989
12	0.0816	0.0844	0.0993	0.1363	0.0951	0.1040	0.0596	0.0990	0.0752	0.2196	0.0144	****	0.8707
13	0.1084	0.1386	0.0965	0.1081	0.0870	0.1072	0.1231	0.1102	0.0788	0.2347	0.1066	0.1385	****

1-Mahara, 2-Ragama, 3-Wattala, 4-Gampaha, 5- Weliweriya, 6- Biyagama, 7- Peradeniya, 8- Galaha, 9- Kandy, 10- Kundasale, 11- Wattegama,
12- Katugasthota, 13- Muruthalawa

The UPGMA dendogram showed that populations from Gampaha health area and Kandy health area were clustered separately (Figure 4.20). Kundasale population represent in the most diverged population.

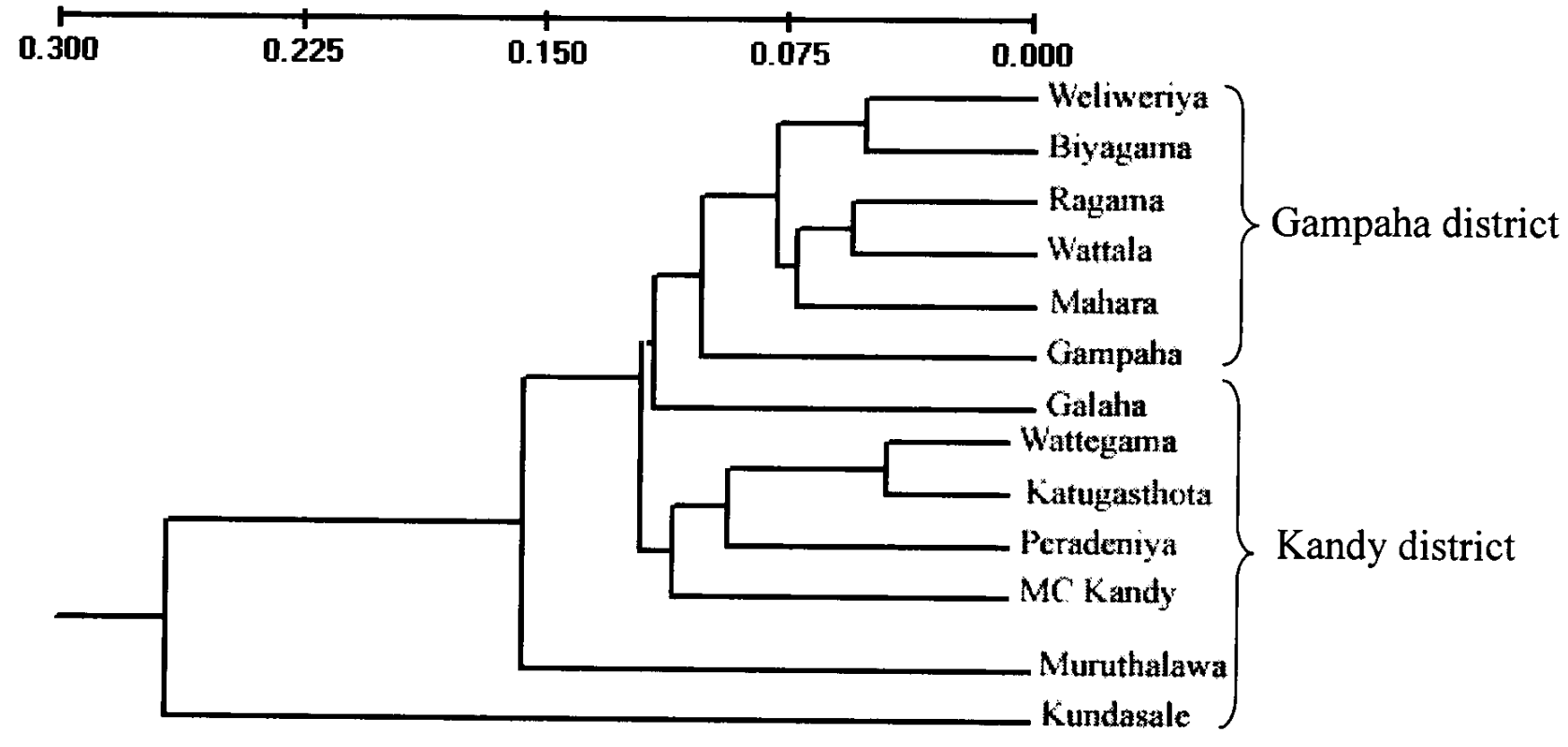

Figure 4.20. Dendrogram based on Nei's (1972) genetic distance among *A albopictus* populations in the Gampaha and Kandy health areas (Table 4.11).

4.5.2.3 Colombo, Gampaha and Kandy health regions

The total gene diversity (Ht) of nineteen *A. albopictus* populations among three districts was 0.3160. Similarly, the overall differentiation among the 19 populations was extremely high (θ = 0.1915; Gst = 0.2969; Nm = 1.1841) (Table 4.3) Pair wise genetic distances varied between 0.0522 and 0.4089 (Table 4.12).

Table 4.12. Pair wise genetic identities (diagonal above) and genetic distances (diagonal below) between nineteen *A. albopictus* populations based on random amplified polymorphic DNA analysis

pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	****	0.9469	0.8852	0.8733	0.8975	0.9438	0.9099	0.9025	0.8768	0.9146	0.9144	0.8837	0.8789	0.8435	0.8585	0.6919	0.9075	0.8668	0.8224
2	0.0546	****	0.8884	0.9017	0.9130	0.9355	0.9398	0.9267	0.9173	0.9122	0.9486	0.9106	0.9234	0.8778	0.8906	0.7352	0.9291	0.9034	0.8695
3	0.1220	0.1183	****	0.9318	0.8831	0.9145	0.8714	0.9122	0.8922	0.8793	0.8991	0.8745	0.8786	0.8561	0.8703	0.7563	0.9183	0.8884	0.8400
4	0.1355	0.1035	0.0707	****	0.8912	0.8946	0.8878	0.8887	0.8917	0.8964	0.9236	0.8831	0.8581	0.8531	0.8607	0.7544	0.8990	0.8539	0.8301
5	0.1082	0.0910	0.1243	0.1152	****	0.9223	0.8510	0.8481	0.8465	0.8798	0.8678	0.8612	0.8527	0.7928	0.8287	0.6644	0.8650	0.8550	0.7820
6	0.0578	0.0667	0.0894	0.1114	0.0809	****	0.9146	0.8978	0.8887	0.8902	0.9313	0.8972	0.8919	0.8740	0.8831	0.7414	0.9064	0.8968	0.8527
7	0.0944	0.0620	0.1377	0.1190	0.1613	0.0893	****	0.9351	0.9248	0.8855	0.9295	0.9007	0.9049	0.8999	0.8698	0.7279	0.9223	0.8999	0.8599
8	0.1025	0.0761	0.0919	0.1180	0.1648	0.1078	0.0671	****	0.9461	0.9012	0.9484	0.9193	0.9019	0.9051	0.8683	0.7625	0.9405	0.8962	0.8332
9	0.1315	0.0863	0.1141	0.1147	0.1667	0.1180	0.0782	0.0554	****	0.8958	0.9331	0.9140	0.8808	0.8814	0.8598	0.7817	0.9192	0.8779	0.8642
10	0.0893	0.0919	0.1286	0.1094	0.1281	0.1163	0.1216	0.1040	0.1101	****	0.9186	0.9118	0.8577	0.8735	0.8445	0.7840	0.8968	0.8468	0.8549
11	0.0895	0.0528	0.1063	0.0794	0.1418	0.0712	0.0732	0.0530	0.0692	0.0849	****	0.9492	0.8951	0.9043	0.8831	0.7526	0.9227	0.8841	0.8749
12	0.1237	0.0936	0.1341	0.1244	0.1495	0.1085	0.1046	0.0841	0.0900	0.0923	0.0522	****	0.8811	0.8738	0.8733	0.7413	0.9168	0.8741	0.8552
13	0.1291	0.0797	0.1295	0.1531	0.1594	0.1145	0.1000	0.1033	0.1269	0.1535	0.1108	0.1266	****	0.8990	0.8864	0.7626	0.9256	0.9094	0.8377
14	0.1702	0.1304	0.1553	0.1588	0.2322	0.1347	0.1054	0.0997	0.1263	0.1352	0.1006	0.1349	0.1065	****	0.8531	0.8626	0.8890	0.8787	0.8529
15	0.1526	0.1159	0.1389	0.1500	0.1879	0.1243	0.1395	0.1412	0.1511	0.1690	0.1243	0.1354	0.1206	0.1588	****	0.7445	0.9201	0.9001	0.8804

The UPGMA dendogram showed that population from three districts clustered separately corresponding to their geographical origin of samples. Among 19 populations analyzed Kundasale population is the highly diverged population.

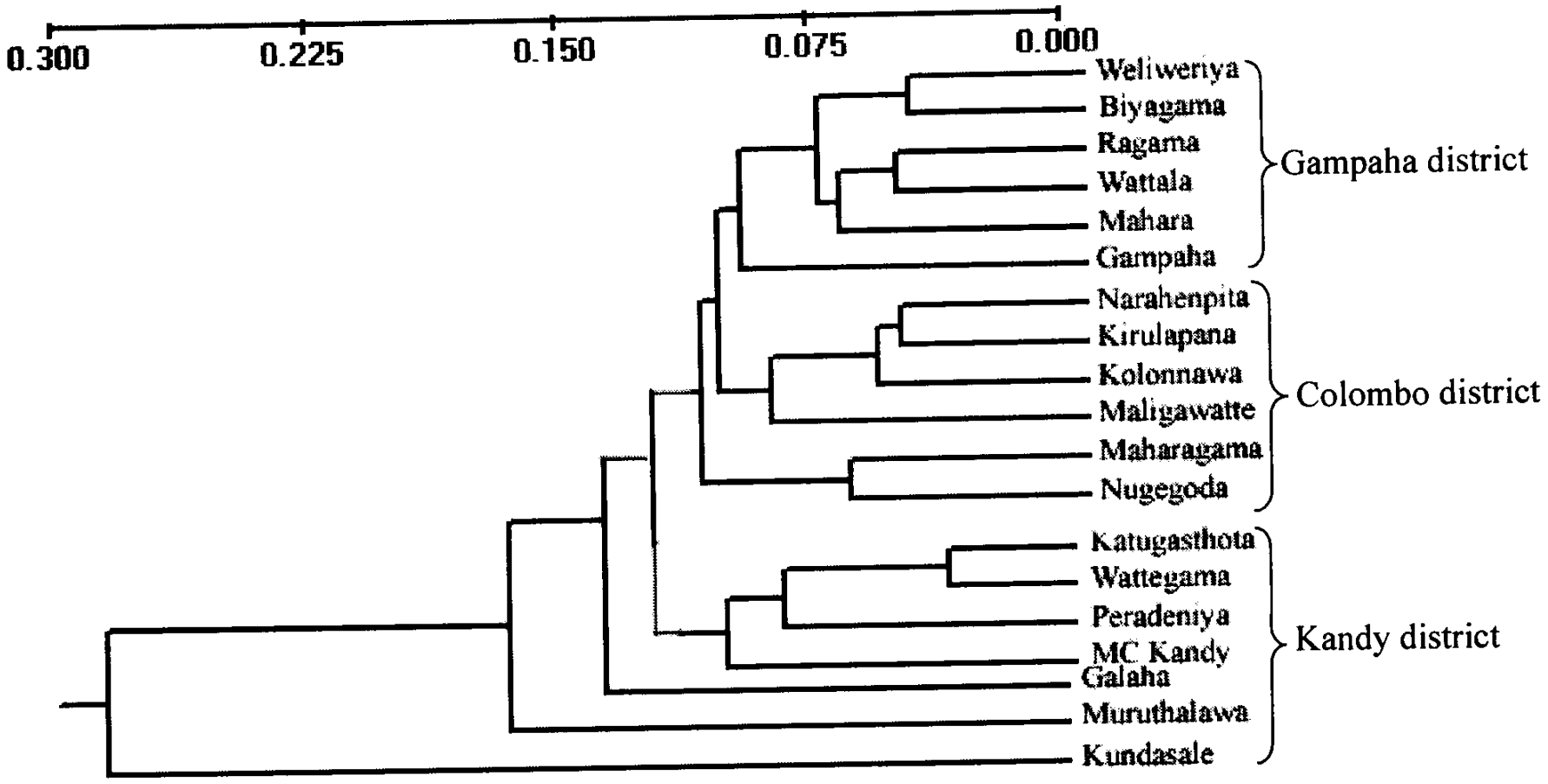

Figure 4.21. Dendrogram based on Nei's (1972) genetic distance among *A. albopictus* populations in all three health areas (Table 4.12).

v. DISCUSSION

5.1 Mosquito collection

The results presented here are the first evaluation of the genetic structure of *A. albopictus* populations in Sri Lanka. Colombo, Gamapha and Kandy districts were selected to examine the local patterns of gene flow in *A. albopictus*, because these districts are the worst affected areas during previous dengue out breaks. Sampling sites in each district were in expanded circular design. This design allows studying gene flow with in and among districts (health regions) under investigation.

Mosquito eggs were collected from 19 sampling sites from the three districts using ovitraps placed for one week period. Although there was a competition in oviposition between the ovitraps and other existing breeding containers such as tree holes, discarded plastic cups, flower vases etc., most of the traps became positive within one week. This indicates the preference of *Aedes* mosquitoes to lay their eggs on black (dark) surfaces. Therefore, black painted containers can be effectively used to collect *Aedes* mosquito eggs (Fay and Eliason, 1966).

In field study, ovitraps were installed both indoors and outdoors. However, compared to indoor sites more of the ovitraps became positive in outdoor sites. This indicates that female *Aedes* mosquitoes show a preference to deposit their eggs in outdoors than in indoors (Dibo et al., 2005).

In the health regions investigated *A. albopictus* was the dominant species found. This is contrary to the results obtained earlier in the literature (Kulathilaka and Jayakura, 1998), where *A. aegypti* was found predominantly in urban areas in Sri Lanka. This suggests a possible recent invasion of regions by *A. albopictus*.

5.2 DNA extraction

Three different protocols were tested for DNA extraction from *A. albopictus* mosquitoes. Although high salt extraction protocol (Sunnucks and Hales, 1996; Aljanabi and Martinez, 1997) is simple, low cost, fast and safe, compared to the protocols based on phenol/chloroform extraction, it yielded poor quality DNA. By considering purity and quantity of extracted DNA obtained, Apostol et al. (1993) procedure was chosen as the best DNA extraction method for the experiment.

~ 80 ng of DNA per mosquito was extracted from Apostol et al. (1993) procedure and produced excellent results in RAPD analysis.

5.3 RAPD-PCR and gel electrophoresis

RAPD method has been employed extensively to detect genetic diversity and genetic differentiations in population studies. The method is fast and does not require any

sequence information. It finds polymorphism in the whole genome. Thus RAPD offers more random and efficient sampling of genome. It has been used as an efficient tool to detect DNA polymorphism in geographically and genetically structured populations (Ballinger- Crabtree et al., 1992; Apostol et al., 1996; De Souza et al., 2001). Ballinger – Crabtree et al. (1992) successfully used a RAPD technique to select a specific group of amplified fragments suitable to differentiate among *A. aegypti*. Apostol et al., (1996) used the same technique to differentiate the 16 *A. aegypti* populations in Puerto Rico city and De Souza et al., (2001) used to estimate levels of intra-specific polymorphism and genetic relatedness of *A. aegypti* populations from Argentina.

Since the RAPD uses random primers, the method must be carefully optimized to get intense and reliable amplification products. Therefore during these work parameters in the RAPD method has been tested and optimized to yield clear, reproducible and intense bands.

The primer concentration is an essential parameter of RAPD - PCR that affects the quality of RAPD profiles obtained. Numbers of amplified RAPD loci were increased with the increase of primer concentration (Figure 4.3). However, very high primer concentrations caused several non-specific amplifications. Conversely, low primer concentration gave low product yield. Therefore, 1.0 mM primer concentration that generated clear intense, reproducible amplification products was used in the RAPD.

Magnesium is another essential component of PCR that affects the quality of RAPD profiles obtained (Munthaly et al., 1992). In general, increasing the amounts of Mg^{2+} will result in the accumulation of non-specific amplification products. Conversely, insufficient Mg^{2+} reduced the yield (Williams et al., 1993). Typical $MgCl_2$ concentrations used in most RAPD analyses reported in the literature ranged from 1- 8 mM (Aitchitt et al., 1993). In the present study, amplification products were detected when $MgCl_2$ concentrations changed from 1.0 to 2.5 mM (Figure 4.4). The number of detectable products increased when $MgCl_2$ concentrations changed from two at 1.5mM to nine at 2.5 mM. Patterns obtained using 2.0 and 2.5 mM $MgCl_2$ were similar. But higher intensity was observed at 2.5 mM $MgCl_2$ concentration. So 2.5 mM $MgCl_2$ concentration was used in the experiment.

An efficient and robust RAPD analysis should be reasonably resistant to variations in template concentrations. Identical profiles were obtained in the experiments over a range of template DNA concentrations from 4 ng to 20 ng in 25 µl reaction volume. Amplification efficiency was increased with the increase of template concentration. When the template DNA concentration reached 12 ng per 25 µl reaction volume, the relative intensity of the RAPD product was well visualized. Therefore, ~10 – 12 ng of template DNA concentration per reaction was used in the RAPD amplification.

The major factor that governs the choice of enzyme concentration is its potential efficiency. An increasing number of bands were observed with increasing enzyme concentration and these bands were more distinct at concentration 0.75 U in 25 µl in the reaction mixture. Devos and Gale (1992) reported a similar observation and suggested that such extra bands however were due to a non-specific amplification.

None of the bands however was amplified at less than 0.25 U per 25 µl reaction. During the study, 0.5 U of Taq. DNA polymerase (Sigma-Aldrich, Inc, USA) in a 25 µl reaction was sufficient to yield good amplification of RAPD bands.

30 primers were screened to select the primers which generate optimum number of reproducible, intense RAPD bands with template obtained from three districts. However, there was low yield or no PCR products amplified with some primers (Figure 4.6). This is probably due to incorrect primer compatibility. Clear intense reproducible band profile was however obtained with compatible primers (Figure 4.5). Five primers, A8, B7, B8, C18 and C19 that produced intense, reproducible, polymorphic bands were selected for the RAPD analysis (Table 3.1).

The different PCR parameters, such as temperature, duration and 'ramping' of denaturation, annealing and extension steps as well as the number of amplification cycles have also been altered to obtain optimal RAPD banding patterns. Denaturation parameters were set to 94°C for 1 min. This step didn't affect the enzyme activity, as Sigma Taq. Polymarase was a highly thermo stable enzyme. Annealing temperature was set to 35°C as recommended by the primer supplier (Operon technologies). Annealing temperatures reported in the literature for arbitrary short primers also range from 34 to 36°C, but can clearly be defined with more precision depending on the melting temperature of each primer. There is an interaction between the time required for primer annealing and the GC content of the primer. All the primers used in this study had GC contents ranging from 60 to 70%. Therefore, 1 minute annealing time appeared to be sufficient to obtain a reproducible RAPD patterns. Sampling of PCR products has been recommended to be carried out during the exponential phase (Cha and Thilly, 1993). This exponential phase of the reaction is defined as the period during which the products accumulate in an exponential manner, beyond which amplification often results in the production of non specific bands. The extension temperature of 72 °C for 10 min gave the clear intense band profile. The same condition was sufficient for extension of products up to 4 kb (Cha and Thilly, 1993).

Thirty five cycles were used in the standard amplification protocol. It was chosen to minimize non specific amplification products.

5.4 Statistical analysis

Mosquito samples, from each population were selected at random and used in the estimation of population genetic parameters, He, Hs, Ht, Gst, Fst (Lynch and Milligan, 1994), Nei's genetic distance D, by assuming Hardy-Weinberg equilibrium.

62 RAPD loci have been revealed during the analysis. Allele frequencies were computed by square root method. This approach has been employed in the present study because the method is widely used to calculate allele frequencies from dominant marker data, and also the defalt method used in many population genetic analysis packages including POPGENE and TFPGA.

He, referred to the average expected heterozygosity, which is an estimate of the extent of genetic variability in the population. Hs, is the weighted average of the expected

heterozygosities (within population gene diversity) across all the subpopulations. Ht on the other hand, referred to the global expected heterozygosity (total gene diversity) over all populations analyzed. The only difference in Hs and Ht was that Ht used global mean allele frequencies rather than subpopulation by subpopulation values for computations.

Differentiation between populations due to population subdivision or fixation index Gst, was calculated for populations using all 62 loci, for which the frequency of both alleles (presence and absence) were greater than or equal to 1%. Gene flow (Nm) was computed from Gst values. Although this method of estimating Nm has been criticized, because of its dependence on assumptions that is inappropriate for most mosquito populations, it was used here as comparative measure with similar estimates from other workers. Dendrograms based on Nei's (1972) original genetic distances were constructed using UPGMA method.

5.4.1 Intra population analysis

5.4.1.1 Colombo health region

The *A. albopictus* populations from Colombo district were genetically much differentiated (Gst = 0.2303; F_{ST} = 0.1104), despite the small geographical distance between them (2.1 – 11.7 km). The levels of gene flow between *A. albopictus* populations in Colombo district is respectively high (Nm = 1.6711). It also estimates low levels of intra population genetic diversity (Hs = 0.2056) and total gene diversity (Ht = 0.2671). The sample from Nugegoda showed a relatively low index of genetic variability than other sites and it lead to low level of intra population genetic diversity.

Colombo is the capital of Sri Lanka. All the sampling sites in the Colombo district are densely crowded urban cities. There are large numbers of available breeding sites for the female mosquito. Therefore, the mosquito population is expected to be high. But eradication campaigns, as well as frequent use of insecticide treatment in these urban areas may tend to disturb the growth of mosquito population. Further the cycle of extinction and recolonization of mosquito population may alter by insecticide application for lead to increase genetic differentiation. Similar observations were reported among *A. aegypti* populations (Paupy et al., 2000; Ayres et al., 2003; Ocampo and Wesson, 2004; Paupy et al., 2005). On the other hand intense human transportation or passive transport has been considered responsible for mosquito dispersal and gene exchange between mosquito populations in Colombo district leading to certain homogenization. Similar observations were reported among mosquito populations in all over the world (Pasteur et al., 1995; Lehmann et al., 1996; Failloux et al., 1997).

Dendogram (Figure 4.14) shows that sampling sites in the Colombo health region were clustered according to their geographical origin. Knowledge of the geographic origin of introduced mosquitoes may be useful to identify current or potential patterns of gene flow. In the present study, the closest relationship was found between Narahenpita and Kirulapana populations, with the lowest genetic distance of 0.0546. The two cities also represent the lowest geographic distance between them. Therefore, they are clustered

together. Maharagama and Nugegoda sites appear to be slightly diverged from other populations. So they are clustered separately.

Therefore, the genetic structure of *A. albopictus* populations in Colombo district would be a combination of two opposite forces. Large genetic drift events resulted from frequent use in insecticide and eradication campaigns tend to differentiate populations at random and gene flow, mediated mainly by passive transport along network of main roads tend to homogenizing it. Some level of short distance, active dispersal would also be evident form. The positive relationship observed between the geographical distance of sites and Fst/1-Fst (R^2 = 0.2428). However, a more detailed sampling design would be necessary to confirm this assumption.

5.4.1.2 Gampaha health region

Gampaha district is the second most populated district in Sri Lanka. The proportion of gene diversity among populations was comparatively low (Gst = 0.1558) and the number of migrants per generation was the highest (Nm = 2.7096). Mosquitoes in the Mahara site showed a relatively high index of genetic variability compared to the other sampling sites in Gamapaha health region. Probably due to its location on A6 Colombo– Kandy highway contributing much to the homogenization of gene by passive transport.

A positive correlation was also found between mosquito oviposition and industrial sites. Large number of industries belonging to export processing zones (Katunayake, Biyagama, Merigama and Wathupitiwala) was located in Gampaha district. They have large number of open water containers and water drainage systems creating large number of breeding sites. As well as people in rural areas in Gampaha district haven't pipe-born water supply. So they store water in open containers. This also facilitates the increase of breeding sites for *Aedes* mosquitoes. Commercial transportation network is well developed in this health region. Therefore, both active and passive transportation media may be responsible for the genetic structure of mosquito population.

According to the dendogram (Figure 4.16) neighboring collection sites appears to cluster together. Geographically close Ragama - Wattala – Mahara and Biyagama – Weliweriya sites group in to two separate small clusters. Lower genetic distance between them explains the gene flow of those clusters. Therefore, active dispersal can be occurring in short geographical distances. Distinct Gampaha population appears to have diverged into a distinct population. The positive relationship between the geographical distance of sites and Fst/1-Fst may provide an evidence for this observation (R^2 =1431) (Figure 4.15).

5.4.1.3 Kandy health region

The Gst value observed in Kandy population was 0.3343, almost twice than that observed in the populations in Gamapaha. Although the relationship between Kundasale and MC Kandy showed the greatest genetic distance (0.2951), it was not as high as those expected based on geographic distance compared to the other sites. Gene flow is poor in

this area (Nm = 0.9955). *A. albopictus* populations from Kandy district are highly differentiated (θ = 0.2127) even among seven sites that are less than 30 km apart and shows no significant correlation with geographic distance (R^2 = 0.0017) (Figure 4.17). This may be due to the micro geographical variations (Ravel et al., 2002). Kandy district is one of the districts in hill country of central province in Sri Lanka. Most of the sampling sites are isolated by ranges of mountains. Mosquitoes can't fly over these natural barriers. Therefore, the variation between genetic distances might be a result of Wahlund effect and is considerably high (0.0373- 0.2951) (Table 4.8).

Most of the houses in Kandy health region haven't pipe-born water supply and they stored water in open containers. This may be another factor that leads to increase availability of local breeding sites for mosquitoes (Table 4.3). Therefore, Kandy health region consists of a highly structured *A. albopictus* population. Similar effects were also observed in Córdoba city in Argentina by Julio et al., 2009 and Ho Chi Minh City in Vietnam by Huber et al., 2004.

According to the dendogram (Figure 4.18) populations are clustered separately due to the natural barriers. Katugasthota and Wattegama population are genetically very closely related. Peradeniya, Galaha, Katugasthota and Wattegama sites are also linked with MC Kandy city site. Therefore, they cluster together and show certain amount of homogenization. Kundasale and Muruthalawa are far apart isolated sites. So they cluster into distinct groups. Of the two sites Kundasale represents the most diverged population.

5.4.2 Inter population analysis

5.4.2.1 Gampaha and Colombo health regions

Low population differentiation (Gst = 0.2248) and high degree of gene flow (Nm = 1.7244) were observed among Gampaha and Colombo mosquito populations. Genetic distances also tend to be comparatively small between these two health regions. Further those health regions were located in adjoining districts in the Western province. Heavy transportation occurs between these two highly populated districts. Therefore, the probability of gene exchange among mosquito populations might be high and tend to homogenize two population sites. This indicates the importance of human activity to mosquito spread.

The dendogram (Figure 4.19) clustered according to the geographical origin of mosquito collecting sites. Gampaha and Colombo populations are separated into two groups. Those groups also further divided according to their geographic distances as mentioned earlier.

5.4.2.2 Gampaha and Kandy health regions

In the present work, relatively high differentiation (Gst = 0.2853) was found among populations in Gampaha and Kandy health regions with restricted gene flow (Nm = 1.2525). Because sampling sites are located far apart in distance between them (116 km)

when compared to Gampaha and Colombo districts. So the geographic distances are much higher than the flight range of the *A. albopictus*. Therefore, passive transport via A-6 Colombo – Kandy highway would be the most influential means of dispersal in these two health regions.

According to the dendogram (Figure 3.20) Gampaha and Kandy populations are also clustered into distinct groups, except Galaha population. They further subdivided according to their geographical distances.

5.4.2.3 Colombo, Gampaha and Kandy health regions

The average heterozygosity among the 62 RAPD loci (Ht = 0.3160) was comparable with an analysis for 57 loci in Puerto Rico (Ht = 0.354) (Apostol et al., 1996). Genetic diversity among 19 Sri Lankan *A. albopictus* populations was significant and shows that these populations are highly differentiated (Gst = 0.2969; Nm = 1.1841; θ = 0.1915) and very polymorphic (Hs = 0.2222). Those values were similar to those described for populations of *A. aegypti* in Brazil (Gst = 0.317; Nm = 0.54; Hs = 0.274) (Ayres et al., 2003); (Gst = 0.208; Nm = 1.90) (Padun et al., 2006) and Argentina (Gst = 0.249; Nm = 0.75) (De Souze et al., 2001).

The high level of genetic differentiation found among the Sri Lankan mosquito samples reveals that the *A. albopictus* populations are highly structured compared with populations of the species in other places of the world. (De Souza et al., 2001; Paduan et al., 2006). The level of genetic differentiation among mosquito populations may also be influenced by the several factors. Mutation, genetic bottlenecks, genetic drifts occur by climatic factors, eradication campaigns, founder effects, wahlund effects, and directional or disruptive natural selection may also favor differentiation, whereas genetic migration due to active or passive transport, purifying natural selection, and balanced or differential natural selection may homogenize the population.

The climatic factors would cause yearly genetic drift events that increase the level of genetic differentiation among mosquito populations (Julio et al., 2009). These mosquito collection sites get seasonal rainfall. South – western monsoon commence in late in late April and North – eastern monsoon rain from October to December. Temporary water containers however, disappear during dry season, forcing female mosquitoes to disperse farther to find suitable breeding sites. This effect may favor homogenization. Conversely, high abundance of breeding site during rainy season restricts the movement of mosquitoes favoring differentiation. However, naturally most of the structured populations show a dynamic equilibrium between these opposing factors (Paduan et al., 2006).

In the cluster analysis (Figure 4.20), population from three districts clustered separately. The population of Colombo appeared to be genetically closely related to Gampaha. Colombo and Gampaha are neighboring districts that has frequent commercial transportation. Kandy is located in the centre of country farther from Colombo and Gampaha. Analysis of *A. albopictus* in Sri Lanka during this study reveals the existence

of two partially isolated groups, representing Colombo- Gamapha region and Kandy region.

Knowledge of the geographic origin of mosquitoes may be useful to identify current or potential patterns of gene flow. In the study, the closest genetic distances were observed between Colombo and Gampaha populations, whereas the greatest difference was Kandy population with the rest. Although the relationships with Kandy showed the greatest distances, they were not as high as those expected based on geographic distance. This factor implies a fast and recent passive dispersal of *A. albopictus* mosquito in the continent through commercial routes.

The observed genetic differentiation may reflect important differences of vector competence, parasite susceptibity, or insecticide resistance. So that populations from different areas in Sri Lanka must be treated as independent genetic units.

vi. CONCLUSION

- *A. albopictus* is the dominant container breeding species found in all the sampling areas investigated.
- *A. albopictus* mosquitoes in Colombo district represents a moderately structured population with limited gene flow.
- *A. albopictus* population in Gampaha district is highly diverse and comparatively less structured.
- *A. albopictus* populations in Kandy district represent highly structured populations with restricted gene flow probably due to micro geographical variations.
- Overall, Sri Lankan *A. albopictus* populations are highly differentiated (structured) and very polymorphic.
- The micro geographical variations, passive transport, climatic factors and insecticide pressure might be the causes for observed genetic structuring (differentiation).
- Genetic distances among *A. albopictus* mosquitoes in Kandy district are much larger. Lowest distances are observed with mosquitoes from Gampaha district.
- Active dispersal of *A. albopictus* mosquitoes may occur to some extent in Colombo and Gampaha district. However passive transport associated with well developed road system, appears to play a key role in homogenizing mosquito populations in all three districts.

vi. REFERENCES

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vii. Problems if any, encountered during the implementation of the project

- Preliminary investigations showed that the dominant mosquito species in the sampled areas were *Aedes albopictus* and not *Aedes aegypti*. Therefore with the recommendation of the evaluators, and the NSF the mosquito species to be investigated during the project was changed from *Aedes aegypti* to *Aedes albopictus*

- The next major problem I faced was to choose a good research student to work on this project. Many though showed interest initially when they find a job with a high salary, they leave the project even without organizing the samples, and the detailed note books they worked with. I experienced this twice during this project as I worked with 03 research assistants in the whole project period.

Section 4

Impact of research results

1) Relevance of results achieved to scientific advancement

This is the first study conducted in Sri Lanka, to determine the population structure of dengue mosquitoes using molecular methods. Therefore new methods on sampling mosquitoes, their DNA manipulations have been developed and optimized for studying our local *Aedes* mosquito populations. Further these methods developed are simple, rapid and cost effective and can be applied even to other mosquito genera found in Sri Lanka.

2) Relevance of results achieved to national / socio-economic development

Dengue is becoming an increasingly important health hazard. Several thousands of cases and significant number of deaths are reported each year. The disease is caused by an arbovirus principally carried by mosquito vectors belonging to genus *Aedes*. Therefore controlling Dengue mainly aimed at reduction of Aedes vector populations.

Vector control programs use number of strategies to control mosquito populations. Removals of breeding sites, insecticide application are some of them. However application of these strategies on mosquito populations might not be effective if their population (breeding) structure, dispersal patterns are not clearly known. Unplanned application of these control strategies on mosquito populations otherwise leads to the development more resistant mosquito variants that may ultimately be difficult to eliminate using currently available strategies.

Therefore our primary aim was to address this important issue and to attempt to uncover their population structure, dispersal patterns in highly threatened areas that can be used to improve the effectiveness of the dengue control programmes.

We have analyzed *A. albopictus* mosquito populations from three districts namely, Colombo, Gampaha and Kandy, which have been highly threatened by this disease. We have found that these populations are significantly structured (grouped). The genetic indices of these populations clearly reveal the extent of their connectivity, migratory patterns and probable epidemiological units that can be adapted for Dengue control programs at national level. These observed differences within and among populations also reflect important clues to their vector competences, parasite susceptibility, insecticide resistance etc.

3) Dissemination / application of research output

- Careful studies on the findings of this project, such as population structure, mosquito dispersal patterns, and localization of epidemiological units of these mosquitoes and their planned manipulation by dengue control programs would help to reduce the mosquito populations immensely and to control the spread of the disease strategically in high risk areas.

- The results of these findings will be published in an internationally recognized peer reviewed scientific journal.

Section 5

Miscellaneous

1) List of major equipment acquired during the project period and their functionality

Item/Description	Status
1. Mini shaker (Votex mixer)	Working
2. PCR Machine	Working
3. Horizontal gel electrophoresis system	Working
4. Microcentrifuge	Working

2) List of publications / communications arising from the project and / or presentations made at seminars, workshops etc.

Manuscripts are in preparation

Section 6

Summary statement of expenditure*

Personnel :	Rs. 676,300.00
Equipment:	Rs. 760,494.53
Consumables:	Rs. 588,648.99
Travel and Subsistence:	Rs. 8,547.00
Miscellaneous:	Rs. 47,040.00

*The original Financial statement and the unspent balance of Rs. 22,919.48 was already sent to the NSF by the University of Kelaniya

Section 7

i) Grantee's Signature :

(Signature /Grantee)

ii) Comments of the Head of the Department and Signature:

progress is Satisfactory.

Recommended & Forwarded

Head/ Dept. of Chemistry
(Signature / Head of the Department)

iii) Head of the Institution's Signature:

(Signature / Head of the Institution)

Professor Sarath [illegible]
Vice-Chancellor
University of Kelaniya
Kelaniya - Sri Lanka

National Digitization Project

National Science Foundation

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