CHARACTERIZATION, DISTRIBUTION AND RISK ASSESSMENT OF THE INVASIVE PAPAYA MEALYBUG (*HEMIPTERA: PSUEDOCOCCIDAE*) IN KWALE, MOMBASA AND KILIFI, KENYA

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Characterization, Distribution and Risk Assessment of the Invasive Papaya Mealybug (*Hemiptera: Psuedococcidae*) in Kwale, Mombasa and Kilifi, Kenya

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DECLARATION

This Thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with our approval as the university supervisors

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DEDICATION

I dedicate this project to my children: Nester Kristine Mapemba and Hans Hobson Heya for their patience, understanding and sacrifice and to my parents Mzee H.H.Heya Kidegho and Mao Abigael Mkango'mbe Mwaisakenyi for their encouragement and prayers.

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ABBREVIATIONS AND ACRONYMS

AEZ	Agro Echological Zones
AFA	Agriculture and Food Authority
APHIS	Animal and Plant Health Inspectorate Service
APU	Arthropod Pathological Unit
ARS	Agriculture Research Service
ATC	Agricultural Training Centre
AUC	Area Under the Curve
BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
CABI	Centre for Agriculture and Bioscience International
Cm	Centimetre
COI	Cytochrome oxidase I
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EPPO	European and Mediterranean Plant Protection Organization
FR	Frequence ratio
G	Grams
GAMS	Generalized Additive Models
GARP	Genetic Algorithm for Rule-set Prediction
GLMS	Generalized Linear Models
GPS	Global Positioning System

HCD	Horticultural Crop Directorate
ICIPE	International Centre for Insect Physiology and Ecology
IITA	International Institute of Tropical Agriculture
INRI	Institut national de la recherche agronomique
IPM	Intergrated Pest Management
IPPC	International Plant Protection Convention
ITS	Internal Transcribed Spacer regions
JAP	Joint Agriculture Secretariat
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEPHIS	Kenya Plant Health Inspectorate Service
KES	Kenya Shillings
KNBS	Kenya National Bureau of Statistics
MATK	Megakaryocyte-Associated Tyrosine Kinase
MaxEnt	Maximum Entropy
MaxEnt	Maximun Entropy
Min	Minutes
mm (s)	Milimetres
MT	Metric Ton
NCBI	National Center for Biotechnology Information
NGST	Next Generation Sequencing Technologies
NPPO	National Plant Protection Organisation
ODK	Open Data Kit
PCA	Principal Component Analysis
PCPB	Pest Control and Products Board

PCR	Polymerase Chain Reaction
PMB	Papaya mealybug
PQBS	Plant Quarantine and Bio-security Station
Rbcl	Ribulose Bisphosphate Carboxylase Large
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
rpm	Revolution per minute
s	Second
μg	Microgram(s)
SDM	Species Distribution Models
UK	United Kingdom
USA	United States of America
USDA	United States Department of Agriculture
V	Volts

ABSTRACT

Papaya mealybug is a serious pest of papaya in South America. In Africa, the pest was first reported in West Africa in 2009. The objectives of this research were to confirm the identity of a pest of papaya mealybug description, which was devastating papaya in Kilifi, Kwale and Mombasa Counties in Kenya; through morphological and molecular characterization, establish its distribution and assess its risk in current and future climatic conditions. Live mealybugs were collected still attached on plants and others in 85% ethanol, transported to Kenya Plant Health Inspectorate Service (KEPHIS) and the International Centre of Insects Physiology and Ecology (*Icipe*) laboratories for morphological and molecular analysis. Morphological analysis was carried out in two ways, useful characteristics were observed on the mealybugs on plants, while specimens in ethanol were mounted on slides and observed under microscope while running through a taxonomy key. Molecular analysis involved DNA extraction, Polymerase Chain Reaction, Gel electrophoresis, sequencing, BLASTing and phylogenesis. Distribution and risk assessment was determined by running occurrence and environmental data in Species Distribution Models., Maximun Entropy (MaxENT) and Genetic Algorithm for Rule-set Prediction (GARP). Morphological analysis confirmed the pest positive for *Paracoccus marginatus*. Molecular analysis confirmed very high similarity of the samples with a Chinese, *Paracoccus marginatus* sample in the GenBank, National Center for Biotechnology Informatio (NCBI). MaxENT and GARP established very high-risk areas of papaya mealybug establishment with current and future climatic conditions in Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

1.1.1 Carica papaya L.

Carica papaya L**.** (papaya, papaw, pawpaw) belongs to family Caracaceae (Saran *et al*., 2014; Srivastava *et al*., 2016). It is an herbaceous, perennial, dicotyledonous polygamous plant having male, female or hermaphrodite flowers on the same plant (Medina *et al*., 2003; Roshan *et al*., 2014). The crop is native to Southern Mexico, Southern and Northern America (Karunamoorthi *et al*., 2014; Srivastava *et al*., 2016) and is now cultivated throughout the Caribbean islands, Florida and several countries of Africa, making it a well-known fruit worldwide (Morton *et al*., 2015; Santana *et al*., 2019). It is an important fruit in many tropical and subtropical Countries (Nguyen *et al*., 2017).

Papaya production is ranked third globally at 11.22 Metric Tones (MT) after Mango and Pineapple at 38.6 MT and 19.41 MT, respectively (FAO 2021). According to geographical areas, Asia is leading in production with 52.55 %, South America with 23.09 %, Africa with 13.16 %, Central America with 9.56 %, the Caribbean with 1.38 % and Oceania with 0.13 % (Edward, *et al*., 2021). In Africa, the major Papaya export Countries are: Côte d'Ivoire 35 124 MT, Egypt 72 517 MT, Kenya 24 132 MT, Senegal 17 301 MT, Mali 12 836 MT, South Africa 7 733 MT and Sudan 788 MT (FAO 2021). In Kenya, the major papaya producing Counties are: Kilifi, Lamu, Machakos, Makueni, Meru, Embu, Kitui, Kwale, Elgeyo Marakwet, Tharaka Nithi, Murang'a, Homabay, Busia, Tanariver, Kisii, Bungoma, Kirinyaga, Baringo, Narok, Kakamega and Migori with annual production of MT 119,449 and value of Kes. 4,121,979,701 (AFA validated horticultural report of 2018-2019).

Papaya is an evergreen latexy tree around 2-10 metres (m) tall, usually unbranched although sometimes branched due to injury. The stem is cylindrical, 10-30 cenctimetre (cm) diameter, hollow with prominent leaf scars and spongy fibrous tissues (Karunamoorthi *et al*., 2014). Leaves are spirally arranged and clustered near the apex of the trunk. Petioles are up to 1 m long, hollow greenish or green- purplish and 25-75 cm in diameter.The plant has an extensive rooting system (Karunamoorthi *et al*., 2014; Santana *et al*., 2019).

Papaya is mostly propagated through seed (Pandit *et al*., 2001; Orwa *et al*., 2009) where germination occurs within 2-4 weeks after sowing and is noted to be slow and variable (Zanotti *et al*., 2014). While seeds may be sowed directly in the farm, some orchards are started with established seedlings, 6-8 weeks after germination (Orwa *et al*., 2009). Direct seeding is practiced where a large number of seeds are sown per planting site. This is also done because the sex of a given plant cannot be determined for up to 6 months after germination (Gangopadhyay *et al*., 2007). Unlike the seeds of many tropical fruit species, papaya seeds is neither, recalcitrant nor dormant hence are classified as intermediate for desiccation tolerance (da Silva *et al*., 2007). Additionally, vegetative propagation of papaya is possible but is not widely practiced except in South Africa where rooting of cuttings is used to eliminate variability in some papaya varieties (da Silva *et al*., 20007).

Papaya grows satisfactorily in a wide range of conditions with its highest production being in warm sunny arears of tropical and subtropical regions preferably below 1500 m (Orwa *et al*., 2009; Santana *et al*., 2019). Strong winds are detrimental, particularly on soils that cannot make up for large transpiration loss. The plant is not frost hardy therefore; exposure to frost or cold wind usually results in leaf damage and subsequent death of the tree. Roots are very sensitive to water logging, in which even short periods of flooding can kill the plant (Orwa *et al*., 2009). Good production temperature ranges from 21 ˚C to 33 ˚C and mean annual rainfall of between 1000-2000 milimetres (mm) (Santana *et al*., 2019). Its production requires well-drained,

permeable, well-aerated, fertile loamy soils rich in organic matter with PH value between 6-7 (Orwa *et al*., *2009*; Roshan *et al*., 2014).

Papaya plant is dioecious or hermaphrodic with cultivars producing only female or bisexual/hermaphroditic flowers. It is sometimes described as 'trioecious' where separate plants bear either male, female or bisexual flowers (Saran *et al*., 2014; Santana *et al*., 2019). The flowers are tiny, yellow, funnel-shaped and either solitary or clustered on the leaf axil (Orwa *et al*., 2009). Female flowers are 3-5 cm long, pear shaped with a large functional pistil and an ovoid shaped ovary. The male plant has a long inflorescence, pendulous panicle bearing many flowers (Saran *et al*., 2014). They have 10 stamens arranged in two rows, a minute calyx with five combined sepals and a long corolla (Orwa *et al*., 2009; Saran *et al*., 2019). Hermaphrodite flowers are waxy, ivory white in colour, cylindrical and larger than males with 5-carpellate ovary (Orwa *et al*., 2009).

Papaya comes into fruiting within 5 months of planting and its lifespan is 4-5 years (Orwa *et al*., 2009). Males produce only pollen and never fruits. Female unless pollinated, produce small, inedible fruits. Pollinating agents include insects such as larger bees, honeybees, long-tongued sphinx moths, humming-bird moths. (Orwa *et al*., 2009). Hermaphrodite can self-pollinate since its flowers contain both male stamens and female ovaries. They are preferred over female or male plants since they produce the most desirable fruits (Saran *et al*., 2014).

Small latex vessels extend throughout the papaya tree and are particularly abundant in fruit that has reached full size but has not yet begun to ripen (Orwa *et al*., 2009).Papaya bears fruits throughout the year, immature fruits are green in colour and deep orange-yellow when ripe (Karunamoorthi *et al*., 2014). The fruits are melon like, oval/oblong to nearly round, somewhat pyriform, or elongated club-shaped, 15-50 cm long and 10- 20 cm thick , weighing up to 9 kg (Medina *et al*., 2003). Fruit formed from female flowers are oblong, spherical, and pear-shaped. Fruits from

hermaphrodite flowers are long, ovoid or pyriform semi wild naturalized (Medina *et al*., 2003).

When the fruit is immature, it is rich in white latex; the skin is green, waxy, thin but fairly tough and hard. As ripening progresses, papaya fruits develop a light- or deep-yellow-orange coloured skin while the thick wall of succulent flesh becomes aromatic, yellow orange or various shades of salmon or red. It is then juicy, sweetish and somewhat like a cantaloupe in flavor (da Silva *et al*., 2007).

1.1.1 Mealy bugs

Mealybugs are small oval shaped, cottony insects with white, waxy protrusions extending from their bodies (Tanwar *et al*., 2007; Moniruzzaman *et al*., 2017). Their family (Pseudococcidae) is a member of the 'advanced scale insects' of an informal group usually called the neococcoids (Cook *et al*., 2002). It is the second largest family of scale insects, with approximately 2000 described species in more than 270 genera (Cook *et al*., 2002; Downie *et al*., 2004).

Mealybug development period is longer than most greenhouse insects with a lifecycle of approximately 60 days, depending on temperature and host plant (Daane *et al*., 2012; Nisha *et al*., 2017) they can have as many as 15 generations per year (Tanwar *et al*., 2007). Eggs of mealybugs vary in size from 0.3 to 0.4 mm in length and their development takes between 3-9 days then hatch into very mobile nymphs called larvae or crawlers. Nymphs of both females and males resemble female adults. Male mealybugs undergo six development stages: egg, first instar, second instar, pre pupa, pupa and a winged adult (Tanwar *et al*., 2007; Daane *et al*., 2012). The first instar is 0.6 mm long and elongate in shape, moves quickly and it is considered the dispersal stage. There are three molts resulting to second instar, third instar and pre pupa, each of these stages resemble, previous one except for increased size and the amount of wax secretions (Daane *et al*., 2012). Adult males are approximately 1.0mm long with elongate oval body, distinct aedegus with ventral lobes that are

broad and cylindrical in dorsal ventral view. They are slightly longer than females (Daane *et al*., 2012; Seni *et al*., 2014).

Female mealybug have five development stages: egg, three crawler stages and adult. They are more sessile than males and are without wings (Daane *et al*., 2012). Mature females lay eggs underneath the body cavity in a white waxy egg sac, one egg sac may contain as many as 600 eggs, majority of which are females resulting in explosive outbreaks (Daane *et al*., 2012). Some mealybug species are ovoviviparous, hatching eggs within the female giving birth to live larvae (Tanwar *et al*., 2007). Adult mealybugs are found in colonies on leaves, fruits, stems and roots covered in white cotton like waxy masses (Daane *et al*., 2012).

Mealybugs feed on plant sap by inserting their stylets into the epidermis of the plant material sucking a large amount of sap depriving plants of essential nutrients (Heu *et al*., 2007). This results in chlorosis, dwarfism, leaf deformation, leaf yellowing, early dropping of leaves and fruits and finally the death of the plant (Heu *et al*., 2007; Moniruzzaman *et al*., 2017) .Excess sap is excreted as honeydew, which attracts fungus growth leading to sooty plant parts. (Tanwar *et al*., 2007; Daane *et al*., 2012; Topagi *et al*., 2014). (Fig 1.1).

Sooty mold on noni fruits
Source: Jorge E Pena, 2015

Leave curling and deformation on papaya
Source: Scot Nelson, 2006

Mealybugs on Citrus

Source: Ann Varela, 2005

Early leaves drop and withering of papaya plant

Source: CABI/EPPO 2012

Figure 1.1: Symptoms of mealybug infestation on various crops

The primary means by which mealybug crawlers disperse are wind, air currents, rain, clothing and vehicles though they can also settle in cracks and crevices of new plants (Tanwar *et al*., 2007). Facilitated passive transportation can occur when farm equipment, animals or people transport wax, which sticks to eggs when they are in the farm (Tanwar *et al*., 2007). Humans through their various activities play a major role in transporting mealybugs from one area to the other (Tanwar *et al*., 2007; Johnson *et al*., 2013). Certain species of ants spread mealybugs. Ants are attracted to the honeydew secreted by mealybugs and in the process of movement, spread mealybug from infested plants to the non-infested plant (Tanwar *et al*., 2007). Long distance movement can be through ferrying of infested plant materials, fresh fruits and vegetables across the Country or from a farm to another (Tanwar *et al*., 2007; Johnson *et al*., 2013). Mealybugs are widely distributed throughout the world and are important pests causing various economic losses of plants in commercial glasshouses, conservatories, and they are important quarantine pests that hinder international trade of plants and their products. (Johnson *et al*., 2013).

1.1.2 Papaya mealybug

1.1.2.1 *Origin and distribution*

Papaya mealybug, *Paracoccus marginatus* (Hemiptera, Pseudococcidae) is an invasive and polyphagous pest that originated from Mexico and or Central America (Goergen *et al*., 2011; Mani *et al*., 2012; Lalitha *et al*., 2015). The first papaya mealybug specimens were collected in 1955 from cassava plant (*Manihot esculenta*) from Mexico. Later on, more specimens were collected from different localities of Neotropical region (Belize, Costa Rica & Guatemala,). Williams and de Willink described the specimens in 1992 and then Miller and Miller re-described it in 2002 (Shepard *et al*., 2009; Galanihe *et al*., 2010).

In the Caribbean, this pest was first reported at Saint Martin in 1995 and since then it has spread to 13 countries in the Caribbean, in Florida in the United States (USA), and three Countries in Central and South America (Walker *et al*., 2002; Tanwar *et al*., 2007). In year 2002, papaya mealybug was reported in the Pacific Islands and in year 2008 in Indonesia and India (Muniappan *et al*., 2009). In year 2009, the pest was reported in Bangladesh and Maldives and later in 2010 , it was reported in Cambodia, Philippines, Thailand and Sri Lanka (Saengyot *et al*., 2011 ; Galanihe *et al*., 2010) and in Malaysia in 2011 (Mastoi *et al*., 2011).

In Africa, the pest was first reported in Benin and Ghana in West Africa in year 2009 (Muniappan *et al*., 2009). Since then it has been reported in other Countries (Muniappan *et al*., 2009; Mani *et al*., 2012). In East Africa, the pest was reported in Tanzania in 2015 (CABI/EPPO, 2016) (Table 1.1).

Table 1.1: Distribution of papaya mealybug; *Paracoccus marginatus* in Africa

Country	Year first reported	Reference
Benin	2009	Muniappan *et al*., 2009; CABI/EPPO, 2012
Ghana	2009	Muniappan *et al*., 2009; CABI/EPPO, 2012
Mauritius	2014	CABI/EPPO, 2016
Reunion	2010	CABI/EPPO, 2012
Tanzania	2015	CABI/EPPO, 2016
Togo	2009	Muniappan *et al*., 2009; CABI/EPPO, 2012
Zanzibar	2015	CABI/EPPO, 2016

1.1.2.2 *Biology*

Papaya mealybughave like other mealybugs have five development stages for females and six for males (Walker *et al*., 2002; Seni *et al*., 2014). Males have a heavily sclerotized thorax and head .They also have very well developed wings (Seni *et al*., 2014). The antenna of papaya mealybug has eight segments with bristles and fleshy setae. Abdomen consists of eight segments and is usually without setae (Seni *et al*., 2014). Females are 2-3 mm long and 1.4 mm wide, greenish yellow in colour, dusted with mealy wax and without wings. They attract males with sex pheromones (Mani *et al*., 2012; Seni *et al*., 2014).

1.1.2.3 *Life Cycle*

Temperature and host plant determines the length of the life cycle of papaya mealybug (Daane *et al.,* 2012). Average life span on papaya is 50 to 60 days) (Figure 1.2).

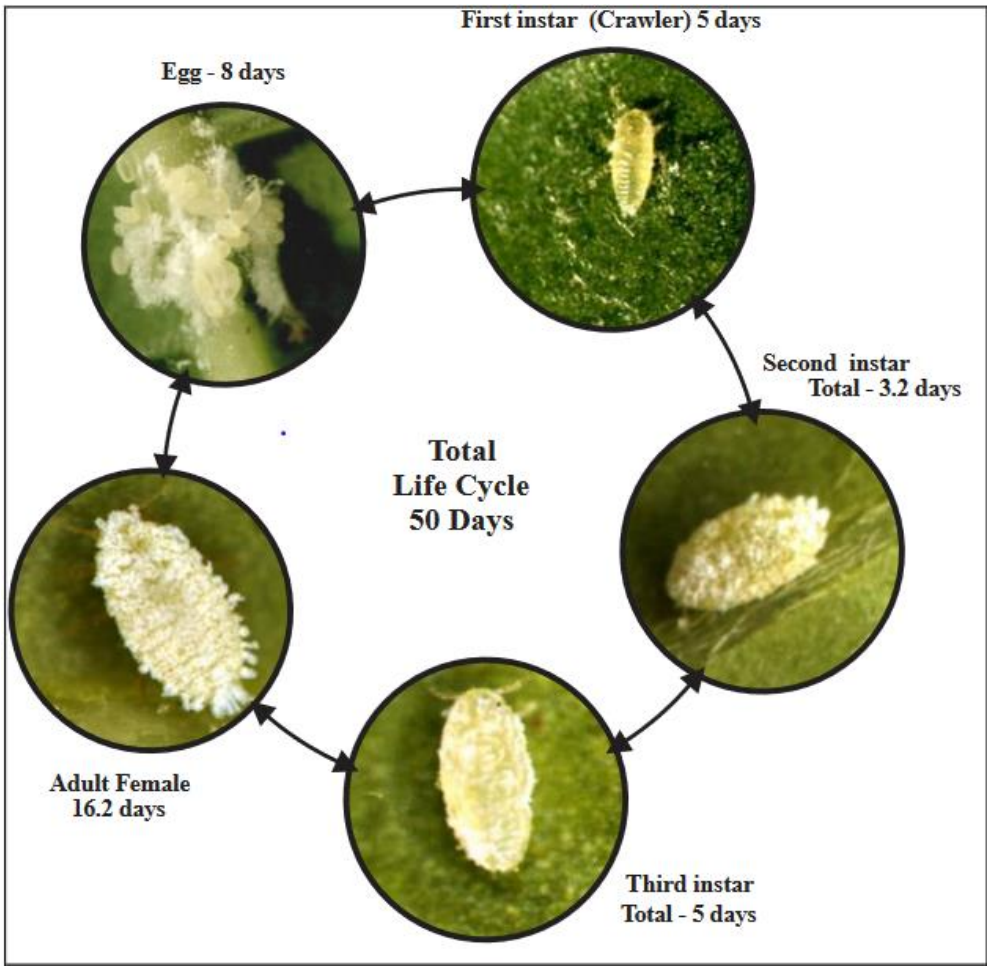

Figure 1.2: Life cycle of papaya mealybug; *Paracoccus marginatus* on papaya. Source: Nisha *et al*., 2017.

1.1.2.4 *Host range*

Papaya mealybug is a very polyphagous pest having been reported in 60 host plants from 25 genera (Walker *et al*., 2002; Goergen *et al*., 2011). The plants include economically important families: Caricaceae (papaya family), Legiminaceae, Verbanaceae , Amaranthaceae ,Malvaceae, Annonacea, Fabaceae, Myrtaceae, Meliaceae, Euphorbiaceae, Solanaceae, Lythraceae, Moraceae , Lamiaceae, Capridaceae , Commelinaceae , Convolvulaceae, Asteraceae , Compositae,

Aizoaceae and Rubiaceae (Heu *et al*., 2007; Galahine *et al*., 2010; Tanwar *et al*., 2010).

1.1.2.5 *Management*

Papaya mealybug control often involves a combination of various methods in an Integrated Pest Management (IPM) approach (Tanwar *et al*., 2010; Seni *et al*., 2014). The reason why IPM should be used in the management of papaya mealy bug is, the insects live in clusters and they are covered with a heavy coating of waxy secretions. These protects them from attack by external factors such as environmental (rains and sunrays), natural enemies and pesticides (Seni *et al*., 2014).

Cultural methods in the management of papaya mealybug include monitoring and scouting of the crop to detect early presence of the mealybug. Others include good crop sanitation, pruning and burning of infested leaves, branches, and removal and burning of crop residues (Tanwar *et al*., 2010). Additional methods include, removal of weeds especially alternative hosts, avoiding moving planting materials from infested areas to other areas, avoiding flood irrigation and encouraging sanitization of farm equipments after every use and before moving them to uninfected crop (Tanwar *et al*., 2010; Seni *et al*., 2014). Papaya mealybugs can be controlled to some extend by directing a powerful jet of water at infested plant parts to dislodge them from the plant thus dying either of hunger or of desidication (Galanihe *et al*., 2010; Seni *et al*., 2014).

Bilogical control like the use of natural enemies is a key component in management for this pest (Galanihe *et al*., 2010). Predators like the larvae of the green lacewings (Neuroptera: Chloropidae) and the adult of the mealybug destroyer, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) were found to have impact on papaya mealybug populations in Central America (Meyerdirk *et al*., 2001). In Peninsular, Malaysia where papaya mealybug is well established, two predators, the mealybug destroyer (*Cryptolaemus montrouzieri*) and green lacewing (*Apertochrysa*

sp) have been reported feeding on papaya mealybugs in its natural environment (Mastoi *et al*., 2011).

United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS) and USDA Agricultural Research Service (ARS) identified five efficient parasitoids (Hymenoptera: Encyrtidae) specific to papaya mealybug in 1999 and Shylesha and team confirmed efficiency of 3 of the 5 in 2010 (Meyerdirk *et al*., 2001; Shylesha *et al*., 2010). The five included *Acerophagus papayae* Noyes and Schauff, *Anagyrus loecki* Noyes, *Anagyrus californicus* (Compere), *Pseudaphycus sp.* and *Pseudleptomastix Mexicana* (Meyerdirk *et al*., 2001). The parasitoids which were found to be efficient in all the areas of release had a 99.7% reduction in papaya mealybug populations in the Dominican Republic and a 97% reduction in Puerto Rico, with parasitism levels of 35.5-58.3% (Meyerdirk *et al.,* 2001 Shylesha *et al*., 2010). All the five parasitoids have been observed attacking second and third instars of papaya mealybug. However, *Acerophagus sp*. emerged as the dominant parasitoid species in both Puerto Rico and the Dominican Republic (Meyerdirk *et al*., 2001). In May 2009, Caribbean, Florida and Hawaii received three parasitoids *(Anagyrus loecki, Acerophagous papayae* and *Pseudleptomastrix mexicana* (Hymenoptera: Encyrtidae) from the USDA APHIS parasitoid rearing facility at Puerto Rico and released them in Sri Lanka in August 2009. The parasitoids have realized 95% to 100% of control of papaya mealybug in some parts of the Country (Muniappan *et al*., 2009).

Chemical management of papaya mealybug is not very effective since papaya mealybugs are protected by thick waxy, cottony surface and are often concealed inside damaged leaves and buds rendering it difficult for the chemical to get into contact with the mealybug (Tanwar *et al*., 2012). Problems of insecticide resistance and non-target effects on natural enemies and pollinaters make chemical control a less desirable control option (Gisi *et al*., 2010). The three insecticides identified in the experiments in Sri Lanka were recommended for use as foliar sprays against the

papaya mealybugs on cultivated crops. They included thiamethoxam 25%WG at the rate of 1g per liter; imidacloprid 200g/l SL at the rate of 1ml per liter and mineral oil (sparrow oil) at the rate of 5ml per liter (Galanihe *et al*., 2010). For larger trees, soil application of thiamethoxam 25%WG at the rate of 1g/l was recommended (Galanihe *et al*., 2010).

Ants that live around mealybug colonies are often associated with mealybugs in a two-way relationship. They benefit by feeding on the honeydew excreted by mealybugs and in return, they protect the mealybugs from predators and parasites (Jahn *et al*., 2003). Without the ants, mealybug populations would be low and slow to invade new areas therefore management of mealybugs includes the control of ants (Tanwar *et al*., 2010). Ant management requires diligent efforts and the combined use of mechanical, cultural, sanitation, and often-chemical control methods (Galanihe *et al*., 2010). Destruction of all heavily infested plant parts, spraying plants near houses and in home gardens with a soap + kerosene oil + water mixture and wrapping polythene/spongy tapes impregnated with insecticides around tree trunks, exclude ants from the canopy and also help in controling mealybugs (Galanihe *et al*., 2010).

1.2 Statement of the Problem

Papaya is an important fruit crop in Kenya for both local and export use. Its production is constrained by both abiotic and biotic factors like diseases and pests. While the area under production increased from 7,000 Ha to 7,969 Ha in 2018 and 2019, production decreased by 8,889 tones representing a 7% decrease. The decrease in production was attributed mainly to pest and disease incidences (AFFA validated horticultural report, 2018-2019). *Paracoccus marginatus* has been reported to cause 100 % yield losses in some Countries (Goergen *et al*., 2011; Galanihe *et al*., 2010). This pest has a wide host range including crops of international economic importance such as avocado, mango, eggplant, pepper and green chilli and papaya.

Additionally, they attack other crops of importance to the local community and its neighborhood. These include cassava, pigeon pea, cowpea, okra, coconut, cashew nut, and neem, which are major food and cash crops to the people of the coastal area. Therefore, this pest has a potential to cause 100% loss that could lead to malnutrition, loss of jobs, food insecurity in the region and export ban (Walker *et al*., 2014).

1.3 Justification of the study

Papaya mealybug is the only one of the 86 described species in the genus (Georgen *et al*., 2011) with the status of an invasive pest outside its home range. Since its first report in Ghana, it has been causing great concern in the major four papaya producing areas of Ghana. First estimates indicated that papaya mealybug caused up to 100 % yield losses and had devastated 85% of the papaya farms in the region. This has led to a number of challenges like loss of foreign exchange through export and loss of jobs (Georgen *et al*., 2011).

Many aspects of this pest have not been understood and this gap had led to its quick spread, multiplication and establishment; the pest management options are also not clearly outlined. Currently, there is no registered control products for papaya mealybug in the statutory organization of Kenya organization that registers pest control products, Pest Control and Product Board (PCPB) database. This deficiency has led to a lot of blind management of the pest in the infested areas some options ending up aggravating its spread, damaging or poisoning the crop. This study was aimed at establishing various aspects of this pest including; its proper identity, genetic diversity, distribution and its risk under current and future Kenyan climatic conditions. The findings from the study will be used in guiding researchers in the development of a management tool to be used by famers in managing the pest thus safeguarding the MT 119,449 of papaya production countrywide and the kes. 4,121,979,701 of revenue through export of papaya. The findings on the current and

future distribution of the pest in the country will be shared with the Counties umbrella body of agriculture the Joint Agriculture Secretariat (JAP) to share with its members to prevent invansion in their Counties or prepare for management in case of invasion. . The findings from this research will also be used in sensitizing farmers and extension officers in the three Counties through local radio channels on detection and reporting of the pest to the respective organizations.

1.4 Hypotheses

i. The pest infesting pawpaw plants in Kwale, Kilifi and Mombasa Counties is not papaya mealybug
ii. Papaya mealybug has no risk of spreading to other areas in Kenya

1.5 General Objective

To characterize, establish the distribution and assess the risk of the invasive pest, *Paracoccu*s *marginatus* Williams and de willink (Hemiptera: Pseudococcidae) in Kwale, Mombasa and Kilifi counties, Kenya.

1.5.1 Specific Objectives

i. To undertake morphological and molecular characterization of *Paracoccus marginatus* infesting pawpaw in Kwale, Mombasa and Kilifi counties, Kenya.
ii. To determine the distribution and do risk assessment of *Paracoccus marginatus* infesting pawpaw in Kwale, Mombasa and Kilifi counties, Kenya.

1.6 Scope of the Study

The study was to determine through morphological and molecular techniques the identity of invasive pest *Paracoccus marginatus* in Kilifi, Kwale and Mombasa counties; determine its current distribution and assess its current and future risk in the

Country. This was carried out between 2017 and 2019 through a survey, which involved random sampling and identification of the pest in the infested Counties.

CHAPTER TWO

LITERATURE REVIEW

2.1 Characterization of papaya mealybug

2.1.1 Morphological characterization

Insects form a large portion of the biological diversity of our planet (Yang *et al*., 2015). Progress in the understanding the composition and functioning of the planet's ecosystems is partly dependent on our ability to effectively find and precisely identify the insects that inhabit them (Valan *et al*., 2019). Authors have applied various combinations of names to various ranks, and none is in common use, in part owing to the inadequate definition of groups, in terms of either their generic composition or their diagnostic morphology (Jiménez *et al*., 2016). Traditionally, morphological traits of different insect groups were used for the identification of insect pests and this generally, depended on adult stage and male genitalia (Jinbo *et al*., 2011; Jalali *et al*., 2015; Tahir *et al*., 2018).

To catalogue the vast numbers of species, naturalists came up with the idea of classifying living beings based on taxonomy, which is a branch of science that helps us to describe a living being based on morphological features (Jalali *et al*., 2015). Although the insects catalog documents more than one million species, a good proportion of them are still undiscovered and though with some training, one can learn how to distinguish higher taxonomic groups. Identification based on

morphology and traditional methods are often difficult since identifying an unknown, cryptic species is time consuming and high level of experience is required for the effective use of taxonomic keys (da Silva *et al*., 2017; Tahir *et al*., 2018). Immature life stages (juveniles, early instars) and pupae are not identifiable by routine taxonomy as most morphotaxonomic keys are useful for adults only (Barrett *et al*., 2005; Tahir *et al*., 2018). Phenotypic plasticity, immense species diversity, millions of undescribed species, and the significant variation within species due to sex, color , morph and life stage further complicates morphological identification (Yang *et al*., 2015; Murugan *et al***.,** 2016; Tahir *et al*., 2018). The task of moving from family level towards species is challenging and complicated because the lower the taxonomic level, the more challenging the identification task becomes, even for experts who are often in high demand (Blaxter 2003; Jinbo *et al*., 2011; da Silva *et al*., 2017).

Different workers at various times have recognized approximately five subfamilies of family Pseudococcidae but none has found practical use (Jiménez *et al*., 2016). Tribal names have been used by some authors but these groups, are not widely used, and often they are equivalent to the subfamily groups of other authors but with a few exceptions, authors like Danzig used tribal rank for taxa which other Coccidologists called subfamilies (Downie *et al*., 2004 ; Jiménez *et al*., 2016). The relationships among many Pseudococcidae genera are poorly known and there is no stable higher-level classification. Occasionally authors have used informal groupings such as the eleven groups recognized and briefly defined by Mckenzie for North America mealybugs genera based on adult female morphology and the six groups recognized based on male morphology (Jimenez *et al*., 2016).

2.1.2 Molecular characterization

2.1.2.1 *Molecular identification*

Identification of specimens at the species level is necessary for understanding the diversity of the species phylogenetic patterns, evolutionary relationship and management of the pest (Tahir *et al*., 2018). Use of molecular tools to discriminate insect populations, and insects' adaptation to various stresses is widely being applied. Deoxyribonucleic acid (DNA) barcoding based approaches have proved to resolve problems related with morphological identification of mealybugs and can also provide valuable information for investigating mealybug associations and interactions with natural enemies (Assefa *et al*., 2018). This method can be the easiest, quickest, reliable tool for insects' identification (Jalali *et al*., 2015; Tahir *et al*., 2018; Madden *et al*., 2019). Being a standardized molecular identification and phylogeny method, DNA barcoding uses species identification markers CO1 gene of the mitochondrial region with numerous applications that has been used extensively to identify immature life stages of animals. It has been used to also identify and assign unknown specimens to species besides facilitating the discovery of new species *(*Jalali *et al.*, 2015; Tahir *et al*., 2018; Madden *et al.*, 2019). In this technique, short standardized gene region of mitochondrial Cytochrome Oxidase sub unit 1 (CO1) is used for discriminating species. This specific sequence (658 bp) is known as DNA barcode and is used as a species tag or barcode tag for each animal taxa. COI gene region is preferably used because it is found in all eukaryotic life forms. Finally, COI fragment bears sufficient sequence divergence to discriminate the closely linked species. In addition, the amplification of this COI fragment is quite easy due to its appropriate sequence, short length and the robust universal primers (Tahir *et al*., 2018*)*. The present results, thus, favour DNA barcoding as a decisive tool in quick and reliable identification of known and unknown species of insects with several researchers in its favour (Jalali *et al*., 2015).

Molecular characterization of mealybugs was successfully performed at Sophia Agrobiotech Institut – INRA (*Institut National de Recherche Agronomique*), Sophia Antipolis, France where at least three individuals from every population collected were analysed. Extraction of DNA was carried out using DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) and amplified from two different loci: the HCO-LCO region of the CO1 (mtDNA) and the 28S Ribosomal gene (nuclear genome). Polymerase chain reactions were carried out using the Qiagen Multiplex PCR kit composed by 23 μL of reaction mix (1X Qiagen buffer, primers at 0.2 μM) and 2 μL of diluted DNA (1–20 ng of DNA). The primers (Forward, Reverse) C-28SLong-F 5′GAGAGTTMAASAGTACGTGAAAC3′ and C-28SLong-R 5′TCGGARGGAACCAGCTACTA3′ (28S-D2) were used for amplifying the 28S gene region and the primers PCO-F1 5′CCTTCAACTAATCATAAAAATATYAG3′ and Lep-R1 5′TAAACTTCTGGATGTCCAAAAAATCA3′ were used to characterize the COI gene region.

Polymerase Chain Reaction (PCR) was performed and their products were sent to Beckman Coulter Genomics (Takeley, United Kingdom) for bidirectional sequencing. New sequences were deposited in GenBank under the accession numbers: KY565026 to KY565046 for 28S and KY687861 to KY687903 for COI (da Silva *et al*., 2017).

Mealybugs infesting vineyards in four production areas in Chile were accurately characterized using molecular techniques. 164 mealybugs were sampled from 26 vineyards and identified by DNA sequencing using two markers (Cytochrome Oxidase I and Internal Transcribed Spacer (ITS) 2). Molecular variability at the COI and ITS2 loci were observed in both *Pseudococcus viburni* and Pardosa *cribata*. (Correa *et al*., 2012).

In a study carried out in Swaziland, a DNA barcoding approach was used to identify mealybug species attacking wild hosts, ornamentals and cultivated crops in the

Country. Molecular identification using fragment of mitochondrial cytochrome oxidase sub-unit I revealed the presence of six mealybug species belonging to four genera (Assefa *et al*., 2018). A significant (0.00-0.68%) within species similarity and between species sequence divergence (6.90-16.80%) was observed. Of the species identified *Phenacoccus madeirensis* and *Phenacoccus solenopsis*, were the dominant and were highly polyphagus (Assefa *et al*., 2018). *P. solenopsis*, was recorded from 12 host plants belonging to six families in three regions, whereas, *P. madeirensis* was recovered from 5 wild host plants growing in Middleveld regions (Assefa *et al*., 2018). Other mealybug species collected were *Saccharicoccus sacchari*, *Planococcus citri*, *Paracoccus burnerae* and *Phenacoccus solani*. The study validated the efficacy of sequence diversity in the COI gene for identifying mealybugs. This was the first DNA based characterization of mealybugs from Swaziland and the findings were aimed at helping in decision making while considering biological control programs (Assefa *et al*., 2018).

2.1.2.2 DNA extraction and PCR amplification

A key element of any DNA-based study of bulk arthropod samples is DNA extraction technique (Nielsen *et al*., 2019). Extraction of high quality DNA is crucial for any molecular genetic analysis. The process of DNA isolation represents a basic step for genetic research since all of the molecular analyses require quality DNA (Pipan *et al*., 2018). High-throughput DNA sequencing offers an efficient tool for assessing the taxonomic content of bulk arthropod samples (Nielsen *et al*., 2019). DNA markers compared to protein markers are important because DNA is more stable and help to detect variation due to mutation on intron or in gene codon (Pipan *et al*., 2018). There are some techniques through which DNA extraction takes 20 minutes from Coleopterans, Dipterans and Hemipterans without any structural damage or discoloration (Pipan *et al*., 2018). It has been found that DNAs of different small insects with short storage time in 95% ethanol can be separated more successfully by a technique, that is simpler, reliable, economical, needs less

equipment and reagents (Pipan *et al*., 2018; Nielsen *et al*., 2019). Like cytochrome COI, gene and nuclear gene (Microsatellites) can be separated from Hymenoptera and amplified by PCR (Asghar *et al*., 2015). The basic criteria that any method of DNA isolation from any sample type should meet includes; efficient extraction of DNA from the sample, production of a sufficient amount of DNA for use in downstream processes, successful removal of contaminants and isolation of high quality and high purity DNA (Dhaliwal *et al*., 2013). There are various DNA extraction strategies available in the market; such as, chemical, thermal, enzymatic, or mechanical lyses, or a combination of all of them. Nevertheless, despite various DNA methods available commercially, reviews of suitable methods of DNA extraction from various food matrices are lacking (Dhaliwal *et al*., 2013).

Polymerase Chain Reaction (PCR) is a simple, yet elegant, enzymatic assay utilized to amplify specific segments of DNA from a complex pool of DNA. It is an application used in many fields, allowing for more than a billion fold amplification of specific target regions (Garibyan *et al*., 2013, Ghannam *et al*., 2018). It is a genetic technique that occurs in vitro, which allows the enzymatic synthesis of large quantities (amplification) of a targeted region of DNA in exponential manner (Garibyan *et al*., 2013). DNA is synthesized in the same manner as that seen in vivo (in the cells) using a DNA polymerase (Ehtisham *et al*., 2016**).** Only trace amounts of DNA are needed for PCR to generate enough copies to be analyzed using conventional laboratory methods. For this reason, PCR is a sensitive assay (Ehtisham *et al*., 2016). The essential components of reaction are :Template DNA, oligonucleotide primers, Thermostable DNA polymerase Deoxynucleoside triphoshates (dNTPs), Divalent Cations, buffer to maintain PH, monovalent Cations and sometimes 10% dimethyl sulfoxide (DMSO) in the Taq polymerase (Garibyan *et al* ., 2013. Ehtisham *et al*., 2016). Each PCR assay requires the presence of template DNA, primers, nucleotides, and DNA polymerase. The DNA polymerase is the key enzyme that links individual nucleotides together to form the PCR product (Garibyan *et al*., 2013). The nucleotides include the four bases; Adenine, (A) Thymine (T),

Cytosine (C), and Guanine (G) that are found in DNA. These act as the building blocks that are used by the DNA polymerase to create the resultant PCR product (Garibyan *et al*., 2013). The primers in the reaction specify the exact DNA product to be amplified. The primers are short DNA fragments with a defined sequence complementary to the target DNA that is to be detected and amplified. These serve as an extension point for the DNA polymerase to build on (Garibyan *et al*., 2013**).** The PCR process involves three major steps; Denaturation of the template by heat, annealing of the oligonucleotide primers to single stranded target sequences and Extension of the annealed primers by thermostable DNA polymerase (Ehtisham *et al*., 2016**).** There are two main methods of visualizing the PCR products: staining of the amplified DNA product with a chemical dye such as ethidium bromide, which intercalates between the two strands of the duplex or labeling the PCR primers or nucleotides with fluorescent dyes (fluorophores) prior to PCR amplification. The latter method allows the labels to be directly incorporated in the PCR product. The most widely used method for analyzing the PCR product is the use of agarose gel electrophoresis, which separates DNA products based on size and charge (Garibyan *et al*., 2013).

The advantages of using PCR are: it is a simple technique to understand and to use, it produces rapid results and it is a highly sensitive technique with the potential to produce millions to billions of copies of a specific product for sequencing, cloning, and analysis (Garibyan *et al*., 2013). Although PCR is a valuable technique, it however has limitations. Since PCR is a highly sensitive technique, any form of contamination of the sample by even trace amounts of DNA can produce misleading results. In addition, in order to design primers for PCR, some prior sequence data is needed (Garibyan *et al*., 2013). Therefore, PCR can only be used to identify the presence or absence of a known pathogen or gene. In addition, the DNA polymerase can incorporate incorrect nucleotides into the PCR sequence, albeit at a very low rate (Ghannam *et al*., 2018). Another limitation is that, sometimes the primers used for

PCR can anneal nonspecifically to sequences that are similar, but not identical, to the target gene (Garibyan *et al*., 2013, Ghannam *et al*., 2018).

2.1.2.2 *DNA Barcoding*

Traditionally, taxonomic identification has relied upon morphological characters. In the last two decades, molecular biology methods, especially DNA barcoding, have been developed for species discrimination (Fiser *et al*., 2013) and are relatively more precise and sensitive in general (Zhang *et al*., 2019). DNA Barcoding is a fast and accurate method of species identification by use of short DNA sequence instead of whole genome .It is used for both Eukaryotes and Prokaryotes (Letchuman *et al*., 2018). The markers in DNA barcoding yield a phylogenetic signal at low taxonomic levels, with an increasing tendency in the DNA barcoding community, particularly among botanical researchers, to use DNA barcode loci to construct whole-community phylogenies (Adamowicz *et al*., 2019).

The short DNA sequence is generated from standard region of genome known as a marker. This marker is different for various species like CO1) for animals, Megakaryocyte-Associated Tyrosine Kinase (MATK) and Ribulose Bisphosphate Carboxylase Large (rbcL) for plants and ITS for fungus (Fiser *et al*., 2013; Letchuman *et al*., 2018).

Barcoding has many applications in various fields such as in agriculture; in helping to identify pests in any stage of life, making it easier to control them thus saving farmers from cost of billion dollars incurred due to pest damage (Madden *et al*., 2019). The global Tephritid barcoding initiative, contributes to management of fruit flies by providing tools to identify and stop fruit flies at the borders (Blacket *et al*., 2012). DNA barcoding is also used in the identification of disease vectors, allowing non-ecologists to identify the vector species that can cause serious infectious diseases to livestock, sustaining natural resources, protecting endangered species, water

quality, and identification of medicinal plants and authentication of natural health products (Letchuman *et al*., 2018).

Information gathered from DNA barcodes can be used beyond taxonomic studies and can have far-reaching implications across many fields of biology, including ecology in rapid biodiversity assessment and food chain analysis, conservation biology in monitoring of protected species, insecurity in early identification of invasive pest species, medicine and pharmacology in identification of active compounds. However, it is important that the limitations of DNA barcoding are understood and techniques continually adapted are improved as this young science matures (Fiser *et al*., 2013).

The ability of DNA barcoding to distinguish species from a range of taxa and to reveal cryptic species has nowadays been well documented (Mat -Jaafar *et al*., 2012). It has proved useful in the study of taxonomically, difficult taxa such as blackflies (Family: Simuliidae), where identification is hampered due to cryptic species or phenotypic plasticity (Hernández *et al*., 2012). Additionally, a global mosquito barcoding initiative in building a reference barcode library that can help public health officials to control these diseases causing vector species more effectively and with very little use of insecticides has been initiated (Letchuman *et al*., 2018).

Moreover, this technique has helped to recognize different developmental life stages of a single species, which could have been impossible by using morphological characters alone. This is more so in the use of DNA sequence data in distinguishing between previously unidentifiable larval stages of some diving beetles (Coleoptera: Dytiscidae) (Fiser *et al*., 2013). Species-level identification is crucial in many applications of economic and social importance where fast identification is highly desirable. Implication of DNA barcoding has been proven successful in rapid biodiversity assessment studies (Fiser *et al*., 2013). DNA barcoding addresses many of the problems inherent to morphological taxonomy. With the number of

taxonomists decreasing and the number of named species increasing, molecular tools have become a mainstay of modern taxonomic analysis (Fiser *et al*., 2013).

Barcoding is advantageous in that, only a small amount of tissue is needed for species determination; the analyses can be performed without prior knowledge of the specimen and can be applied to all stages of development (Fiser *et al*., 2013). Significantly, it emerges as a cost-effective standardized approach for rapid species identification and is currently being used widely in almost all types of organisms (Fiser *et al*., 2013; Adamowicz *et al*., 2019).

2.1.2.3 *2.1.2.5 Sequencing of DNA*

The process of DNA sequencing utilizes biochemical methods in order to determine the correct order of nucleotide bases in a DNA macromolecule using sequencing machines (Kchouk *et al*., 2017). The first method of sequencing came about half a century ago, and since then, sequencing technologies have continued to evolve especially in the appearance of the first sequencers. The First Generation of Sequencing, especially Sanger sequencing was dominant for three decades, however, the cost and time taken was a major obstacle (Kchouk *et al*., 2017). The Next Generation Sequencing Technologies Technology (NGST) was introduced in 2005 and changed the view of the analysis and understanding of living organisms. The strengths of Second Generation Sequencing Technology included, generation of many millions of short reads in parallel, speed up of sequencing process compared to the first generation and low cost of sequencing. In addition, its output was directly detected without the need for electrophoresis (Kchouk *et al*., 2017). NGST is now the starting point for several areas of research based on the study and analysis of biological sequences (Kchouk *et al*., 2017; Li *et al* ., 2019).

In the wake of constant improvements in sequencing technologies, numerous insect genomes have been sequenced (Li *et al*., 2019). Currently, 1219 insect genome - sequencing projects have been registered with the National Center for Biotechnology

Information (NCBI). This included 401 that have genome assemblies and 155 with an official gene set of annotated protein-coding genes (Li et *al*., 2019). The increasing availability of insect genomic resources is beneficial for developing alternative pest control methods. However, many opportunities remain for developing data-mining tools that make maximal use of the available insect genome resources. Although rapid progress has been achieved, many challenges remain in the field of insect genomics (Kchouk *et al*., 2017; Li et *al*., 2019).

2.2 Distribution and Risk assessment

A key issue in ecology and conservation biology is to determine how species are distributed in space. Species distribution models (SDMs) are receiving such an increase in attention in conservation and bio geographical studies, that they are currently one of the most widely used scientific approaches for the identification of potential climate change effects on biodiversity (Berzitis *et al*., 2014; Chlond *et al*.,2015; Urbani *et al*., 2017). These models are successfully and widely applied to assess the ecological and evolutionary forces that shape the geographical distribution of species and the suitability of their habitat (Elith *et al*., 2006; Bosso *et al*., 2013; Zhu *et al*., 2013). Several studies on different predictive spatial distribution approaches have in fact demonstrated the critical role of the impacts of climate change on species distributions (Rebelo *et al*., 2010; Bellard *et al*., 2012; Devictor *et al*., 2012; Travis *et al*., 2013) and in particular on sensitive or threatened species distributions like those endemic (Escalante *et al*., 2009; Bosso *et al*., 2013; Kamino *et al*., 2012). Modeling studies are of great importance in biological conservation; their predictive capabilities shed light on several issues, such as reduction of the distribution area of protected species or the possible extension of invasive species (Fourcade *et al.*, 2014; Urbani *et al.*, 2017).

Spatial and temporal distribution of species is ffected by the quality and quantity of habitats. Several statistical models exist to predict the distribution of a species

(Fourcade *et al*., 2014). SDM's are generally used to predict the habitat potential and spatial distribution of a species according to the occurrence data and different environmental variables (Elith *et al*., 2006).

These models have been widely used for many different purposes in ecological and conservation studies to evaluate the relationship between species occurrence and environmental variables (Elith *et al*., 2009; Fourcade *et al*., 2014).

Spatial distribution could provide important information for conservation planning, reclamation projects of degraded habitats, and prediction of anthropogenic and climatic impacts on habitat potential of a plant species (Mousazade *et al*., 2019).

Although a variety of statistical and probabilistic models is currently used to determine the spatial distribution of species (Fourcade *et al*., 2014), however the common SDMs used in the recent literature are the statistical models such as Generalized Linear Models (GLMs) and Generalized Additive Models (GAMs) and probabilistic models such as Maximum Entropy (MaxEnt) and frequency ratio (FR) (Syphard *et al*., 2009) ; Mousazade *et al* ., 2019). Maxent has been proven suitable to predict the habitat potential of species based on presence-only occurrence data (Mousazade *et al* ., 2019). The principle of SDM is to relate known locations of a species with the environmental characteristics of these locations in order to estimate the response function and contribution of environmental variables and predict the potential geographical range of a species (Fourcade *et al*., 2014). These models estimate the fundamental ecological niche in the environmental space. This is through assessment of species response to abiotic environmental factors and projecting it onto the geographical space to derive the probability of presence for any given area or, depending on the method, the likelihood that specific environmental conditions are suitable for the target species (Mousazade *et al*., 2019). To estimate the most suitable areas for a species and infer probability of presence in regions where no systematic surveys are available conservation practitioners use distribution

models (Fourcade *et al*., 2014; Mousazade *et al*., 2019). SDMs' can also be used to assess the potential expansion of introduced species in newly colonized areas and estimate the future range of a species under climate change which could assist in reserve planning (Liu *et al*., 2019). MaxEnt has been described as the most efficient SDM to handle complex interactions between response and predictor variables as well as being less sensitive to small sample sizes. This and its extreme simplicity of use, has made it the most widely used Species Distribution Models algorithm (Mousazade *et al*., 2019).

Species Distribution Models are now commonly implemented in conservation-oriented studies where regional or continent-wide studies are facilitated by the recent availability of global datasets (Fourcade *et al*., 2014). Environmental layers, such as the global climate variables developed in the WorldClim project offer continuous description of very large areas

(Waltari *et al* ., 2014). Similarly, the development of open biodiversity databases (see for example the Global Biodiversity Information Facility, GBIF, http://www.gbif. org) increases manifolds the spatial coverage of fieldwork observations that could have been collected by a single project. Such databases usually provide presence-only data that can be handled by modeling methods like Maximum Entropy Model (MaxEnt) (Mousazade *et al*., 2019).

MaxEnt has been shown to perform better with small sample sizes relative to other modeling methods. MaxEnt uses presence-only data to predict the distribution of a species based on the theory of maximum entropy (Fourcade *et al*., 2014). The program attempts to estimate a probability distribution of species occurrence that is closest to uniform while still considering environmental constraints (Qin *et* al., 2017). This model is a machine learning/data mining program that evaluates the distribution probability of a species in relation to environmental factors. It has a general-purpose approach to estimate the probability distribution of a species, proven

to work well in practical studies. In addition, it attempts to predict the habitat suitability for a species. MaxEnt could support categorical and continuous predictor data varying from the lowest 0 to 1 as the highest suitability (Mousazade *et al*., 2019).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

This study was carried out in Kwale, Kilifi and Mombasa Counties (**Fig 3.1)** in coastal side of Kenya. The climate in these counties is warm where all stages of mealybugs may be present throughout the year (Flint *et al*., 2016). Farms formed sampling units, sampling method was random and a sample size of 42 sites was computed using the proportion sample size determination formula given by Mugenda wa Mugenda (2003).

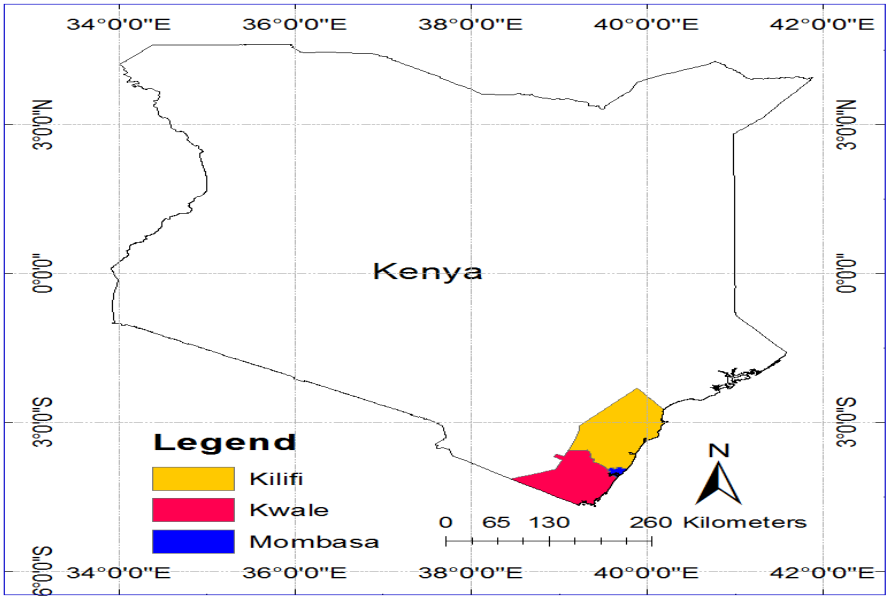

Figure 2.1 -Map of Kenya highlighting Kwale, Mombasa and Kilifi Counties Source: Obed Ogega, 2016.

3.1.1 Kwale County

Kwale County borders Taita Taveta County partly to the North and partly to the North West, Kilifi County partly to the North and partly to the North East, Mombasa and Indian Ocean to the East and Tanzania to the South. The County lies between latitude 4.181630 3‘South and longitude 39.4606 East. The total area of the County is 8270.2 $Km^{2.}$ With a population of 713,488 people (KNBS 2020). The main food crops in this county include maize, cassava, beans, peas, green grams, semi commercial crops are coconut, papaya and mangoes .Cash crops grown are cashew nuts, sugarcane, cotton, simsim, bixa and tobacco. (Kwale County Integrated Development Plan, 2018-2023). Kwale County is relatively warm, with average temperature of 27. 4℃, average humidity of 77 %. Precipitation ranges from 200 mm in the dry months and 1000mm during rainy months (Climate data .org).

3.1.2 Kilifi County

Kilifi County borders Kwale to the South West, Taita taveta to the West, Tana River to the North, Mombasa to the South and Indian Ocean to the East. The County lies between latitude 3.5107 South and longitude 39.9093 East and it covers 12,609.7 Km^2.The main cash crops grown in the county include: Coconuts, Cashew nuts, sisal, mangoes and pineapples. The County has five agro ecological zones (AEZ): Coconut -cassava zone, cashew nut -cassava zone, livestock- millet zone, lowland ranching zone and coconut – cashew nut –cassava zone (Kilifi County official website 2021). Kilifi County receives an average annual rainfall of about 900mm to 1100mm .The average temperature is 26˚ C and 78 % humidity (Climate data.org).

3.1.3 Mombasa County

Mombasa County borders Kilifi County to the North, Kwale County to the South West and the Indian Ocean to the East. It covers 219 km^2 with a population density of 4,292 people per kilometer (KNBS 2020). The County lies between latitude -

4.0547 South and longitude 39, 6636 East. The County experiences a tropical wet and dry climate with only a slight seasonal temperature variation with high temperatures ranging from 28.8 ˚C to 33.7˚C; about 997 mm of annual precipitation falls annually and average annual humidity of 70 % (Mombasa County official website).

3.2 Research design

The study used a descriptive design, which provided an accurate picture of the situation/ phenomenon as it naturally happened. The design identified variables that existed in the given situation and described the relationship that existed between the variables. The design used survey method, which involved conducting brief interviews with farmers, taking their opinion, thoughts and feelings where sampling was done and captured in a questionnaire in a form of an Open Data Kit. Random survey was the technique used to collect standardized information. The survey represented a probe into a given state of affairs that existed at the given time. Direct contact was made with the individual farmers whose characteristics were relevant to the investigation. This study was a detection survey, which focused on establishing the presence, distribution and risk assessment of a new pest; a mealybug devastating papaya in Kilifi, Kwale and Mombasa Counties. Sample information was captured through ODK. Randomly, infested plant samples were collected from the three Counties and transported to the laboratory for analysis.

3.3 Sample size determination

The purpose for this research was detection, to establish the presence or absence of a new pest reported in Kwale, Mombasa and Kilifi Counties in Kenya determine its distribution and establish its risk. The sample size was determined by population size computed using the proportion sample size determination formula given by Mugenda wa Mugenda (2003).

NF= n/ 1+ (n/N).

According to the above formula:

Nf: Desired sample size when the population is less than 10,000

n- Desired sample when the population is more than 10,000

N-Estimate of the size

Kilifi -15 (estimates sites)

Kwale -15 (estimates sites)

Mombasa -15 (estimates sites)

Total = 45 Sites

Nf = 384/1+384/45= 40 Sites

The 40 sites were shared between the three counties giving us an average of 13.3 that was rounded off to 14 sites per County.

3.4 Sampling design

Sampling design was guided by the sample size determination results of the 14 sites per County (section 3.4). However, since papaya is not grown in an organized manner and the crop is not found in all the farms in the area, sampling for the pest was carried out randomly in the14 sites guided by the availability of infested plants. The 14 sites were distributed across the Counties and physical movement from one area to the other was involved. Visual observations of farms for infested papaya plants was done and when an infested plant was detected, permission was sought to

enter the farm to sample. During sampling the farmer was interviewed and information captured in a guided e- questionnaire in a form of an Open Data Kit (ODK) .Live samples on plants were collected in Khaki bags ,others picked using a camel hairbrush into vials with 85% ethanol, both were labelled and shipped to the Laboratory for sorting, counting, tabulating and analysis.

3.5 Data Collection

To undertake morphological and molecular characterization of the pest, some mealybugs were gently collected using a camel hairbrush into vials with 85% ethanol. The vials were clearly labeled in hard and black pencil with sample codes (for example Kilifi 1, Mombasa 1, Kwale1) with adhesive labels. In addition to what was collected in ethanol, additional random live specimens were collected while still attached on plant materials (stem and leaf) in khaki bags for obseravation of characters in colonies before slide preparation. The detailed information of the codes on vials was captured in ODK. The information included sample code, County, collection site, Sub County, Ward, farmer's name, GPS Coordinates, collection date and collector's name. All the samples were taken to the Kenya Plant Health Inspectorate Service (KEPHIS) entomology laboaratory for sorting. At the KEPHIS laboratory, under the stereomicroscope Leica MZ125, the live samples were observed for useful characters and identified. Under the same stereomicroscope Leica MZ125, while using camel hairbrush and soft forceps, mealybugs, were separated into nymphs, adult males and adult females, counted and tabulated. Fifty-five females were picked at random for slide mounting for morphological analysis at KEPHIS while the rest were transported to the International Centre of Insects Physiology and Ecology (*Icipe*) for molecular analysis. The nymphs were discarded. To determine the distribution of the mealybug on papaya, GPS Coordinates collected during sampling were used in developing the distribution map using Google map app. For risk assessment of the pest, occurrence GPS coordinates obtained during

sampling together with environmental data in Species Distribution Models was used to predict the current and future risk of the pest.

3.6 Morphological characterization

The mealybugs were positively identified as the papaya mealybug, *Paracoccus marginatus* at KEPHIS- PQBS -Entomology laboratory. Using stereo microscope with an in built camera (Leica EZD Stereomicroscope; Leica Microsystems (UK) ltd).Live samples were observed for useful characters in their colonies while still attached to plant materials following features described by (Walker *et al*., 2002; Muniappan *et al*., 2008; Galanihe *et al*., 2010; Seni *et al*., 2014). For slide preparation, fifty-five adult females in ethanol were randomly selected and slide mounted following the process described by (Wu *et al*., 2014) with additional details from the protocol described by Williams, de Willink. (a)after making a small incision on the back of the mealybug , one specimens were placed in cold 10% Sodium hydroxide (NaOH) for 24 hours to soften body contents , (b)the specimens were transferred from NaOH solution into a watch glass of distilled water to rinse off and neutralize NaOH, , (c)using a small spatula, the specimens were gently pressed to expel the body contents through the incision until they became translucent while gently spreading the legs and antennae for shaping , (d) translucent specimens were transferred to 90% ethanol for 24 hours to toughen the cuticle, neutralize NaOH and dissolve wax , (e)the specimens were transferred to xylene and soaked at room temperature until all fat/wax was dissolved, (f) the specimens were put in half full watch glass of glacial acetic acid and two drops of acid fuchsin stain and covered overnight, (g)the specimens were removed and rinsed in 100% ethanol to remove surplus stain and fully dehydrate them , (h)the specimens were transferred from ethanol to clove oil and covered overnight , (i)2mm of clove oil was put at the centre of a clean microscope slide and the specimen was transferred into the drop using thick mounted needle and the mealybugs oriented ventrally , (j)a drop of a canada balsam diluted with xylene DPX was placed on the specimens to prevent the

antennae and legs from collapsing, (k)a cover slip was gently lowered onto the drop of canada balsam and labels put on either side of the cover slip, (l) the right hand label details included : sample code , County, Sub County, ward, farmer's name, collection date, host plant and collectors name ,(n)the slides were then placed on a slide drier for a month , (m)using Euromex Compound Microscope BS.1152-EPL mounted with Euromex Camera DC.1355F050, features of the mealybug were observed and identified done based on the detailed taxonomic keys by Williams and de Willink (1992) and Miller & Miller (2002). After identification, a label with information on the genus and the species of the specimen was put on the lesft side of the specimen.

3.7 Molecular characterization

The molecular analysis samples were taken to the Arthropod Pathology Unit (APU) of *Icipe*, Nairobi Kenya for analysis. (a.) individual insect was surface-sterilized using 3% Sodium hypochlorite (NaOCl) and rinsed three times with distilled water, (b) Genomic DNA was extracted from individual whole mealybug using the ISOLATE II Genomic DNA Kit (Bioline), following the manufacturer's instructions: individual insect was put in an eppendorf tube and 180 µl lysis buffer added and the insect crashed using a pestle. 25µl of protein kinase was added to lyophilize the sample, vortexed one minute at 11,000-x g and put in 56-℃ water bath overnight. 200 µl of Lysis buffer was added to the sample and put in a 70℃ water bath for 10minutes. The sample was transferred to collection tubes with a column where 210 µl of absolute ethanol was added and the sample centrifuged for one minute. The sample was then washed two times with 600 µl wash buffer and dried. 25 µl of elution buffer was added to the sample, incubated at room temperature for one minute., centrifuged and purification and concentration of the sample taken , (c) purity and concentration of the resultant extracted DNA was determined using a NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific) by passing 2 ul of the sample to purifying columns of the Nanodrop Spectrophotometer, (d) Polymerase

Chain Reaction (PCR) was done to amplify the D2 region of 28S rDNA (28S) region, using LepD2 Forward 5′3′and LepD2 Reverse 5′ 3′ markers (Campbell *et al*., 1993; Goolsby *et., al.*,2001), (f) PCR was carried out in total reaction volumes of 20 *µl* containing 5× My *Taq* Reaction Buffer (Bioline; 5 mM dNTPs, 15 mM MgCl2, stabilizers and enhancers), 10 µmole of each primer, 0.5 mM MgCl2, 0.0625 U/µl My *Taq* DNA polymerase (Bioline) and 15 ng/*µl* of DNA template ,this reaction was set up in Nexus Mastercycler gradient PCR machine (Eppendorf), (g) ycling conditions included initial denaturation for two minutes at 95.0°C, 40 cycles of 30 s at 95.0°C, 45-s of annealing at 58.8°C and 1 minute at 72.0°C and final elongation at 10 min at 72.0°C ,(h) the amplified PCR products were resolved through a 1.2% agarose gel, DNA bands on the gel were analyzed and documented using KETA GL imaging system trans-illuminator (Wealtec Corp), (i)successfully amplified products were excised and purified using ISOLATE II PCR and Gel Kit (Bioline) by manufacturer's instructions, (j)the purified samples were shipped to Macrogen Netherlands (Macrogen *Europe B.V.*), for bidirectional sequencing ,(k)successful sequences were assembled and edited using Geneious version 8 (http://www.genei ous.com;Kearse *et al.,* 2012), (l)the primer sequences were identified and removed from the consensus sequences generated from both the forward and reverse reads, (m)for conclusive identification of the species, both similarity and phylogenetic analyses were conducted, similarity searches were done by querying the consensus sequences via Basic Local Alignment Search Tool (BLAST) at GenBank database hosted by the National Centre of Biotechnology Information (NCBI), Bethesda, Maryland, USA, (n)Maximum likelihood method was used to run the phylogenetic and molecular evolutionary analyses which were conducted in MEGA version X (Kumar *et al* , 2018), (o) the reliability of the tree was assessed using 1,000 bootstrap replications , (p)estimates of evolutionary divergence between sequences were conducted using the Kimura 2-parameter model (Kimura, 1980) in MEGA X , (q)initial trees) for theheuristic search were obtained automatically by applying neighbour-joining and BioNJ algorithms to a matrix of

pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log-likelihood value, (r)estimates of evolutionary divergence over sequence pairs between groups were calculated using the Kimura 2-parameter (K2P) distance model (Kimura, 1980) in MEGA X (Kumar *et al.*, 2018), Principal Component Plot was then developed from the genetic distance matrix by GenAlEx 6.5 (Peakall *et al.*, 2012).

3.8 Distribution

A field survey was conducted in Kilifi, Kwale and Mombasa Counties in Kenya based on the methodology designed and implemeneted by Tanga, Ekesi, Govender and Mohamed (Tanga *et al* ., 2015), and additional information by methodology designed and implemented by Bertin, Cavalieri and Bosco (Bertin *et al* .,2010). A destructive sampling technique was used during the survey, whereby infested host plant parts (leaves, stems and fruits) were randomly picked. Infested plant materials were collected in khaki bags labelled and sent to the laboratory for analysis. Others were collected with a hairbrush in 85% ethanol vials labelled and sent to the laboratories for analysis. Occurrence data including GPS coordinates (Longitude, Latitude and Altitude) was captured in ODK. To generate a comprehensive map on the distributional range of *P .marginatus* , GPS data was downloaded from the ODK data, checked and validated by entering the geographic coordinates in Google Maps (www.google.com/maps), thereby avoiding erroneous geographic coordinates and uncertain locations, which could have been introduced during sampling. A distribution map was developed by following several steps (a)the occurense GPS coordinates were typed in excel spreadsheet and saved in CSV (Comma delimited) format in a folder on the desktop of the computer , (b)Google website was opened and displayed google maps icon (c)google maps icon was clicked and displayed " Menu " icon , (d)" Menu" icon was clicked and displayed " your places" icon (e) " your places" icon was clicked and displayed a maps icon (f) maps icon was clicked and displayed create a map icon ,(g)map icon was opened and displayed add

layer icon (h)add yayer icon was opened and CSV (Comma delimited) file prior saved on the desktop uploaded and ,(i)columns to position placemarks ticked for longitude and latitude (j)the data was electronically picked run on the system and a map was developed , (k)the map was aligned and saved .

3.9 Risk assessment

In assessing the risk of this invasive species, softwares: Maximum Entropy Modeling (MaxEnt) and Genetic Algorithm for Rule Set Production (GARP) models were used to predict the current and future risks of the pest depending on set climatic conditions . Parameters used were, occurrence data (GPS Coordinates and environmental variables, Bioclim variables obtained from the World Clim Global Climate Database.

3.10 Data analysis

Data analysis for the mealybug DNA concentration and purity (quantity and quality) and absorbance values in ng/µl $A_{260/280}$ from different samples was performed using (NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific). DNA bands of the target gene weight, 575 base pairs was analysed and documented using KETA GL imaging system trans-illuminator (Wealtec Corp). Purity and concentration of the amplified products in ng/µl $A_{260/280}$ was done using ISOLATE II PCR and Gel kit (Bioline). Data obtained from sequenced samples was analyzed, assembled and edited using Geneious version 8 (http://www.genei ous.com; Kearse *et al*., 2012). Quality control of the sequences was carried out to remove poor quality reads at the beginning (10-20) and towards the end (above 600) using Geneious version 8 (http://www.genei ous.com; Kearse *et al*., 2012). High quality sequences from both forward and reverse reads were used to generate consensus sequence, which was used to compare other sequences available at (NCBI) using BLAST. Qualified *Paracoccus marginatus* identities of close similarity with Sequence KP69233 *Paracoccus marginatus* in the database were used to run phylogenetic and molecular evolutionary analyses in MEGA version X (Kumar et al., 2018) using Maximum

likelihood method. The reliability of the tree was assessed using 1,000 bootstrap replications. Estimates of evolutionary divergence between sequences of values between 0.0135 and 0.0900 were conducted using Kimura 2-parameter model (Kimura, 1980) in MEGA version X. Checking for the quality of occurrence data and its validation was carried out using Google Maps (www.google.com/maps). Quality of the ability of two models MaxENT and GARP models to predict current and future risk at > than 0.5 was done by evaluating their Receiver Operating Characteristics – Area under Curve (ROC –AUC) using the research data and training data for control . MaxEnt scored 0.97/1.0 and GARP 0.96/1.0 for reliability in predicting the research samples in Kenya.

CHAPTER FOUR

4.0: RESULTS

4.1 Morphological characterization

To positively identify the live mealybugs on plants collected in Kwale, Mombasa and Kilifi Counties were *Paracoccus marginatus* Williams and de Willink(1992), important characteristics were observed under Microscope with an in built camera (Leica EZD Stereomicroscope; Leica Microsystems (UK) ltd. As shown in Figure 4.1.1, clusters of cotton like masses of different stages of tiny, soft cream -white crawlers and mature mealybugs were seen on the stem of the papaya plant. As shown in Fig 4.1.2, yellowish-cream mealybugs with a white thin layer of wax, which appeared thinner between the segments giving the body a barred appearance. Also observed was the the surface wax on the dorsum of the mealybug which showed transverse creases between the body segments without any longitudinal depressions. In addition to that, the margin had a series of very short white waxy filaments spaced evenly along each side. As shown in Fig. 4.1.3, the adults were yellow- green body in colour ,without wings and the abdomen was without setae.As shown in Fig.4.1.4, the mealybugs were oval in shape without longitudinal depressions .These features matched the morphological characteristics described for *P.marginatus* by Muniappan *et al*., 2008, Galanihe *et al*., 2010 , Daane *et al.,* 2010 and Wu *et al*., 2014. There were no males identified in the samples collected.

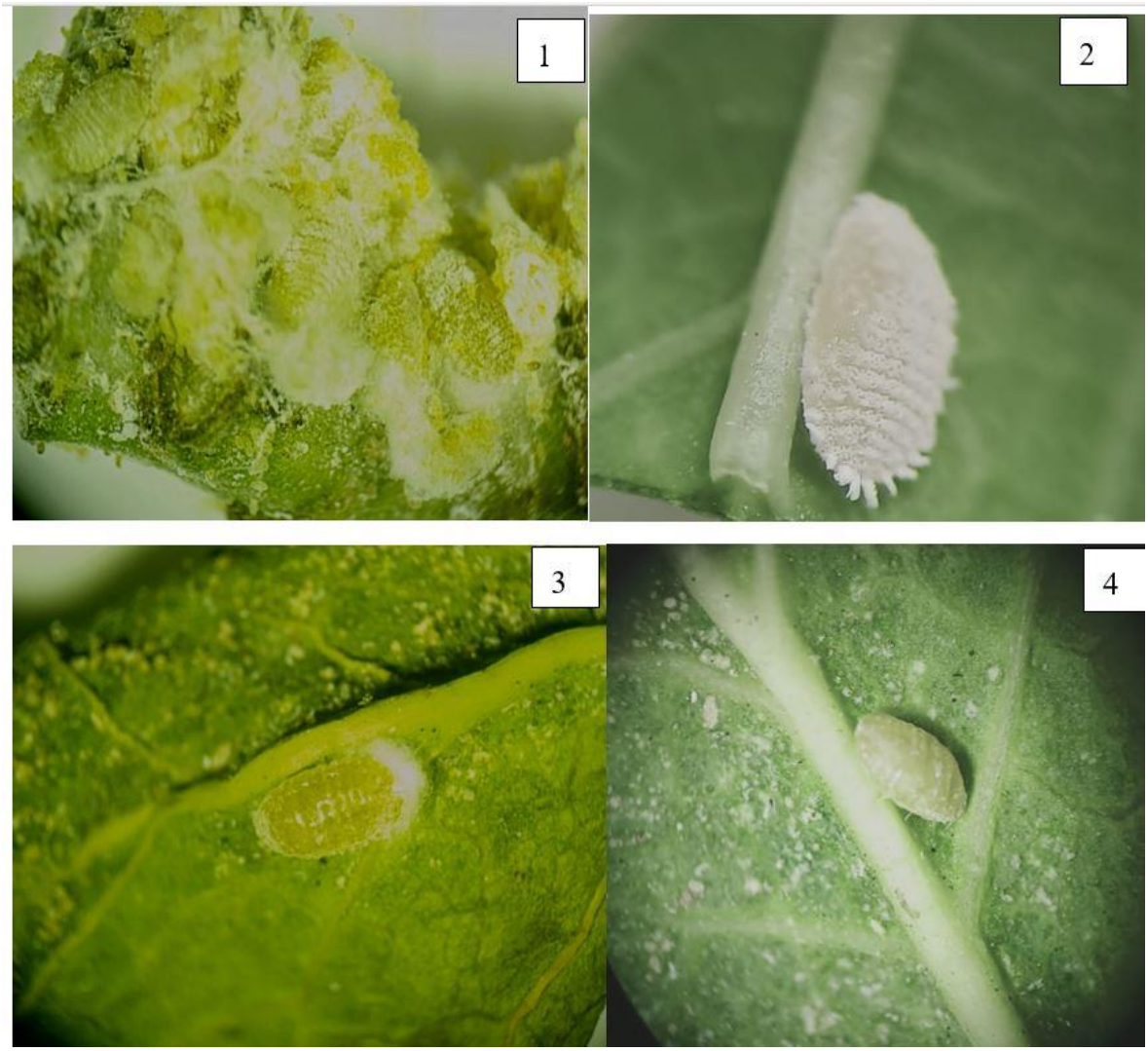

Fig. 4.1: Characteristics of live papaya mealybugs on papaya stem and leaves. Source: Helen Heya, 2021

Key: Clusters of cotton like masses with different stages of *P.marginatus* on papaya stem (1). Adult female *P.marginatus* covered with white wax showing tranverse creases between body segments and a series of short waxy filaments alog the margin (2). Oval, yellow green adult P. *marginatus* female (3). Adult female *P. marginatus* showing absence of longitudinal depressions and setae (4).

To positively identify the mealybugs collected in 85% ethanol and mounted on slides as *P.marginatus*, specimens were observed under Microscope with an in built camera (Leica EZD Stereomicroscope; Leica Microsystems (UK) ltd. In addition to this, female adult mealybugs were mounted on slides and observed under Euromex Compound Microscope BS.1152-EPL mounted with Euromex Camera DC.1355F050 and ran through a taxonomic key by Williams & Granara de Willink (1992) and Miller and Miller (2002). As shown in Fig.4.2.1, specimens killed in 85% Ethanol without dipping in hot water had turned black.As shown in Figure 4.2.2 the mealybug had 8 antennal segments with bristles and fleshy setae. As shown in Figure.4.2.3 there was presence of oral rims. As Shown in Figure.4.2.4, the samples had a ventral anal lobe. As shown in Figure. 4.2.5, the hind coxa had numerous transluscent pores. As shown in Figure.4.2.6 the specimens had 17 pairs of cerarii. As shown in Figure.4.2.7, the dorsum had oral rim tubular ducts restricted to marginal areas. As shown in Figure.4.2.8, the ventral side had multilocular pores in the centre of the abdomen and absent on the margins. These features matched with features used by Heu et al., 2007, Seni *et al*., 2014, Galanihe *et al*., 2010 and Wu *et al*., 2014.


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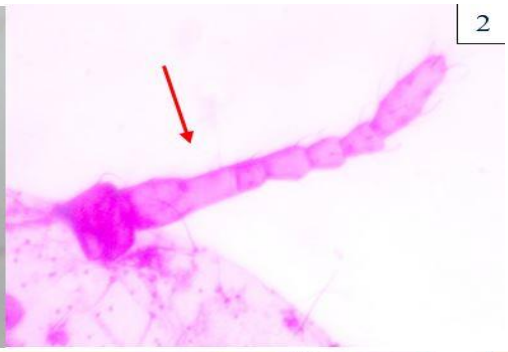
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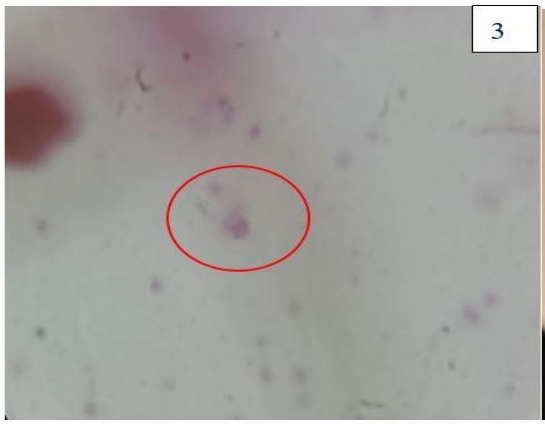
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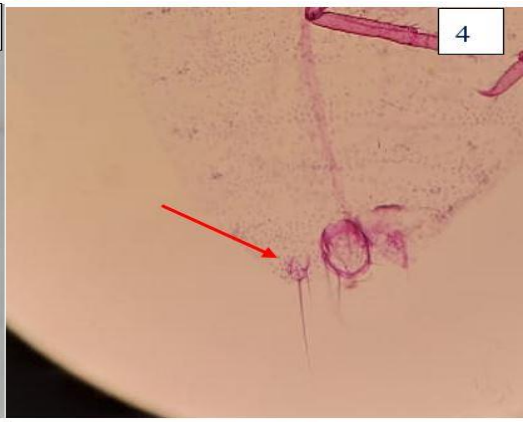
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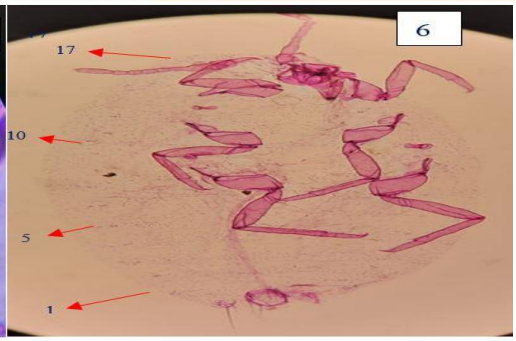
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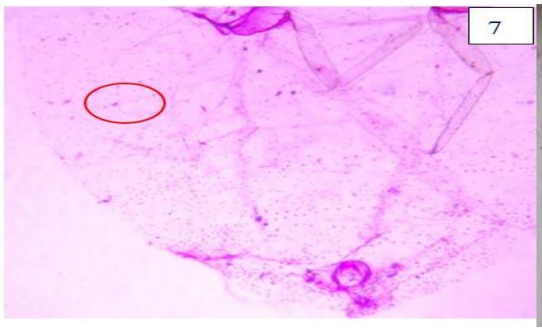
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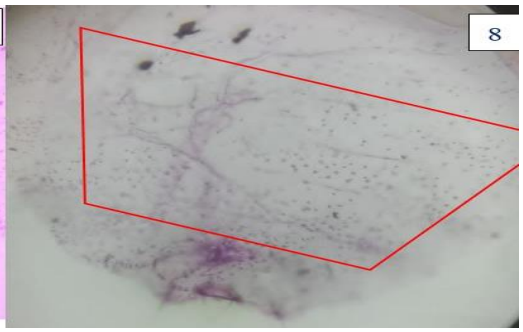
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Figure 4.2: Identification characteristics of slide mounted papaya mealybug Source: Helen Heya, 2021

Key: Black *P.marginatus* adult females killed in 85% Ethanol without dipping in hot water (1), eight antennal segments (2), presence of oral rims (3), presence of ventral anal lobe (4), presence of transluscent pores restricted to the hind coxa (5), 17 pairs of cerarii (6), presence of dorsal oral rim tubular ducts (7), presence of oral rims on the body (8) and presence of multilocular pores on the lower body (8). Photo Source: Helen Heya, 2018.

4.2 Molecular characterization

To determine the genetic characteristics of the mealybugs collected from Kilifi, Mombasa and Kwale and positively confirm them as *P.marginatus*, Deoxyribonucleic acid (DNA) was extracted from the mealybugs collected in 85% ethanol and amplified using Polymarase Chain Reaction (PCR). The PCR product was then resolved in 1.5 % agarose at 70 volts, analysed and documented using KETA GL imaging system trans-illuminator (Wealtec Corp).

As shown in Figure. 4.3, a partial sequence of about 350-450 bp size of the 28s gene region of rDNA was obtained from positively amplified samples collected from Kilifi, Kwale and Mombasa.

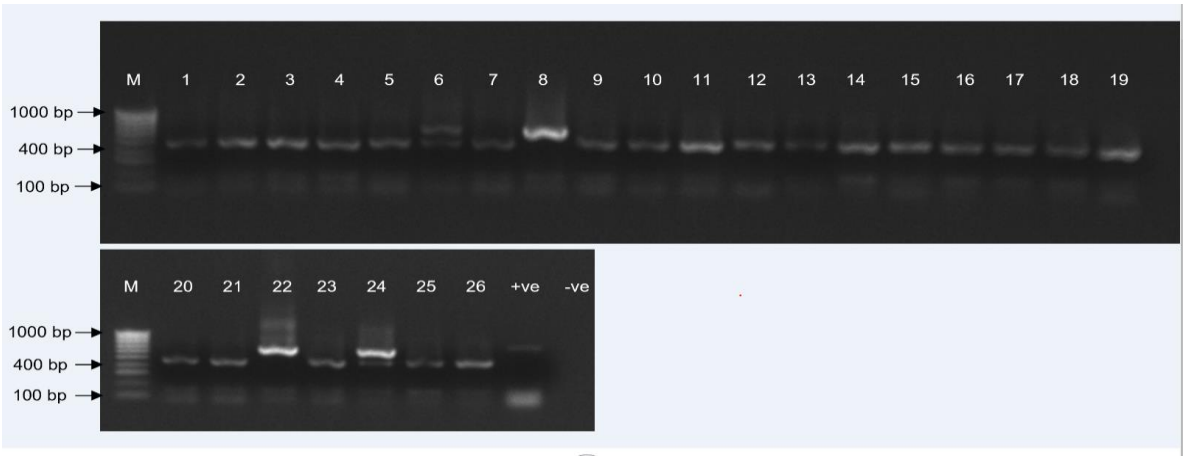

Figure 4.3: A 1.5% agarose gel electrophoresis image of papaya mealy bug samples amplified using Lep D2 primers. M: 100 bp ladder (Bioline, London, UK). Lanes 1 – 10: Samples collected from Mombasa. Lanes 11 – 20: Samples collected from Kwale. Lanes 21 – 26: Samples from Kilifi. +ve: Positive control sample for the PCR (in this case FAW). –ve: Non-template negative control (distilled water).

To determine the evolutionary similarity between the samples collected from the three Counties and *P. marginatus* in the world genebank (NCBI), GenBank accession KP692333 (GenBank identification *Paracoccus marginatus* isolate S3-668.1) and the rooting sample *Planacoccus citri* GenBank accession KP692399, estimates of evolutionary divergence over sequence pairs between groups were calculated . The calculation was done using Kimura 2 parameter (K2P) distance model in MEGA and estimates of evolutionary divergence tabulated. As shown in Table 4.1, low divergence was observed among all the samples collected in the three Counties and in addition to this, low divergence was observed between the samples and the GenBank *P marginatus*. The divergence ranged between 0.0135 and 0.0900. However, high divergence was observed between the samples collected in the three Counties and GenBank *Planococcus citri* . Also observed was, high divergence between the Genbank *P.marginatus* and *P. citri* .The divergence of all the *P. marginatus* samples and *P. citri* ranged between 0.1667and 0.2490. Estimates

divergence results confirmed that samples collected from the three Counties were similar to each other and similar to the GenBank *Paracoccus marginatus*.Estimates

Table 4.1: Estimates of evolutionary divergence between *Paracoccus marginatus* sequences

	Kwale_2	Kwale_4	Kwale_3	Kwale_6	Kilifi_1	Mombasa_5	Mombasa_1	Mombasa_2	Mombasa_4	Mombasa_6	Kwale_5	Mombasa_3	Kwale_1	Kilifi_2	KP692333.1Paracoccus_marginatus	KP692399.1Planococcus_citri
Kwale_2	0.0000															
Kwale_4	0.0135	0.0000														
Kwale_3	0.0320	0.0182	0.0000													
Kwale_6	0.0321	0.0274	0.0181	0.0000												
Kilifi_1	0.0136	0.0273	0.0181	0.0181	0.0000											
Mombasa_5	0.0273	0.0413	0.0461	0.0461	0.0273	0.0000										
Mombasa_1	0.0367	0.0509	0.0557	0.0366	0.0367	0.0273	0.0000									
Mombasa_2	0.0511	0.0656	0.0706	0.0608	0.0511	0.0461	0.0510	0.0000								
Mombasa_4	0.0414	0.0460	0.0366	0.0366	0.0273	0.0556	0.0557	0.0804	0.0000							
Mombasa_6	0.0227	0.0366	0.0319	0.0320	0.0135	0.0366	0.0461	0.0656	0.0319	0.0000						
Kwale_5	0.0704	0.0853	0.0752	0.0703	0.0556	0.0556	0.0654	0.0366	0.0752	0.0702	0.0000					
Mombasa_3	0.0608	0.0755	0.0755	0.0656	0.0559	0.0414	0.0557	0.0135	0.0853	0.0704	0.0366	0.0000				
Kwale_1	0.0656	0.0804	0.0804	0.0704	0.0606	0.0461	0.0605	0.0273	0.0903	0.0753	0.0508	0.0135	0.0000			
Kilifi_2	0.0654	0.0801	0.0801	0.0802	0.0605	0.0508	0.0752	0.0556	0.0901	0.0751	0.0801	0.0509	0.0556	0.0000		
KP692333.1Paracoccus_marginatus	0.0604	0.0751	0.0751	0.0653	0.0556	0.0605	0.0702	0.0702	0.0702	0.0604	0.0900	0.0653	0.0801	0.0703	0.0000	
KP692399.1Planococcus_citri	0.2189	0.2306	0.2371	0.2311	0.2253	0.2068	0.2120	0.2183	0.2253	0.2242	0.2491	0.2125	0.2311	0.2259	0.1667	**0.0000**

To confirm the similarity between the genetic characteritics of the samples collected in the three Counties and the GenBank *P.marginatus* and GenBank *P. citri*, a Principal Component Analysis (PCA) was done and a Principal Component Plot developed from the genetic distance matrix. As shown in Figure 4.4, the first two PCA axes generated from the distance matrix accounted for 69.1% of variation (first axis 41.9%, and the second axis 27.2%) between all the samples. Furthermore, the PCA clustered the samples into two, where all the *P. marginatus* samples clustered together irrespective of collection sites, and a cluster of the *Planococcus citri* at a distance.This analysis confirmed that, the samples collected in the three counties are similar to the GenBank *P.marginatus.*

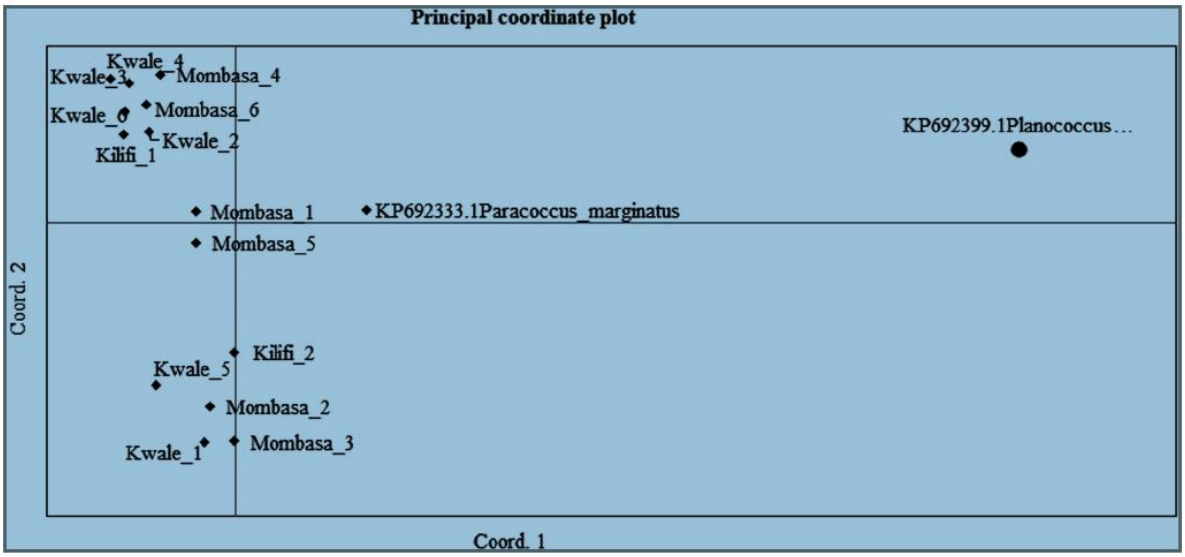

Figure 4.4: Principal component analysis (PCA) plot for the *Paracoccus marginatus* species calculated using GenAlEx.

To determine the phylogenetic relationship between the samples collected in Kilifi, Kwale, Mombasa, and the GenBank *P.marginatus*, the samples were BLASTed searched through NCBI and phylogenetic analysis carried out by Maximum likelihood method, based on Kimura @-parameter model for *Paracoccus marginatus* samples and rooted by *Planococcus citri*. As shown in Figure 4.5, percentage

similarity between the samples and *P.marginatus* GenBank accession number KP692333.1, ranged between 93 % and 99%. In addition to that, the phylogenetic tree had two distinct clusters. One cluster consisted of the samples and the

GenBank *P. marginatus* and the other consisted a distance branch of a GenBank accession of *P. citri* (KP692399.1), which rooted the samples.The Percentage of trees in which the association taxa clustered together was shown.Phylogenesis confirmed that the samples collected in the three Counties are similar to the GenBank *Paracoccus marginatus*

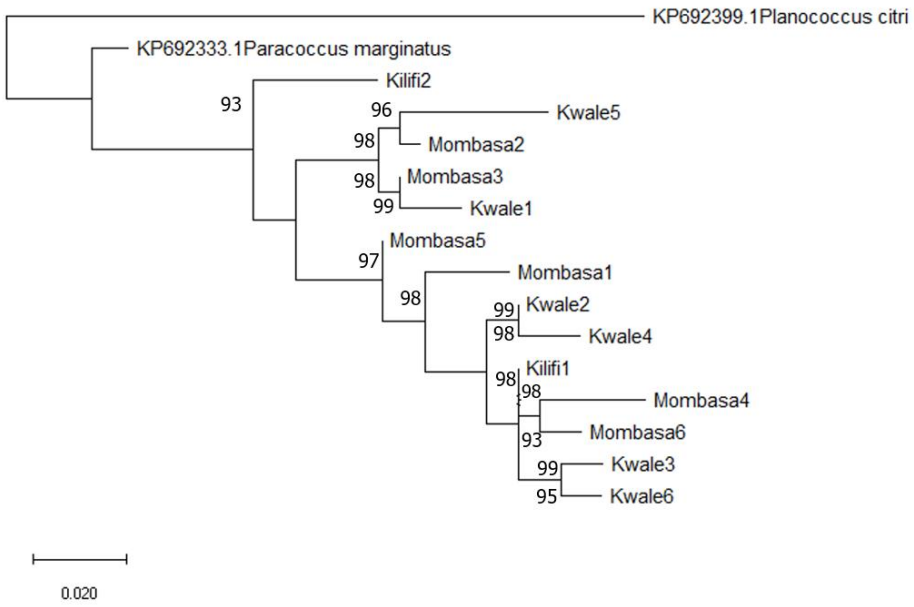

Figure 4. 5: Phylogenetic analysis by Maximum Likelihood method based on the Kimura 2-parameter model for *Paracoccus marginatus* samples and rooted by *Planococcus citri*.

4.3 Distribution of *Paracoccus marginatus*

To determine the current distribution of *Paracoccus marginatus*, a field survey was conducted in Kilifi, Kwale and Mombasa Counties based on the methodology designed and implemeneted by Tanga, Ekesi, Govender and Mohamed and

additional information information by Bertin, Cavalieri and Bosco. With the methodology guidance by Mugenda wa Mugenda, 14 sites per County were sampled and specimens transported to the Plant Quarantine Laboratory at the Plant Health Inspectorate Service (KEPHIS), Muguga, Kenya for sorting, counting, tabulating and morphological identification under stereomicroscope (Leica MZ 125 Microscope, Leica Microsystems, and Switzerland). As shown in Table 4.2, papapa mealybug, *Paracoccus marginatus* was distributed in all the 42 sites sampled.

Table 4.2: Distribution of sampling sites and the number of mealybugs collected per site

County	Site	Sample Code	Coordinates		No. of Mealybugs sampled		
			East	South	Nymphs	Adult ♀	Adult ♂
Kilifi	Mariakani	Kilifi 1	39°29'19.4	3°52'04.3"	60	15	0
Kilifi	Kambe	Kilifi 2	39°36'32.4"	3°51'12.6"	100	25	0
Kilifi	Kaloleni shopping centre	Kilifi 3	39°37'26.5"	3°49'37.3"	45	22	0
Kilifi	Ribe	Kilifi 4	39°38'33.4"	3°56'48.8"	60	30	0
Kilifi	Shimo la tewa	Kilifi 5	39°43'23.9	3°58'16.0"	45	22	0
Kilifi	Takaungu	Kilifi 6	39°51'53.3"	3°40'51.7"	23	13	0
Kilifi	Bamba	Kilifi 7	39°30'54.4"	3°34'07.9"	31	15	0
Kilifi	Ganze, migumo miiri	Kilifi 8	39°39'13.7"	3°34'41.5	22	20	0
Kilifi	Ganze /jaribuni	Kilifi 9	39°44'31.9"	3°37'38.5"	30	12	0
Kilifi	Watamu	Kilifi 10	39°58'25.2	3°19'56.8"	59	35	0
Kilifi	Malindi	Kilifi 11	40°06'16.4"	3°13'15.5"	110	33	0
Kilifi	Magarini West	Kilifi 12	39°50'32.9"	3°07'48.8"	60	23	0
Kilifi	Magarini East	Kilifi 13	40°03'54.3"	3°01'49.4"	21	15	0
Kilifi	Mtwapa ATC	Kilifi 14	39°43'37.3"	3°56'03.2"	50	40	0
Mombasa	Tudor	Mombasa 1	39°39'47.4	4°02'05.7"	22	12	0
Mombasa	Shimazi	Mombasa 2	39°38'59.9"	4°03'07.6"	15	13	0
Mombasa	Magongo	Mombasa 3	39°37'27.6"	4°01'23.4"	21	10	0
Mombasa	Jomvu Kuu	Mombasa 4	39°35'40.4"	3°58'43.8"	40	15	0
Mombasa	Port reitz	Mombasa 5	39°36'52.4	4°01'07.0"	23	12	0
Mombasa	Miritini	Mombasa 6	39°35'28.8	3°59'54.5"	23	5	0

Mombasa	Nyali	Mombasa 7	39°42'08.0	4°02'56.5"	75	22	0
Mombasa	Shanzu	Mombasa 8	39°44'51.9	3°57'49.3"	76	16	0
Mombasa	Mwakirunge	Mombasa 9	39°40'32.0	3°56'15.8"	100	26	0
Mombasa	Kongowea	Mombasa 10	39°41'15.7"	4°02'21.4"	24	9	0
Mombasa	Frere town	Mombasa 11	39°41'38.7"	4°01'28.6"	15	5	0
Mombasa	Likoni	Mombasa 12	39°39'30.1"	4°05'06.2"	55	12	0
Mombasa	Mtongwe	Mombasa 13	39°39'04.1"	4°05'01.2"	49	14	0
Mombasa	Kiembeni	Mombasa 14	39°41'59.8	3°59'15.4"	22	7	0
Kwale	Golini	Kwale 1	39°27'30.4	4°08'05.6"	33	19	0
Kwale	Matuga	Kwale 2	39°34'12.9"	4°10'26.8"	15	15	0
Kwale	Tiwi	Kwale 3	39°34'44.5"	4°13'59.2"	66	17	0
Kwale	Shimbahills	Kwale 4	39.3941	4.36703	80	15	0
Kwale	Ndavaya	Kwale 5	39°09'54.2"	4°15'40.5"	70	22	0
Kwale	Kinango	Kwale 6	39°19'05.8"	4°08'26.5"	67	23	0
Kwale	Mackinon Road	Kwale 7	39°02'04.1"	3°43'39.5"	52	21	0
Kwale	Lunga lunga	Kwale 8	39°07'19.3"	4°33'20.0"	90	33	0
Kwale	Mazeras	Kwale 9	39°33'02.1"	3°57'54.9"	88	19	0
Kwale	Kikoneni	Kwale 10	39.36168	4.41355	180	33	0
Kwale	Vanga	Kwale 11	39.20203	4.66622	150	46	0
Kwale	Ukunda	Kwale 12	39°34'24.8"	4°17'15.3"	56	21	0
Kwale	Mivumoni	Kwale 13	39.51736	4.34658	49	14	0
Kwale	Kwale town	Kwale 14	39°27'15.5"	4°10'46.1"	26	12	0

mine the distribution of papaya mealybug, a comprehensive map of *Paracoccus marginatus* was developed using google maps application with the use of the occurrence global positioning system (GPS) data for 14 sites per County as guided by Mugenda wa Mugenda. As shown in Figure, 4.6, papaya mealybug is currently distributed in Mombasa, Kwale and Kilifi.

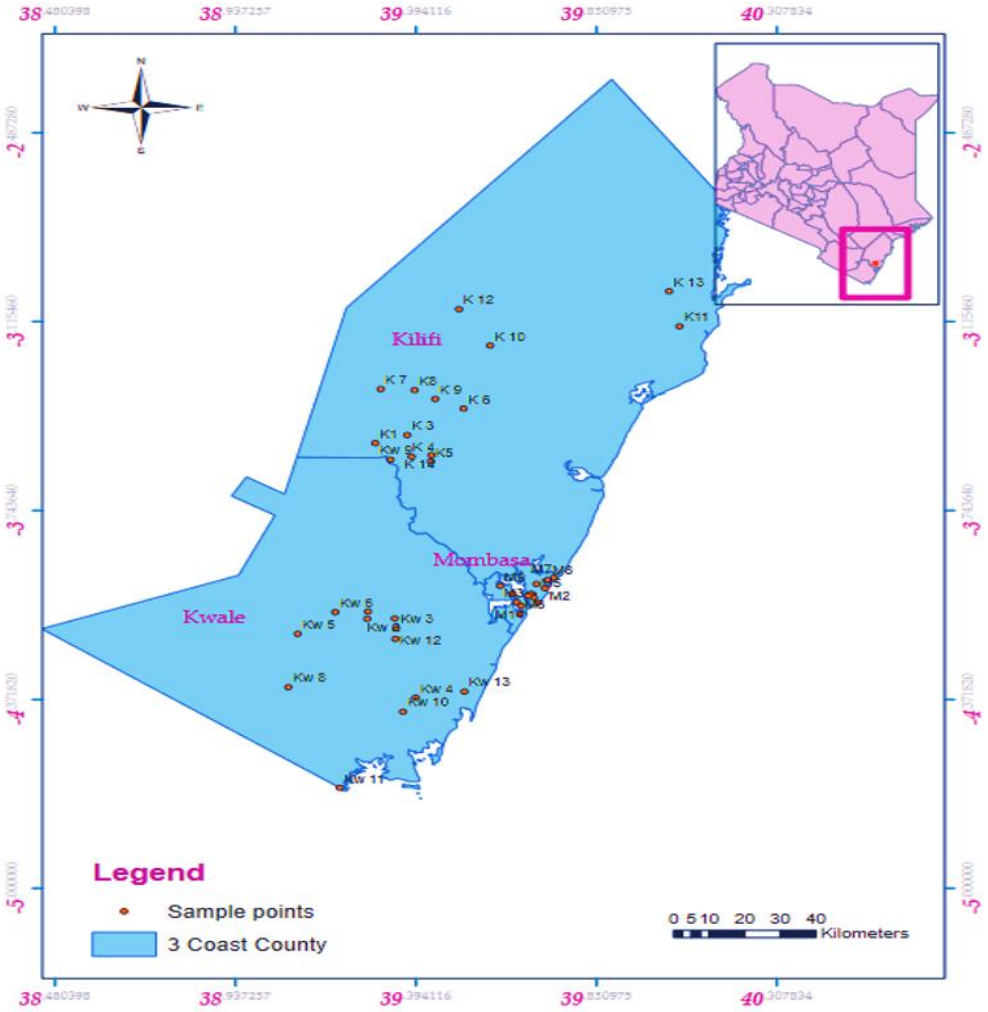

Figure 4: 6: A map of Kenya showing the current distribution of *Paracoccos marginatus*

4.4 Risk assessment

To determine the habitat suitability for potential geographical distribution of *Paracoccus marginatus* in papaya cropping in Kenya, Species Distribution Models; Maximum Entropy (MaxEnt) and Genetic Algorathim for Rule Set (GARP) models were used for both under current and future climate change scenarios. Bioclimatic variables influencing the geographic distribution of *P. marginatus* were: *I*sothermality (Bio-3), temperature mean diurnal range (Bio-2), temperature

seasonality (Bio-4), temperature annual range (Bio-7) and annual precipitation (Bio-12). Isothermality (Bio-3) was the most important abiotic variable that influenced the geographic distribution of *P. marginatus*. As Shown in Figure .4.7, the projections of the potential distribution of *P. marginatus* in both models under the respective thresholds were slightly similar.They indicated high reliability in the generated predictions. In addition, all the areas along the coastal belt, Garissa, Machakos, Kitui, Kajiado and Makueni were identified as areas of potential suitability for this mealybug species establishment. As shown in Figure. 4.7a MaxEnt predicted a more expanded area for *P. marginatus* invasion with significantly higher risk compared to GARP model which was somewhat restrictive in its prediction of potential area for establishment. MaxEnt model revealed additional areas like Marsabit and a small portion of Wajir to be low to moderate area for possible establishment of *P. marginatus*, which differed from that predicted by GARP algorithm, which additionally predicted Meru, Tharaka Nithi, Embu, Kirinyaga, Muranga, Kiambu, Kitui and Makueni.

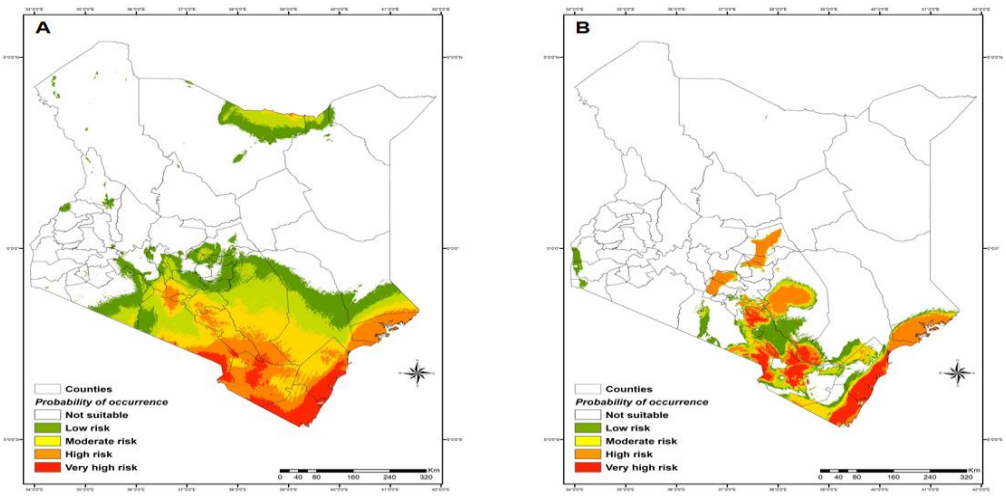

Figure 4.7: Habitat suitability for the potential geographic distribution of *Paracoccus marginat*us *s* in papaya production areas of the country under current climate change scenarios using (A) maximum entropy modelling (MaxEnt) and (B) genetic algorithm for ruleset production (GARP) models.

As shown in Figure 4.8, both distribution models of *P. marginatus* showed that the probability of occurrence of this species decreased considerably in Counties in which the mean temperature of the coldest quarter was below 15°C and the maximum temperature of the warmest month above 33°C. Regarding rainfall, the standard curves of the model indicate that the annual precipitation strongly influenced the distribution of the species with annual rainfall ranging between 80 - 2,000 mm. In addition, both models highlighted a South-North range where the models did not predict the presence of the species, delimiting a discontinuity in the potential distribution of *P. marginatus*. In Northern Kenya, both models predicted potential suitability of *P. marginatus* establishment lie in a wide range along most of the northern part of Marsabit and Wajir counties. Both models predicted Mandera as low risk zone. As shown in Fig. 4.8 a, under future climatic scenarios, the MaxEnt model predicted more extensive areas for the occurrence (higher environmental suitability) of *P. marginatus* almost throughout the North Eastern region of Kenya. As shown in Figure 4.8 b, GARP model was more conservative in terms of predicted areas under future climate change. The intensity of the algorithm was much higher in Kilifi, Kwale, Taita Taveta, Tana River, Kajiado and Kiambu. In addition to this, it revealed Siaya, Homa Bay and Migori Counties as potential areas for future invasion by *P. marginatus*, which differed with the prediction by MaxEnt algorithm. Future climate change model using GARP algorithm predicted a contraction of *P. marginatus* suitable climate areas for potential spread, while MaxEnt showed an expansion of the pest's ecological niche. The climatic suitable areas with substantial reduction as expressed by GARP algorithm under climate change scenario included Meru, Tharaka Nithi, Embu, Kirinyaga, Murang'a, Kitui and Lamu.

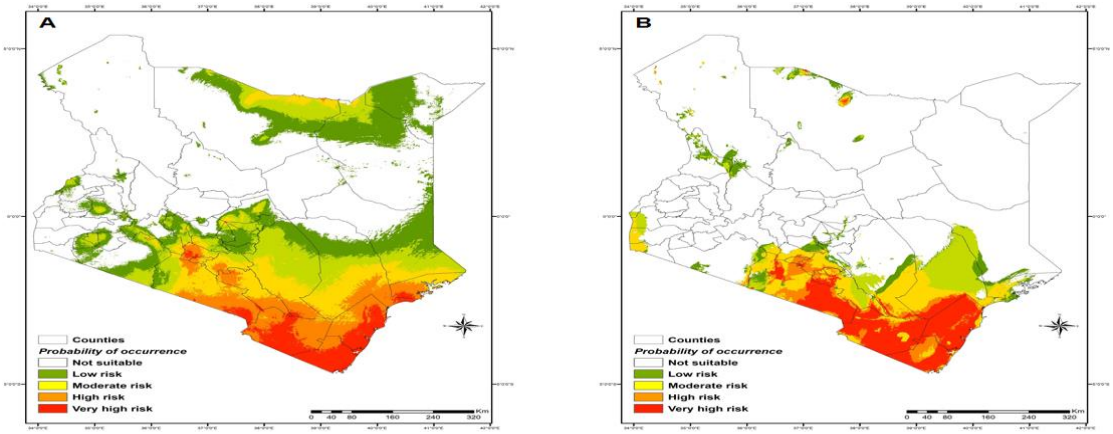

Figure 4.8 Habitat suitability for the potential geographic distribution of *Paracoccus marginatus* in papaya production areas of the country under future climate change scenarios using (A) maximum entropy modelling (MaxEnt) and (B) genetic algorithm for ruleset production (GARP) models.

Under current or future climatic conditions, MaxENT and GARP models demonstrated that there is a risk of this invasive pest, *Paracoccus marginatus* spreading to other parts of Kenya.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Morphological characterization of the Mealybugs collected from infested farms within the coastal strip of Kenya was carried out by observing live specimens and mounted slides. This is in line with similar studies carried out when characterizing mealybugs infesting Brazilian vineyards (da Silva *et al*. 2014), papaya mealybug in Malaysia (Matsoi *et al.* 2011) and Sri Lanka (Galanihe *et al*. 2010). Using molecular procedures to confirm the morphologically characterized specimens is in corroboration with the characterization carried out for African Anopheline mosquitoes (Erlank *et al*., 2018).

Extraction of Deoxyribonucleic acid using the isolate II genomic DNA Kit corroborates the preparation of mealybugs for genetic research carried out in California, USA (Bahder *et al*. 2015). Determination of the purity and concentration of extracted DNA using Nanodrop 2000/2000c Spectrophotometer corroborates with accurate determination of DNA yield from individual mosquitoes for population genomic applications research carried out in the UK (Wilding *et al*. 2009).

Amplification of the D2 region of 28S rDNA (28S) region using LepD2 Fw 5’ AGTCGTGTTGCTTGATAGTGCAG 3’ and LepD2 Rev 5’ TTGGTCCGTGTTTCAAGACGGG 3’ markers and using PCR conditions: initial denaturing for 2 minutes at 95 °C, 40 cycles of 30 seconds at 95 °C, annealing for 45 sec at 58.8 °C and 1 min at 72 °C and final elongation at 10 min at 72 °C corroborates with similar conditions used in molecular characterization of mealybugs in Brazil vineyards. (da Silva *et al*. 2014).

Resolving the amplified PCR products using 1.5% agarose gel and analyzing DNA bands in the gel using KETA GL imaging system trans-illuminator corroborates with using DNA barcoding to improve invasive pest identification research carried out at U.S.A ports-of-entry (Madden *et al*. 2019).

BLASTing the specimens' sequences to determine the relationship with specimens stored in the National Centre for Biotechnology Information (NCBI) data base is line with research on confirming the first record of mealybug *Pseudococcus jackbeardsleyi* (Hemiptera: Pseudococcidae) in Costa rica (Jiménez *et al* 2016) . Running sequences derived in BLAST

into Mega software for phylogenesis corroborates with studied on preliminary phylogeny of the scale insects based on nuclear small - subunit ribosomal DNA research carried out in Australia (Cook *et al*. 2002) and phylogenetic analysis of mealybugs based on DNA research carried out in South Africa (Downie *et al*. 2004).

Using MaxENT species distribution models to predict the risk of papaya mealybug under current and future climatic conditionsin Kenya corroborates with prediction of future trends in the distribution of high altitude endemic insects in response to climate change research carried out in Italy (Urbani *et al*. 2017) .This also corroborated with prediction of impacts of climate change research on the potential distribution of Thuja sutchuenensis Franch, an extremely endangered conifer in SouthWestern China (Qian *et al*., 2007). Using of MaxENT model to predict the risk of papaya mealybug in Kenya corroborates with using of Maximum Entropy Modeling (MaxEnt) to predict future trends in the distribution of high altitude endemic insects in response to climate change research in Italy (Urbani et al., 2017). Using of GARP species distribution models to predict the current and future risk of papaya mealybug in Kenya corroborates with using

of GARP in predicting the spatial distribution of non-vagile invertebrate species in North Carolina, USA (Stockman *et al.* 2006).

5.2 Conclusion

1. This study positively identified through morphological methods that the invasive pest devastating papaya in the three counties: Kilifi, Kwale and Mombasa is *Paracocsos marginatus*. The ability to collect and prepare microscopic slides of female mealybugs and observe the features taxonomic characteristics under the compound microscopic allowed quick identification of the mealybug.
2. This study further confirmed through molecular characterization that the invasive pest devastating papaya in the three counties: Kilifi, Kwale and Mombasa is *Paracocsos marginatus*. Through a recently developed technique for species-level identification that involved the use of short, standard DNA sequences as species labels offered an effective complement to traditional taxonomic classification based on morphology.
3. This research confidently outlined the current distribution of the new invasive pest in the three Counties .Using occurrence data, google map app was used to clearly outline the areas where the pest was present in the Counties.
4. Using species distribution models; Maximun Entropy and Gene Algorithm for Rule Set Production, this research was able to determine the current and future risk of the invasive mealybug, Paracoccus *marginatus in* Kenya.

This study supports the use of both taxonomic/morphological and DNA tools in identification of invasive pests .The study support use phylogeny analysis for the confirmation of invasive pests in relation to what is in the world gene database and use of MaxE*nt and* GARP models in establishing current and future risks of establishment of invasive pests.

5.3 Recommendations

1. Morphological/taxonomic tools can be used to reliably identify invasive pests. This method is fast and very useful where a quick decision on the identify of a new is required to determine the fate of an import plant consignment. For example when National Plant Protection Organizations (NPPO) are carrying out plant inspections at the Countries exit and entry points. This toll is also very useful there is neither molecular equipment nor expertise available to do molecular analysis.

2. Molecular characterization is a very important tool in confirming occurrence of invasive pests. This method is sensitive, specific and it has increased precision. In addition to this Phyogenesis by BLASTing research specimens in the world species gene bank to compare similarity with what is stored in the bank is very important to conclusively confirm invasive species relationship with similar species in other parts of the world.

3. Google Map app is a reliable tool to be used in outlining the distribution of a new pest in a place. Species distribution models; MaxENT and GARP are reliable tools in predicting the risk of an invasive pest spread in a Country in current and future climatic conditions.

4. The mealybug devastating papaya in the three Counties has been fully identified therefore there is need for:

I. The Kenyan NPPO, KEPHIS to report the new pest with the International Plant Protection Convention (IPPC) to advise its member countries on Kenyan Papaya import requirements
II. There is need for researchers to carry out efficacy trials for papaya mealybug management pestcides, register them with the Pest Control and Products Board

(PCPB) and make them available for farmers in the coastal Counties to use for management. There is need for researchers to also do research on natural enemies, which have worked in ther parts of the world on management of papaya mealybug, import, masss produce and release in the infested areas.

III. There is need for the three Counties (Kilifi, Kwale and Mombasa Counties) to create awareness to the extension officers and farmers in these areas by preparing technical materials (brochures, posters, identification guides, pest identification cards) and sharing them to help them with proper identification of the pest. There is need for the Counties to create awareness using Counties social media platforms (face book, whats up groups, twitter and Instagram) and air programs on the pest in the Counties local radio station like Kaya Fm and Pwani Fm.

IV. Risk assessment software's predicted which Counties have the capability of invasion

by the invasive and therefore there is need for the Counties agriculture umbrella, Joint Agriculture Secretariat (JAP) to allocate funds to Counties highlighted for them to take prevention measures like establishing early warning systems .

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