MOLECULAR CHARACTERIZATION AND DISTRIBUTION OF *'CANDIDATUS'* LIBEROBACTER SPECIES/STRAINS IN MACHAKOS AND KAKAMEGA DISTRICTS OF KENYA

MASTER OF SCIENCE (M.Sc) THESIS

BY

MAGOMERE TITUS OBIDI

B.Sc (Agric), M.A. U.O.N.

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THIS THESIS IS SUBMITED IN PARTIAL FULFILMENT FOR THE REQUIREMENTS FOR MASTERS OF SCIENCE DEGREE IN GENETICS AND PLANT BREEDING.

DEPARTMENT OF CROP SCIENCE

FACULTY OF AGRICULTURE

UNIVERSITY OF NAIROBI

SIGNATURE PAGE

I declare that this thesis is original work and has not been presented for a degree in any other university. All references are duly acknowledged.

STUDENT: Magomere Titus Obidi
BSc

________________________Date ______________

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

1. Dr. Silas. D. Obukosia
PhD

________________________Date______________

2. Prof. Eunice W. Mutitu
PhD

________________________Date______________

3. Dr. Christopher Ngichabe
PhD

________________________Date______________

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DEDICATION

I dedicate this work to my father (Nathan Fitzjoji Magomere) and my mother (Athibeta Mwenesi Magomere) for their strong belief in education as the root of life and the endless and tireless effort that they dedicate towards educating the youth.

EPIGRAPH

There is therefore no doubt that for the whole of the characters involved in the experiments the principle applies that the offspring of the hybrids in which several essentially different characters are combined exhibit the terms of a series of combinations, in which the developmental series for each pair of differentiating characters are united. It is demonstrated at the same time that the relation of each pair of different characters in hybrid union is independent of the other differences in the two original parental stocks.

Gregor Mendel (1865)

Experiments in Plant Hybridization

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LIST OF ABBREVIATIONS

µl	Micro litres
µM	Micro molar
A, T, C, G.	Adenine, thymine, cytosine, guanine.
ABI	Applied Biosystems
AEZ	Agro-ecological Zones
ANOVA	Analysis of variance
BLAST	Basic logical alignment sequencing tool
BLO	Bacterial like organism
Bp	Basepair
DDBJ	DNA databank of Japan
ddNTP	Dideoxyribonucleoside triphosphate.
DNA	Deoxy ribonucleic acid
dNTP	Deoxyribonucleoside triphosphate.
EDTA	Ethylene diamine tetra acetate
EMBL	European Molecular Biology Laboratory Nucleotide Sequence Data Library
et al	and others
g	Grams
HLB	Huanglongbing
Kb	Kilobase
Kg	Kilogram
LM3	Lower midland 3
LM4	Lower midland 4
LM5	Lower Midland 5
M	Molar
mAB	Monoclonal antibody
ml	Mililitre
mM	Milimolar
NCBI Gen Bank	National center for biotechnology information gene bank
ng	nanograms
PCR	Polymerase chain reaction
RCF	Relative centrifugal force.
rDNA	Ribosomal deoxy ribonucleic acid
RPM	Rotations per minute
SDS	Sodium dodecyl sulphate
s.e.	Standard error
Taq	*Thermus aquaticus*
TBE	Tris borate buffer
TE	Tris EDTA
TEMED	Tetra methyl ethylene diamine
Tm	Melting temperature
UM3	Upper midland 3
UM4	Upper midland 4

ABSTRACT

The current decline in citrus production in the country is attributed to a great extent to Huanglongbing (HLB) (greening) disease, caused by a phloem limited Liberobacter species belonging to the alpha sub-division of the class *Proteobacteria*. A study was undertaken to characterize bacteria, which cause the HLB disease in citrus plants in Machakos and Kakamega districts lower highlands (LH, 1800-1900mASL); upper midlands (UM, 1390-1475mASL), lower midlands (LM, 1000-1390m) and to determine the distribution of the HLB disease across agroecological zones (AEZs) and varieties in the two districts. In the surveys farms and citrus trees were randomly sampled, scored and bacterial DNA isolated from the leaf samples. PCR was done to amplify specific ribosomal regions on the bacterial genome and these regions were sequenced and systematically analysed for homology.

There were significant (P<0.05) differences in the distribution of the HLB disease among the four citrus varieties in Machakos district. The washington navel oranges had the highest incidence with a mean visual score (MVS) of 2.62 and a mean PCR score (MPS) of 0.81. There were significant (P<0.05) differences in the distribution of HLB disease among the agro-ecological zones of Machakos district. The UM4 AEZ had the highest incidence (MVS of 2.33, MPS of 0.67). In Kakamega the MVS showed significant (P<0.05) differences while the MPS did not in the distribution of the HLB disease among citrus varieties. The distribution of HLB disease among the agro-ecological zones of Kakamega district was significant (P<0.05) based on the MVS and not significant (P<0.05) based on MPS. MPS and the MVS were highly correlated in Machakos and Kakamega (r^2 of 0.703 and 0.661 respectively). The distribution of the psyllid (*Trioza erytreae*) across AEZs and citrus varieties showed significant (P<0.05) differences in Machakos district and the differences were insignificant (P<0.05) in Kakamega district. LH4 had the highest psyllid infestation with a score of 0.833 while LM5 had the least psyllid infestation score (0.167).

The PCR reactions with 16SrDNA, 16S/23SrDNA and L10/L12 ribosomal protein rDNA primers produced 1100bp, 800bp and 716-720bp DNA fragments respectively from diseased plants. The L10/L12 ribosomal protein rDNA consisted of; 536 bp L10 protein DNA; 44 bp of DNA intergenic region and 136 basepairs of DNA that partially encodes the L12 protein. Sequences of rpL10/L12 protein genes from Kenyan strains were 99% and 83% similar to the South African "*Candidatus* L. africanus Nelspruit" and the Asian "*Candidatus* L. asiaticus" strains, respectively. The intergenic rDNA sequence of Kenyan strain from UM and LM showed 84% similarity with "*Candidatus* L. africanus Nelspruit" and 50% similarity with "*Candidatus* L. asiaticus" strain. However, the LH strain from Kakamega had an 11- basepairs deletion, while the LM4 from Machakos had a 5-basepair deletion in the intergenic region compared to all other strains.

The strains of HLB bacteria in Machakos are different from those in Kakamega. They are close descendants of South African strains, suggesting the origin of the disease. PCR primers can be developed from the unique intergenic regions for early indexing for different strains of the bacteria. This technique can be adopted by the Kenya Plant Health Inspectorate Service to curb the cross border movement of the HLB diseased citrus. In addition, the tolerance exhibited by the rough lemon can be harnessed and introduced to high yielding citrus varieties. Finally, control of the HLB disease and the psyllid vector should be emphasized in agroecological zones that lie above 1400mASL.

CHAPTER 1

1.0 INTRODUCTION

1.1 Background

The important citrus species and related genera are primarily evergreen species of sub-tropical and tropical origins belonging to the order Geraniales and family Rutaceae (Swingle and Reece, 1967). Sweet orange (*Citrus sinensis*) is the most important cultivated species of citrus worldwide and in Kenya, followed by limes (*Citrus aurantifolia*), grapefruit (*Citrus paradisi*), lemons (*Citrus Limon*) and tangerines (*Citrus tangarina*) (Anon, 1982; Button and Kochba, 1977; Ziegler and Herbert, 1961).

1.2 Importance of citrus

The citrus fruit, as opposed to many other fruits has both nutritive and medicinal value. Fresh citrus fruits are rich in ascorbic acid (35-36µg/100ml), thiamine (60-145µg/100ml), riboflavin (11-90µg/100ml), vitamin B-6 (25-80µg/100ml), niacin (200-330µg/100ml), folacin (120-330µg/100ml), pantothenic acid (130-210µg/100ml), potassium (300mg/178ml), iron, magnesium, zinc and calcium (Araujo, 1977; Ting, 1980). In addition, citrus contains a host of 170 active phytochemicals that also protect our health. Fresh fruit consumption provides a protective effect on cancer development (Block *et al.,* 1992). Citrus has more than 60 flavonoids, which possess anti-inflammatory and anti-tumor antioxidant properties, inhibition of blood clots and strong antioxidant activity (Attaway, 1994). The flavonoids, tangeretin, nobiletin and limonoids are known to be potent inhibitors of tumor cell growth and can activate the detoxifying P-450 enzyme system (Lam *et al*., 1994). The increased intake of antioxidant vitamins such as vitamin E and C reduces morbidity and mortality from coronary artery disease. These antioxidants in oranges limit vascular disease and stroke (Diaz *et al*., 1997).

Most Citrus fruits produced globally are consumed as either fresh fruit or processed to provide fruit juice, canned fruit for salads, preserved fruit (pickles), pulp for soft drinks or confectionaries, essential oils for flavourings and the peel is used for cattle feed, mollases or it is processed to pectins and flavonoids (United Nations, 1969). The fresh fruit is a necessary supplement for various essentials and non-essential vitamins and minerals.

In Kenya, fresh fruit is either consumed locally or exported. Export of fresh fruits to Europe and Djibouti used to be a lucrative source of foreign exchange (Anon, 1982). In addition, the citrus industry provides direct or indirect employment in both rural and urban centres. In the rural areas employment is created in the form of farm labour, while in the urban centres, citrus related employment activity is present in processing industries and fresh fruit marketing. Unfortunately, citrus demand in the country far outweighs the current production that is below 25% of the production potential. In 1990 citrus was planted on 18280 hectares of land with a total production of 137100 tonnes (Ministry of Agriculture, 1990). Yields are below 25% of the production potential (Waithaka, 1991; Obukosia and Waithaka, 2000).

Huanglongbing (HLB) disease (greening disease of citrus) has caused the greatest harm to citrus production in the tropics (Anon, 1982; Garnier *et al.,* 1984; Da Graca, 1991; Garnier *et al.,* 2000). This disease together with other diseases, insect pests, poor orchard management and lack of adequate planting material (Obukosia *et al.,* 1999b) have led to the current situation where, more than 30% of the citrus planted in Kenya is non-productive. The disease has affected most of the orchards in the highlands and in regions above 700 m above sea level. Huanglongbing disease has destroyed most of the citrus orchards in Kakamega district (Obukosia *et al.,* 1999a). Though, it is difficult to obtain accurate estimates of yield losses due

to HLB disease in a perennial crop such as citrus, infected branches produce fruits of little or no marketable value (Obukosia *et al.,* 2000). The HLB disease is caused by an obligate, rod-shaped, gram negative, phloem-restricted bacterium of the alpha sub-division of proteobacteria (Garnier *et al.,* 1984; Jagoueix *et al.,* 1994). However, there are no reports on the strains and species of the bacteria that cause the disease in Kenya. The bacteria that cause the HLB disease in Asia and Africa are characterized into two major species, "*Candidatus* L. asiaticus" and "*Candidatus* L. africanus" respectively (Jagoueix *et al.,* 1996). The diversity of the HLB bacteria strains present in Kenya could be wider than the five strains identified elsewhere, which include Poona (India), Nelspruit (South Africa), Fugian (China), Lipacity (Philippines) and Nakhon Pathom (Taiwan) (Jagoueix *et al.,* 1994). In Africa, apart from the strains characterized in South Africa namely Nelspruit (Jagoueix *et al.,* 1994) and subspecies Capensis (Garnier *et al.,* 2000) no other strains have been reported. The immense impact of the disease has necessitated research to find methods for alleviating or necessarily eradicating the disease, but with scanty information on the causative agent progress on the control of the disease is severely hampered.

Information on the current geographical distribution of the HLB disease in the country is insufficient. The two species of the HLB disease are distributed according to their temperature requirements (Garnier *et al.,* 1987), such that "*Candidatus* Liberobacter africanus" from South Africa is found to be heat sensitive and the disease symptoms do not develop in Asian hot climates where temperatures above 30°C are reached for several hours a day. The second form "*Candidatus* L. asiaticus" from Asia is not found in South Africa. A report on the survey of citrus diseases in the country shows that Huanglongbing disease is more prevalent in highlands in Kenya and the distribution of the affected orchards is within the high altitudes, which are

favourable for the proliferation of the Liberobacter species (Anon, 1982). The report shows that the affected orchards are predominantly in areas where the psyllid vector thrives well (Anon, 1982).

The geographical distribution of the greening bacterium and the disease on citrus varieties in Kenyan agroecological zones is not known. Available reports on citrus varietal susceptibility to the HLB disease seem to vary in different countries (Oberholzer *et al.,* 1965; Miyakawa, 1980; Gonzales *et al.,* 1972; Fraser, 1978), though all reports concur on the idea that, some of the citrus varieties have some inherent tolerance to the greening bacterium (Manicom *et al.,* 1990; Da Graca, 1991). Nevertheless, there exists no report showing citrus varieties with resistance to the HLB bacteria. Similarly, there is no report on the susceptibility of the different varieties in Kenya to the HLB disease. This condition stalls the use of citrus breeding and the use of genetic interventions to remedy the HLB disease situation in the country through incorporation of such tolerance or resistance genes into commercially important citrus varieties.

1.3 Research objectives

The objectives of this study were;

1. To determine the geographic distribution of the Huanglongbing disease in Machakos and Kakamega districts of Kenya.
2. To determine the distribution and severity of the Huanglongbing disease on important citrus varieties in Machakos and Kakamega districts of Kenya.
3. To characterize bacteria which cause the Huanglongbing disease in citrus plants in Machakos and Kakamega districts of Kenya.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 The citrus crop

2.1.1 History and Taxonomy

The term citrus originated from the Latin form of 'Kedros' a Greek word denoting trees like cedar, pine and cypress. The smell of citrus leaves and fruits was reminiscent of that of cedar, thus the word "citrus" was increasingly used to define all members of the citrus genus. Linnaeus grouped all citrus species known to him in the citrus genus (Spiegel-Roy *et al*., 1996). In Greek mythology citrus fruits were referred to as "The Golden Citron from the Garden of Hesperides". The fruit was a gift from Tellus (Greek goddess of the earth).

Citrus belongs to the family Rutaceae, which is one of the twelve families in the sub-order Geraniineae. It belongs to the order Geraniales. The species within Rutaceae generally have four important characteristics; presence of oil glands; raised ovary on a floral (nectary) disk; pellucid dots present in the leaves and axil placentation inherent in the fruits (Swingle and Reece, 1967). Most true citrus belong to the sub-family Aurantioideae. Nevertheless edible and non-edible citrus varieties constitute a complex and closely related cluster of genera that hybridize readily, giving it no clear reproductive separation of species (Swingle and Reece, 1967). Classification of the sub-genus Citrus has proven particularly difficult due to several factors including comparative ease of hybridization, nucellar polyembryony, presence of numerous cultivars, hybrids and spontaneous mutants. Nevertheless, Swingle (1943) characterized the sub-genus citrus by pulp vesicles nearly free from oil droplets and never containing acrid oil; the petioles having narrow wings or remaining wingless, presence of large fragrant flowers (2.5-4.5 cm in diameter), with stamens clustering in bundles.

A common feature of polyembryony identifies most of the species in the Rutaceae family. Members of this family can produce both zygotic seedlings and several vigorous nucellar seedlings, which are somatic and thus genetically identical to the mother plant (Davies and Albrigo, 1998). Cultivated citrus varieties belong to the sub-family Aurantioideae in which all species are trees or shrubs with evergreen leaves. They have white and fragrant flowers. Most of the genera in this family have fruits with a green yellow or orange peel dotted with numerous oil glands. These fruits have well defined pulp vesicles into delicious juicy tissue, making it the most delicious fruit known to man (Swingle and Reece, 1967). Though such common characteristics are used to describe citrus fruits, it is worthwhile to note that there are several varieties, which differ in fruit size, fruit shape, size of peel (albedo and flavedo), fruit colour and fruit taste. The nutritive compositions of different citrus also differ among varieties (Ting, 1980). The wide variability inherent in the sub-family Aurantioideae, allows it members to grow in a wide range of climatic and environmental conditions, from the tropical to the temperate regions (Ziegler and Herbert, 1961).

2.1.2 Geographical Distribution

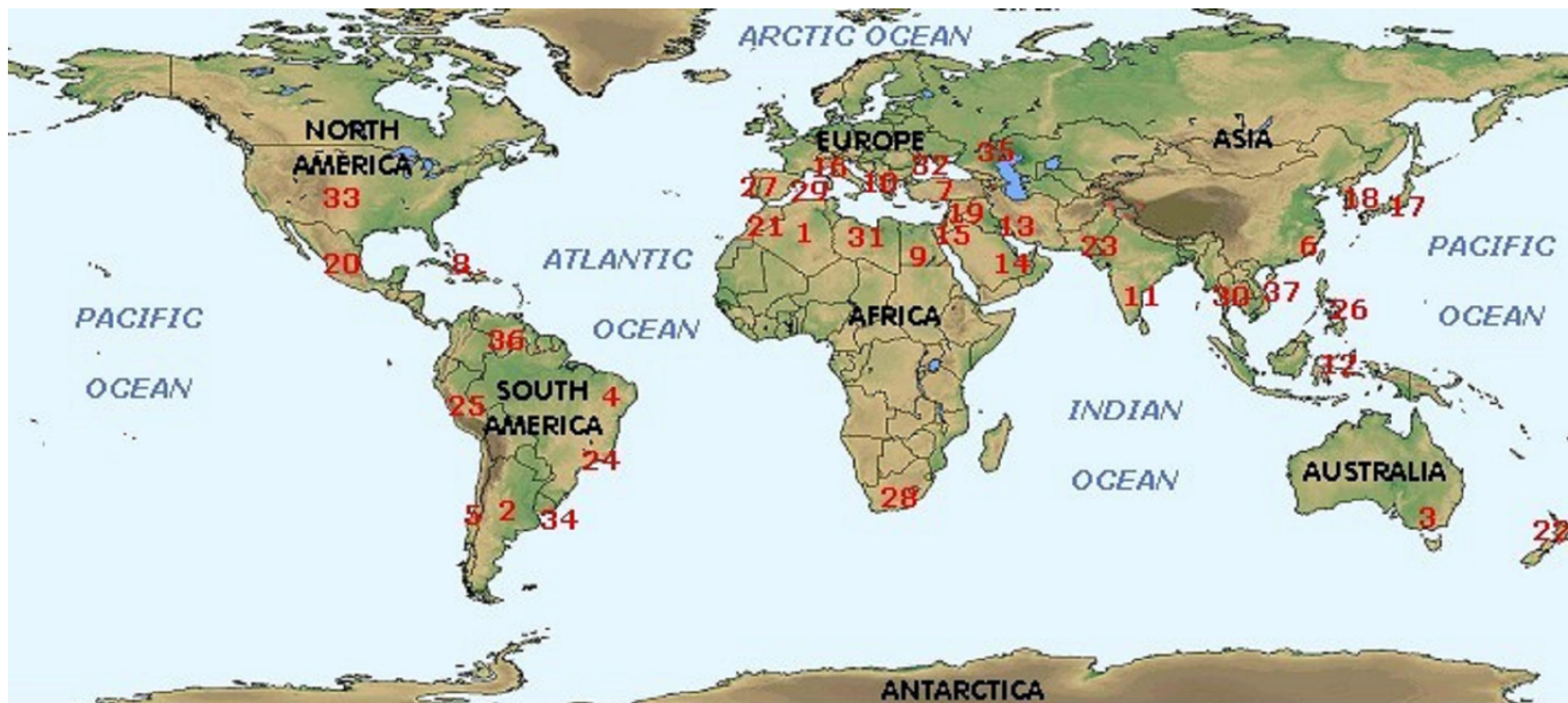
The general area of origin of Citrus is believed to be South Eastern Asia, including the region that covers Eastern Arabia (Figure 2.1). Different varieties have shown characteristics that suggest different areas of origin. The sweet orange is believed to be from Southern China and possibly spread as far as Indonesia (Reuther *et al.,* 1967). Chinese fishermen took citrus fruit with them as food and distributed it both northerly and southerly directions. With improved navigation and sailing equipment, trading with neighbouring countries expanded in all directions. Eventually their ships went as far south as India. The grape fruits appear to have

originated as a mutation or hybrid of shaddock in the West Indies (Davies and Albrigo, 1998), while the lemons origin remains unknown. The lemon is possibly a hybrid between citron and lime creating an intermediate species (Chapot, 1975; Barret and Rhodes, 1976). With the spread of agriculture these varieties have spread widely to their current distribution.

2.1.3 Citrus Production

Total world citrus production in 1990 – 1991 was 67.9 million tonnes, out of which 72.9% were oranges, 12.2% were tangerines (mandarins), 9% were lemons and 5% were grape fruits and pummelo. The northern hemisphere produced 47.6 million tonnes, while the southern hemisphere produced a total of 20.3 million tonnes. Brazil and the United States were the highest producers with 14.3 million tonnes and 9.99 million tonnes respectively (FAO, 1991). Globally citrus is produced in regions of varied climatic conditions, ranging from latitude over 40° north (Japan) to almost 40° south (New Zealand); from equatorial, hot-humid climates through warm –subtropical and even cooler maritime climates (Figure 2.1).

In Kenya about 16400 hectares of land is under citrus cultivation with an annual output of 170,000 tonnes valued at US$1.1 million. Citrus is cultivated in Coast, Nyanza, Western, Rift Valley, Eastern, North Eastern and Nairobi Provinces. Citrus yields range from 4 – 10 tonnes per hectare, while the potential is up to 80 tonnes per hectare (Waithaka*,* 1991). Eastern and Coast provinces are the most important regions for citrus production in Kenya. Within Eastern province, Machakos district has the highest citrus production. Citri-culture is practised on both large and small scale in this district, the products of which are marketed both internationally and locally. In Eastern province citrus is the most important fruit with an output of 37,744 tonnes followed by bananas and mangoes (Ministry of Agriculture, 1995).



1. Algeria	7. Cyprus	13. Iran	19. Lebanon	25. Peru	31. Tunisia	37. Vietnam
2. Argentina	8. Cuba	14. Iraq	20. Mexico	26. Philippines	32. Turkey	
3. Australia	9. Egypt	15. Isreal	21. Morocco	27. Portugal	33. United States	
4. Brazil	10. Greece	16. Italy	22. NewZealand	28. SouthAfrica	34. Uruguay	
5. Chile	11. India	17. Japan	23. Pakistan	29. Spain	35. Former USSR	
6. China	12. Indonesia	18. Korea	24. Paraguay	30. Thailand	36. Venezuela	

Figure 2.1. Major citrus producers globally showing citrus production from equatorial, hot-humid climates through warm – subtropical and even cooler maritime climates

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2.1.4 Constrains in citrus production

The major constraints in citrus production are diseases, insect pests, poor orchard management and lack of adequate planting material (Obukosia *et al.,* 1999, Obukosia *et al.,* 2002). These constraints have led to the current situation where, more than 30% of the citrus planted in Kenya is non-productive (Anon, 1982). Diseases impede citrus production in Kenya. Important viral diseases in Kenya include tristeza viral disease transmitted by the aphid *Toxoptera citricidus*, psorosis, veinenation, woody gull, and ring spot (Anon, 1982). Common fungal diseases include, phytophthora gummosis, armillaria root rot, heart rot and scab (Whiteside *et al.,* 1988), while the bacterial diseases include citrus canker caused by *Xanthomonas campestris* (Civerolo, 1981), citrus variegated chlorosis caused by xylem limited bacteria *Xylella fastidiosa* (Lee *et al.,* 1991) and Huanglongbing disease caused by "*Candidatus* Liberobacter" species (Jagoueix *et al.,* 1994). These diseases are found in different areas of the country with some being more prevalent in the lower altitudes while others in high altitudes.

Pests of citrus trees include mites, insects and nematodes (Talhouk, 1975; Smith *et al.,* 1997). Mites damage citrus fruits and leaves by their feeding and sucking mouthparts. The mites include, citrus rust mite (*Phyllocoptruta oleivora* Ashm) broad mites (*Hemitarsonemus latus* Banks) and spider mites (*Panonuchus citri* McGregor) (Davies and Albrigo, 1998). The insect category is the largest with the most important insects belonging to 17 families. The most important include false codling moth, orange dog, leaf miners (Lepidoptera), aphids (Aphididae), scales, white flies and psyllids (Homoptera). These insects reduce citrus production and diminish the quality of the fruit harvested by transmitting diseases and leaving

fruits with bruises, lesions and ugly scars, which diminish their quality and thus lower the market prices (Smith *et al.,* 1997).

Poor orchard management has contributed greatly to the decline in citrus production. Farmers fail in spraying, fertilizer application, judicious irrigation, proper spacing, and sanitation of orchards by destroying diseased material. This exacerbates the poor health of citrus plants in orchards (Obukosia *et al.,* 1999). Lack of adequate planting materials is a major problem in all citrus growing areas in the country. Farmers obtain poor quality seedlings from local nurserymen and nurserywomen, most of which are diseased and of inappropriate varieties for their local climatic conditions. Planting and maintaining these seedlings becomes expensive and costly. At maturity these seedlings yield lower than their expected potential (Obukosia *et al.,* 1999).

2.2 Huanglongbing disease

2.2.1 Brief History & Distribution

The Huanglongbing (HLB) disease formerly known as citrus greening disease is a severe and a widespread disease of citrus in Asia and Africa. It was first reported in China towards the end of the 19th century, and its effects in citrus production were not felt until 1925 in the same country (Anon, 1982). The effect of the disease is felt more in three large citrus growing areas of the world, (i) the Indian subcontinent, Southeast Asia and China, (ii) South and East Africa and, (iii) the Arabic Peninsula (Figure 2.2). The disease is a major limiting factor for citri-culture in these regions. It also poses a serious threat for South and Central America (Villechanoux et *al.,* 1992; Coelho *et al.,* 2002).

In Africa HLB was first reported in South Africa in 1929 (Moll *et al.,* 1978) and its severity has been reported in Swaziland, Zimbabwe, Ethiopia, Sudan, Kenya, Malagasy and Tanzania (Anon, 1982). The presence of the disease in Kenya was suspected as early as 1982 (Anon 1982) however, the failure to demonstrate transmission or signs of the disease by conventional serological methods, led to the conviction that greening symptoms were manifestations of nutritional deficiencies. The disease was first diagnosed and reported in 1975 by a FAO consultant based on four proofs. First, the symptoms exhibited matched those of the HLB disease elsewhere in the world. Secondly, the distribution of affected orchards was within a temperature range favourable for African bacterial strains. Thirdly the affected orchards were found predominantly in areas where the psyllid vector *T. erytreae* thrived well and lastly, it was reported that the first grafted mother trees in Kenya originated from South Africa where the HLB disease was already documented and its effects were severe (Schwarz, 1975). Geographically, in Kenya the disease was reported in Thika, Kamiti, and Kitale. All the three areas are at a high altitude that provides optimal conditions for the vectors to multiply (Seif and Whittle, 1984). The disease is found in all citrus growing areas in the highlands (Muriuki, 1989), thereby culminating in a situation where the estimates of losses are about 25% but in some areas citrus culture has collapsed (Seif, 1991).

2.2.2 Transmission

Bacteria that causes HLB is transmitted from one plant to another through two major channels. The first is the use of infected bud wood (Chen, 1943). Chen (1943) suggested that on the basis of graft inoculations yellow shoot (HLB) might be a viral disease. This assertion was held for sometime when McClean and Oberholzer (1965) confirmed that HLB was indeed graft transmissible. Transmission has been reported irrespective of whether the scion is infected and

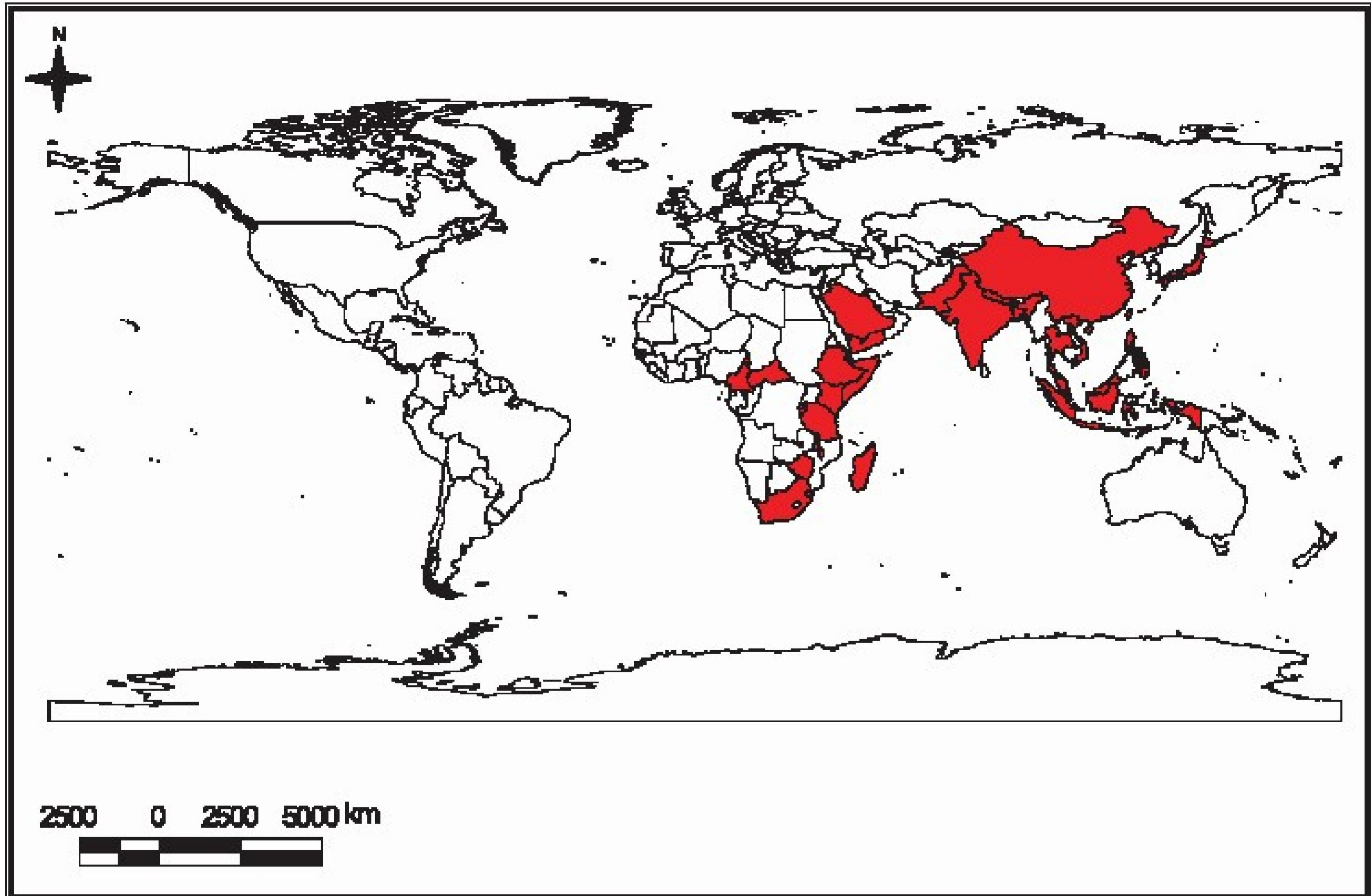


Figure 2.2. Geographic distribution of the citrus greening disease (Courtesy; Coelho and Marques, 2002)

the rootstock is not prior to budding or vice versa. No infection is obtained when budding material from apparently healthy sectors of diseased trees are used (Schwartz, 1970). In 1964 it was reported that undiseased seedlings exposed to adult of psylla species *Trioza erytreae* in a greening infected orchard developed disease symptoms and these symptoms continued to spread in the orchard if the insects were not controlled (Schwartz, 1970). Schwarz later showed a positive correlation between the degree of greening infection, the number of psylla, and the rate of transmission. *Trioza erytreae* (Del Guercio) (Homoptera: psyllidae) is a confirmed principle vector of the greening disease in Africa (McClean, 1974). The phloem feeding adult psyllid ingests the organisms, which penetrates the gut during the latent period of about 21 days and proliferate in the insect haemolymph. The organisms can be injected with saliva into the next plant during feeding. *T. erytreae* can acquire the inoculum within five days of feeding and transmit the disease in less than 60 minutes of feeding (van Vuuren and daGraqa, 1977). Salibe and Cortez (1966) demonstrated that an insect vector found to transmit the HLB disease in India was another Psylla, *Diaphorina Citri* (kuwayama). This psylla can transmit the pathogen to an uninfected plant in 15–30 minutes with a latent period of 8–12 days (Salibe and Cortez, 1966). Both psylla are strongly attracted by yellow green light of wavelength 550nm, making diseased trees attractive targets and thereby increasing the proportion of disease-carrying insects (Samways, 1987).

T. erytreae is sensitive to heat and temperatures of 32ºC kill all stages of the insect including nymphs while 27ºC allows rapid development of the insect (Moran and Blowers, 1967; Catling, 1969a; Catling, 1973). The insect prefers cool, moist upland regions. Their eggs and the first instars are particularly vulnerable to heat (Catling, 1969b). *Diaphorina Citri* is best suited in Asia since it is more resistant to extreme temperatures and is more sensitive to high rainfall and

humidity (Catling, 1973; Aubert, 1987; Regmi and Lama, 1988; Xia *et al.,* 1987). There does not appear to be any specificity between the psylla species and the greening type. The African HLB forms have been shown to be transmitted by both psylla in Reunion Islands (Catling, 1973). However, Indian greening has been experimentally transmitted by *T. erytreae* while the African greening by *D. Citri* (Massonie *et al.,* 1976).

2.2.3 Etiology

After confirming that HLB was graft and insect transmissible most scientists believed that a virus was responsible. McClean *et al.* (1965) in South Africa showed that tristeza and greening could readily be distinguished since the aphid *Toxoptera citricidus* transmitted tristeza but not greening and the psylla transmitted greening and not tristeza.

Lafle'che and Bové (1970) observed mycoplasma-like-organisms (MLOs) in infected citrus phloem tissue. The organisms measured 100 – 200 nm in diameter. These organisms were also shown to be present in the haemolymph and salivary gland of infected *T. erytreae* and *D. citri.* Through electron microscopy (Garnier *et al.*, 1984) showed that the organism that causes greening was not a mycoplasma-like-organism (MLO) since the envelope that surrounded the organism comprised of three zones. A dark inner zone, a dark outer zone and an intermediate electron-transparent zone. The thickness of the three zones was approximately 250 Aº. Each of the two dark zones could be resolved into a triple-layered unit membrane 90 – 100 Aº thick. The inner membrane appeared as the cytoplasmic membrane and outer membrane as a cell wall. Whereas MLOs are surrounded by a true unit membrane 10nm thick, the HLB organism has an outer envelope of 20nm thick. Treatment of the greening affected plants with penicillin

resulted in the remission of symptoms, suggesting the presence of a peptidoglycan (PG) layer in the envelope of the organism (Garnier *et al.*, 1984).

Electron microscopy combined with cytochemistry revealed that the HLB organism is surrounded by a peptidoglycan conforming to the membranous cell wall of the gram-negative bacterial type. This renders the organism susceptible to penicillin, which inhibits a late step in peptidoglycan synthesis. Due to the thickness of the envelope of the organism and the effect of penicillin the HLB causing organism was thought to be a bacteria and not a mycoplasmal organism (Garnier *et al.*, 1984).

In further cytochemical experiments the envelope of the HLB organism was shown to be different from the envelope of *S. aurens* (gram-positive) but similar to the *E. coli* (gram-negative) envelop, thus it was deduced that it is a true bacterium of gram-negative type (Garnier *et al.*, 1984). Jagoueix *et al.* (1994) showed that the organism was in fact a phloem limited bacterium that is a member of the α sub-division of the proteobacteria. This conclusion was reached at after amplification of the 16S rDNA of the Asian and African strains of the HLB organism, and sequencing of the product. The sequence was then compared with 16S rDNA sequences from the Gene Bank database. Even though the closest relative to the HLB organism are members of the alpha-2 subgroup, these bacteria are distinct from this subgroup as there is only 87.5% homology between the 16S rDNA of the HLB organism and those of their relatives. Therefore Jagoueix *et al.* (1994) postulates that the bacteria studied are members of a new lineage in the α sub-division of the proteobacteria.

Jagoueix *et al.* (1996) further characterized the HLB organism from the sequence of its 16S/23S ribosomal intergenic region DNA. They characterised it into a new "*Candidatus*" genus, Liberobacter, in the alpha sub-division of proteobacteria. They further recognised two "*Candidatus* L. asiaticus" and "*Candidatus* L. africanus" by amplification of a 1,160 bp fragment of the 16S rDNA of the HLB organism and digestion with x*bal* restriction enzyme.

2.2.4 Host Range

Huanglongbing disease is primarily a disease of *Citrus sinensis* Osbeck (sweet orange). This includes valencia orange and washington navel orange among other navels. It also infects tangerines (*C. tangarina*) and rough lemon (*C. jambhiri*). The disease also infects sour orange (*C. aurantium* L.) and grape fruit (*C. paradisi* Macf). In India the rough lemon, sweet lime (*C. limettoides*) and pomelo (*C. grandis* Osbeck) are fairly tolerant (Da Graca, 1991).

Lemons are found to serve as a very efficient reservoir of the disease, since its frequent flushes of yellowish new growth render them very attractive to the insect vectors, but rough lemon is found to be fairly tolerant to the disease (Miyakawa, 1980; Gonzales *et al.,* 1972; Fraser, 1978). In some cases the rough lemon rootstock has been found to induce some degree of tolerance in the sweet orange scion in greenhouse trials (Kapur *et al.,* 1984). The susceptibility of common citrus cultivars to the HLB ranges from; severe (in sweet orange, tangelo, mandarin), moderate (in grape fruit, lemon, sour orange) and tolerant (in lime, pomelo and trifoliate orange) (Manicom *et al.,* 1990). The HLB bacterium has been experimentally transmitted to many citrus species including the trifoliate orange and other rutaceous plants. The disease has also been experimentally transmitted by dodder to periwinkle (*Catharanthus roseas*) in which it induced marked yellowing (Ke *et al.,* 1988).

2.3 Methods of detecting and Characterizing HLB

2.3.1 Conventional methods of detection and characterization

There are eight methods reported for conventional detection of the HLB disease. These include; Use of morphological symptoms (Martinez, 1972), bacterial isolation on nutrient media (Garnett, 1984), indicator seedlings (Matsumoto *et al.,* 1968), biochemical inference (Schwarz, 1968), light microscopy (Wu, 1987), electron microscopy (Anon, 1982), immuno electron microscopy (Ariovich *et al.,* 1988) and cytochemistry (Garnier *et al.,* 1976; Garnier *et al.,* 1984). Though the Liberobacter species exhibit strong resistance to culture on artificial nutrient media (Manicom, 1984), making it impossible to carry out Koch's postulates, considerable work has been done on the use of conventional methods for detection of the bacteria.

2.3.2 Use of morphological symptoms

Huanglongbing disease is associated with peculiar primary and secondary symptoms on leaves, fruits and roots (Da Graca, 1991). Primary symptoms are characterized by yellowing of normal sized leaves along the veins (interveinal chlorosis) and sometimes by the development of a blotchy-mottle. Secondary symptoms depict leaves that are upright, small and with a variety of chlorotic patterns resembling those induced by zinc and iron deficiencies (Figure 2.3). Analysis of affected leaves shows higher potassium content and lower calcium, magnesium, and zinc levels (Aubert, 1979). The infected fruits are small, lopsided, and have a bitter taste, which could be as a result of higher acidity and lower sugars (Kapur *et al.,* 1984). Many of the fruits fall prematurely, while those that remain on the tree do not colour properly, remaining green on the shaded side (Figure 2.3). The seeds in such severely infected plants are often abortive. Fruit yellowing occurs from the peduncular end rather than the astylar end, which is the reverse colouring of normal healthy fruits.

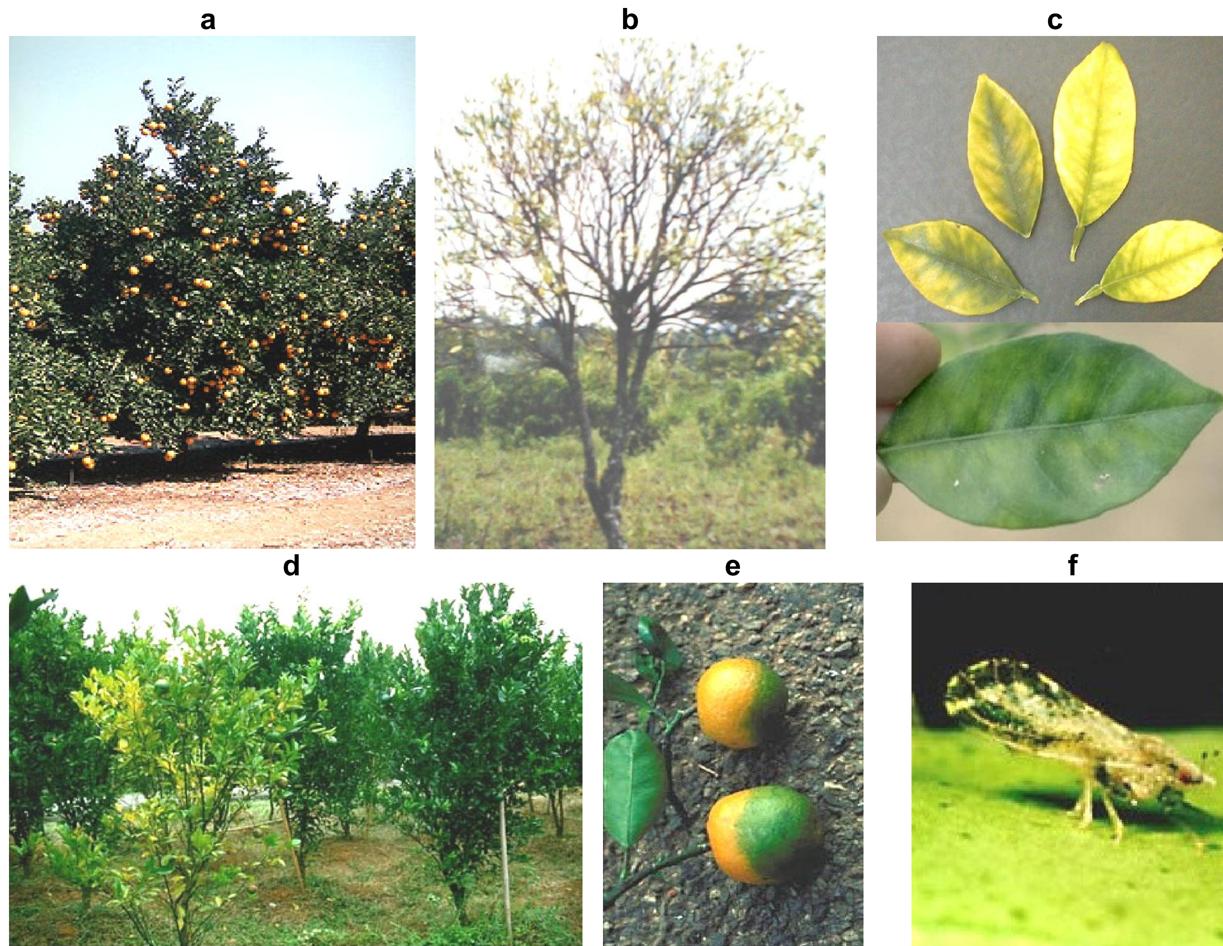


Figure 2.3: Huanglongbing disease symptoms on various parts of citrus trees
(a) Healthy fruiting sweet orange in orchard
(b) Secondary symptoms: upright, small leaves
(c) Leaf symptoms showing blotchy mottles and interveinal chlorosis
(d) Diseased citrus among healthy plants (Courtesy; Coelho. and Marques, 2002)
(e) Fruit symptoms showing irregular yellowing Courtesy; Coelho and Marques, 2002)
(f) Psyllid vector feeding on citrus. (Courtesy; Coelho and Marques, 2002)

Root systems are poorly developed with relatively few fibrous roots, possibly because of root starvation, new root growth is often suppressed and the roots often start decaying from the rootlets (Zhao, 1981). Cytopathic studies have revealed pockets of necrotic phloem, excessive phloem formation, abnormal cambial activity and accumulation of starch in plastids. Cytoplasmic membranes in infected plants form invaginations of the plasmalemma; aberrations of the chloroplast thylakoids and mitochondrial collapse (Wu and Faan, 1988). Symptoms manifest throughout the tree, especially if the infection occurs at or soon after propagation (Mc Clean, 1970). If the infection occurs latter the symptoms and the casual organisms are partially confined leading to sectoral infections. Infected trees or branches suffer heavy leaf drop followed by out-of season flushing and blossoming, with die back (Martinez, 1972).

2.3.3 Bacterial isolation on nutrient media

Liberobacter species are still not culturable on artificial nutrient media (Manicom, 1984). However there are reports on the isolation of micro-organisms that were thought to cause the greening disease (Ghosh *et al.,* 1971; Ghosh *et al.,* 1975; Nariani *et al.,* 1975). These studies isolated micro-organisms from Indian greening infected citrus. All showed characteristics of true microplasma like organisms and were probably contaminants. Though the microplasmas were re-infected in citrus and caused foliar symptoms similar to those of HLB disease, the Koch's postulates were not conclusive since several organisms are known to cause transient leaf blotches (Manicom, 1984). Thus initial attempts to culture the bacteria were in vain.

A long rod-shaped gram-negative bacterium was isolated from African greening infected citrus midribs (Garnett, 1984). The bacteria formed small round colonies with predominantly long

rod-shaped cells near its edges. Antibodies raised against this isolate gave positive ELISA results only with greening infected plant material. Garnett (1984), recovered more isolates from greening infected samples from China, Taiwan, Reunion, South Africa, India and Philippines for comparative studies. The isolates shared common serological and protein profiles. They also showed high percentage DNA homology, but completion of Koch's postulates was not reported. Manicom (1984) in an attempt to confirm Garnett's (1984) work isolated several species but none resembled the Liberobacter species. Further, Garnier *et al.* (1987) found that antibodies against the organisms cultured by Garnett (1984) failed to react with greening infected tissue. This showed that the isolated organisms were not causal agent for HLB disease.

2.3.4 Indicator seedlings

Indicator seedlings are the earliest methods used to detect the presence of greening disease in citrus. In this procedure a variety of citrus is selected that gives uniform symptoms irrespective of the scion used. Graft sticks or buds are grafted on to the indicator stems using about 20 seedlings per test. Different varieties have been used as indicator seedlings in different countries e.g. in South Africa valencia sweet orange or orlando tangelo are used (Schwarz, 1968), in Taiwan ponkan mandarin is preferred (Matsumoto *et al.,* 1968) while in India Mosambi sweet organge or Dargeeling orange are used (Ahlawat *et al.,* 1988). The limitation to this technique is that symptoms appear in 3-4 months in a greenhouse at 21-23^0 C.

2.3.5 Biochemical inference

Use of biochemical inference in detection of the HLB disease is quicker and less cumbersome than conventional and morphological markers such as morphological markers and indicator

seedlings. It is based on the biochemical changes that occur in an infected plant. Schwartz (1968), reported that a fluorescent substance later identified as gentisoyl-β-glucoside was detected in ether extracts of greening infected bark and albedo, but not in healthy ones. When this substance was separated by paper or thin layer chromatography using chloroform-methanol, a bright violet-blue spot fluoresced under ultra violet light (365nm). The test is not only specific for greening disease and the gentisoyl-β-glucoside concentrations are found to vary with seasons and plant part used (Da Graca, 1991). This test has been variously modified by using water extraction instead of ether and by employing acid hydrolysis to liberate gentisic acid, which produces a clear spot (Burger *et al.,* 1984).

2.3.6 Light Microscopy

Light microscopy diagnostic procedure for HLB disease has been developed (Wu, 1987). The procedure involves the examination of sections of infected leaves either by fluorescent microscopy or by staining sections with safarin. Fluorescent microscopy reveals a yellow florescence in infected phloem that is absent from healthy, virus infected and nutrient deficient tissues. Sections stained in safarin, shows red patches in infected phloem (Wu and Faan, 1987). However this technique was inconclusive since it did not show the structure of the HLB bacteria (Wu and Faan, 1987).

2.3.7 Electron Microscopy

Electron microscopy has been a strong tool in diagnosis and characterization of the HLB bacteria (Anon, 1982). This technique was used to view the HLB pathogen from ultra thin sections of leaf tissues obtained from citrus growing districts in Kenya. The presence of a prokaryotic organism resembling bacteria was found only in samples expressing primary and

secondary symptoms of the disease. The study also revealed that the HLB causing organisms had bacterial-like-shape which had slightly pleomorphic particles with definite cellular envelope (Anon, 1982). The EM studies further showed that the bacteria were more concentrated in the younger tissues than in the lower mature leaves showing mottling. The bacterial cells appeared slightly larger and more pleomorphic in older tissues. The bacterial cells were found to be 1000nm to 1200nm in length and 285nm to 430nm in diameter. They also had a cell wall of approximately 35nm confirming its bacterial nature (Anon, 1982). These cells were similar in morphology with those viewed from the heamolymph of psylla vectors (Moll *et al.,* 1973).

2.3.8 Immuno- electron microscopy

The bacterium exhibits pleomorphic properties therefore electron microscopy is not sufficient for detection and identification of the organism (Ariovich *et al.,* 1988). Gold labelled immunglobulin (IgG) complexes have been used successfully for detection of plant viruses in post-sectioning and immuno-staining at the ultra-structural level. In the procedure polyclonal antisera were raised against the bacteria isolated from greening-infected citrus. The IgG fractions were adsorbed onto collodial gold particles and used to detect the bacteria in electron microscopy. To specifically identify greening bacteria in infected material, antibodies against the cultured greening organism were raised in rabbits and gold-labelled IgG complexes were prepared for the detection of the greening bacteria in crude plant extracts. The gold-IgG probe was tested against three bacterial species: *Bacillus subtillis*, *Ewinia liquefasciens* and *Escherichia coli* but no labelling occurred. This technique required lengthy preparation procedures and some of the antigenicity was lost due to the fixation of the material,

nevertheless the technique was found to be most useful because the serological reaction could be visualized (Ariovich *et al.,* 1988).

2.3.9 Cytochemistry

Neither EM nor immunogold EM showed the divisions within the bacterial cell wall. However through cytochemistry Garnier *et al.* (1984) showed that the envelope surrounding the organisms comprises of three zones: a dark inner zone, a dark outer zone and intermediate electron-transparent zone. The thickness of the three zones was approximately $250A^0$. Each of the two dark zones could be resolved into a triple-layered unit membrane $90\text{-}100A^0$ thick (Garnier *et al.,* 1976; Garnier *et al.,* 1984). The inner membrane appeared as a cytoplasmic membrane while the outer as a cell wall. No peptidoglycan (PG) layer could be demonstrated between the inner and outer membranes of the HLB pathogen. However penicillin, which inhibits a late step in PG synthesis showed remission of symptoms when applied to diseased citrus. The thickness of the envelope and the effect of penicillin confirmed the greening organisms to be bacteria (Garnier *et al.,* 1984).

In attempt to confirm the presence of PG in the cell wall of the bacteria Garnier *et al.* (1984) used the protocols by De Petris (1967) to treat the bacterium with papain. After papain treatment the greening bacterium, like *E. coli* exhibited 3 membranes but *Staphylococcus aurens* a gram-positive bacteria showed two membranes. In addition, when both papain treated *E. coli* and the greening bacteria were submitted to lysozyme digestion the intermediate P.G. layer revealed after papain treatment was lost. This evidence suggested that the bacterium is of the gram-negative type (Garnier *et al.,* 1984).

2.4 Molecular methods of detection and characterization

There are five molecular methods currently used to detect and characterize the HLB bacteria. These include; monoclonal antibodies (Gao, 1988; Garnier *et al.,* 1987), radioactive DNA probes (Villechanoux *et al.,* 1992), non-radioactive DNA Probes (Hocquellet *et al.,* 1997), PCR methods and DNA sequencing (Leblond *et al.,* 1996; Jagouiex *et al.,* 1996; Jagoueix *et al.,* 1997;). Based on the use of these methods the greening organism has been classified as a rod shaped, phloem limited gram-negative bacteria of Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae. It has two species "*Candidatus* L. africanus" and "*Candidatus* L. asiaticus" (Jagoueix *et al.,* 1996). One subspecies has been published "*Candidatus* L. africanus" subspecies Capensis (Garnier *et al.,* 2000). The bacteria were given the "*Candidatus"* status following (Murray and Schleifer, 1994) who proposed the concept of a waiting position for putative taxa in a category called "*Candidatus*", which would have indefinite rank. This was established for certain putative taxa that could not be described in sufficient detail to warrant establishment of a novel taxon. Murray and Stackebrandt (1995) suggested the "*Candidatus* concept for bacteria whose genomic information such as sequences apt to determine the phylogenetic position of the organism, structural, metabolic, and reproductive features are described, together with the natural environment in which the organism can be identified by in situ hybridization or other similar techniques for cell identification. "*Candidatus"* concept is encouraged for well-characterized but as yet uncultured organisms (Murray and Stackebrandt, 1995). This section shall dwell on the enormous amount of scientific work that has been done to characterize and detect these bacteria by molecular methods.

2.4.1 Monoclonal Antibodies against the HLB bacteria.

Monoclonal antibodies (mAb) against the bacteria have been developed and used to detect the bacteria in citrus (Martin-Gros *et al.,* 1987; Gao, 1988; Garnier *et al.,* 1987). In the procedure Myeloma cells (X63653 Ag 8) were fused with spleen cells from a mouse immunised with a phloem homogenate of HLB bacteria infected periwinkles. In one fusion, 562 hybridomas were obtained and screened by differential ELISA. Immuno-fluorescence for the HLB-specific clones was performed. Two hybridomas, 10A6 and 2D12, gave a positive ELISA with vascular bundle homogenates from HLB bacteria infected periwinkle plants and a negative ELISA with bundle preparations from healthy plants (Garnier *et al.,* 1987). The two hybridomas showed specificity to the HLB bacteria from citrus or from infected periwinkle plants. The two were able to detect either the South African or the Asian form of the bacteria but none of the other organisms tested, including spiroplasmas (*S. citri*) or mycoplasma-like organism of apple proliferation, aster yellows, clover phyllody or tomato stolbur.

Garnier *et al.* (1987) tested the possibility of detecting the HLB bacteria in citrus by immunoflouresence with sweet orange seedlings infected with greening from different geographical origins. The two hybridomas gave positive fluorescence reaction with citrus infected with various forms of greening but not with healthy citrus or citrus infected with tristeza virus. Garnier *et al.* (1987) double cloned the hybridomas 10A6 and 2012 and found the immunoglobulins produced to belong to isotypes IgG1 and IgG2a respectively. The hybridomas were amplified *in vivo* and *in vitro* and thus were used in large-scale detection of citrus greening in citrus nurseries and orchards. The use of monoclonal antibodies in detecting greening had a limitation because the mAbs were strain specific. They reacted specifically with a given homologus bacterial strain e.g. mAbs against the Poona strain could not recognise other

strains from Africa, China etc. The greening bacteria have several serotypes, while the available mAbs were too few and too specific for the detection of all or most strains of the bacteria (Garnier *et al.,* 1987).

2.4.2 DNA probes used in detection and characterization of the bacterium

DNA probes were developed to detect and characterize the HLB bacteria (Villechanoux *et al.,* 1992). In this procedure, DNA probes were prepared by purifying DNA of the (Indian) Poona strain of the organism from the phloem tissue of infected periwinkle plants. The purified DNA was then restricted by *Hind* II endonuclease and the fragments cloned in the replicative form of bacteriophage M13MP18. Differential hybridisation involving DNA from healthy and infected periwinkle plants was then performed. Three recombinant phages containing bacterial DNA inserts were selected. The bacterial DNA inserts (In – 2.5, In – 1.9 and In – 0.6) were purified and the viral replicative forms were used as probes. Southern and dot hybridisation were performed and it was shown that In – 2.6 and In – 1.9 recognised all Asian strains while In – 0.6 reacted only with the Indian bacterial strain. This shows that the three inserts do not cross-hybridise and have different specificities towards various strains of greening bacteria (Villechanoux *et al.,* 1992).

2.4.3 Non-radioactive Probes

The use of radioactively labelled probes is not easy in most of the countries where HLB is present, because of stringent disposal requirements and difficulty in procurement of radioactive 'hot' nucleotides. This led to the development of non-radioactive probes for the detection of the HLB bacteria in infected plants (Hocquellet *et al.,* 1997). These non-radioactive probes were labelled with digoxigenin via PCR incorporation of digoxigenin – 11 – dUTP (DIG-UTP).

The DIG-labelled probes have sensitivity equivalent to that of the radioactive probes. These have an advantage in that they can be used even in regions where disposal of radioactive material is a problem. Liberobacter species have several serotypes and thereby require many probes for their efficient differential identification. Secondly, DNA extraction for dot-blot hybridisation is time consuming and tedious. This led to the use of PCR in detection of the bacteria and thereby the disease (Jagoueix *et al.,* 1994).

2.4.4 PCR methods in detection and characterization

Polymerase chain reaction is a powerful tool developed in 1985 by Karl Mullis (Mullis and Faloona, 1987). The procedure involves *in vitro* replication of specific DNA sequences through repeated thermal cycles while using a thermal stable DNA polymerase with special primers. The technique is used to detect and characterize several organisms using uniquely conserved DNA sequences (e.g. ribosomal DNA) within their genomes (Weisburg *et al.,* 1991; Barry *et al.,* 1991; Jagoueix *et al.,* 1997).

The sequence of the 16S ribosomal region is useful for differentiating species and also strains within species of prokaryotes (Leblond *et al.,* 1996). Using the 012C and 011 PCR primers Jagouiex *et al.* (1996) were able to amplify an 1160 fragment of greening Liberobacter 16S rDNA from purified infected plant DNA irrespective of whether the Asian or the African strains were present. The 1160 bp PCR fragment obtained with the 16S rDNA was similar whether "*Candidatus* L. africanus" or "*Candidatus* L. asiaticus" were present in the plant sample. To differentiate the two species X*bal* restriction enzyme was used to hydrolyse the 16S rDNA of "*Candidatus* L. africanus" into three fragments of 520bp, 506bp and 130bp and that

of "*Candidatus* L. asiaticus" into only two fragments of 640 bp and 520 bp (Jagouiex *et al.,* 1996). Albeit this important step identification of various strains still remains a problem if X*bal* hydrolyses is used.

The 16S/23S intergenic region is important in detection and characterization of the bacteria (Ferris *et al.,* 2003; Jagoueix *et al.,* 1997; Luz *et al.,* 1998; Rocap *et al.,* 2002). The intergenic region has been used to provide polymorphism within species that correlate to biologically significant traits. The sequence of the intergenic region is relatively conserved within species and it is variable when the phylogenetic gap widens. This region is highly variable at both interspecies and intraspecies levels (Barry *et al.,* 1991). The 16S/23S ribosomal intergenic regions of "*Candidatus* L. asiaticus" Poona and "*Candidatus* L. africanus Nelspruit have been sequenced and analysed for polymorphism. These sequences have been deposited in the Genbank database under accession numbers U61359 and U61360 (Jagoueix *et al.,* 1997)

2.4.5 Characterization of the bacterium by DNA sequencing.

Although radioactive probes, DIG probes and the PCR methods adduced to have to some extent been used for detection and identification of the BLO, they have not permitted differentiation of the various serotypes that occur in each species (Jagoueix *et al.,* 1997). To further characterise the two Liberobacter species at the molecular level and to find genomic polymorphism the intergenic 16S/23S rDNA spacer region known to be highly variable at both the interspecies level and the intraspecies level, was cloned and sequenced (Jagoueix *et al.,* 1997). The sequences of the two species "*Candidatus* L. asiaticus" and "*Candidatus* L. africanus" were 100% homologous, which implied that the PCR was not able to identify different strains.

Jagoueix *et al.* (1997), noted that though the 16S/23S intergenic region is varied and thus useful for differentiating strains of prokaryotes, it is limited in the genus "*Candidatus* L. asiaticus" since strains poona and fuzhou showed 100% sequence homology. Thus monoclonal antibodies remain the only reagents that allow identification of BLO strains within the species (Jagoueix *et al.,* 1997).

2.5 The ribosomal DNA

Ribosomes are the sites of protein synthesis in both eucaryotes and procaryotes. They are found in abundance in the cytoplasm either free floating or attached to the endoplasmic reticulum (E.R) (Stryer, 1988). All ribosomes constitute two subunits of unequal size. The smaller (30S) subunit contains a 16S rRNA molecule and 21proteins, while the larger (50S) subunit contains two rRNA molecules (5S and 23S). The DNA that codes for these proteins and transcribes to the three rRNA molecules have been studied and found to have conserved and polymorphic regions within species (Gutell *et al.,* 1994). The intergenic spacer regions have been used to characterize organisms (Ferris *et al.,* 2003; Rocap *et al.,* 2002). The 16S rRNA is widely used to study polymorphisms within species and a ribosomal database project is maintained (Olsen *et al.,* 1991; Olsen, and Woese 1993; Maidak *et al.,* 2001).

2.6 Control of the Huanglongbing disease

Various methods have been used in the control of the citrus greening disease including; thermotherapy, chemotherapy, biological control of vector, cultural practises, breeding for resistance and tissue culture (Nariani *et al.,* 1980; Van Vuuren, 1977; Davies and Albrigo, 1998; van Lelyveld *et al.,* 1998; Obukosia *et al.,* 2000; Waithaka *et al.,* 1988; Navarro, 1984). Most of these methods exhibit minimal positive results, which have been mainly ephemeral or

too expensive to be applied in orchards, with the exception of tissue culture which is currently in use to rejuvenate farmers' orchards by providing clean planting material.

2.6.1 Thermotherapy

This method involves the use of high temperatures on seedlings or on bud wood. It has been used in India in treating of bud wood. Temperatures of 47ºC for two hours reduced disease incidence by 50% and longer treatments eliminated the pathogen (Nariani *et al.,* 1980). Infected trees covered for two to five months with polyethylene-covered fibreglass exhibited a dramatic reduction in symptoms.

2.6.2 Chemotherapy.

Chemotherapy involves treatment of HLB infected plants with antibiotics such as penicillin, tetracycline hydrochloride and penicillin-carbendazin. The realisation that the HLB pathogen was a prokaryote with a peptidoglycan (PG) layer on its envelope prompted researchers to use various antibiotics including penicillin, which inhibits a step in PG synthesis. In South Africa this reduced disease symptoms in the next crop from 60% to 20%. Trunk injection of tetracycline hydrochloride has also been successful in Taiwan, China and Reunion (Da Graca, 1991). Van Vuuren (1977) reported that though tetracycline hydrochloride suppresses the symptoms, it also has phytotoxic characteristics. Buds immersed in concentrations of over 250 ppm did not survive after grafting. A derivative of tetracycline hydrochloride, n-pyrrolidinomethyl tetracycline (PMT) is more soluble in water, thus giving slightly better control of greening and causes no foliar phytotoxicity (Van Vuuren, 1977).

2.6.3 Insect vector control and cultural practices in HLB disease control.

Field control of psyllid vectors can profoundly remedy the HLB disease condition. These vectors can be controlled by cultural methods, biological methods, chemical methods, use of resistance varieties and integrated pest management (Davies and Albrigo, 1998; Pyle, 1977; Aubert, 1987). Systemic and contact chemicals used to control insects on citrus include organochlorines, organophosphates, carbamates, pyrethroids, botanicals, and bio-pesticides, and they vary in efficacy with specific chemicals being recommended in most major production areas (Davies and Albrigo, 1998). Use of Chemicals such as fenitrothion, dimethoate, diazinon, endosulfan and pyrethroids has been recommended in the region, however, judicious use is emphasized so as to destroy psyllids, protect beneficial insects, avoid environmental contamination and avoid pest resistance to chemicals (Pyle, 1977; Smith *et al.,* 1997).

Biological insect control is the use of natural enemies to control psyllids. Several strategies are used, this include use of predatory animals and insects, use of parasitoids and the use of pathogens. Ectoparasites, such as *Tetrastichus dryi* and *T. radiatus*, from South Africa, have been used successful to control the two citrus psyllids, *T. erytreae* and *D. citri* in the Re-Union Islands (Aubert, 1987; Catling, 1969b). Several fungi and bacteria have been used to control insects. The fungus *Cladosporium oxysporium* has been shown to have considerable impact on homopterans especially *T. erytreae* (Samways and Grech, 1986). Cultural insect control methods are less hazardous to the environment than use of chemicals. They include crop rotation, trap cropping, sanitation, manuring, soil cultivation, strip farming and intercropping (Buitendag, 1988). All these methods give varied results and should be used together with other methods for maintenance of the insect population under the economic threshold level.

Integrated pest management has great potential in controlling of insects and at the same time reduce the adverse effects of the control mechanisms used.

2.6.4 Breeding for disease resistance.

This is a technique of using the natural variability in citrus to incorporate genes for resistance or tolerance to the HLB bacterium in commercial citrus varieties. Tolerance to the greening disease has been associated with citrus varieties that express high perioxidase activity (De Lange *et al.,* 1985; van Lelyveld *et al.,* 1988). Some Tahiti limes, which show high perioxidase activity, are being used to create hybrids with sweet oranges and the hybrids and cybrids tested for possible resistance (De Lange *et al.,* 1985; van Lelyveld *et al.,* 1998). Citrus breeding through these methods is difficult and time consuming because most citrus and related species are very heterozygous and few important traits show single gene inheritance patterns, therefore F1 hybrids and cybrids tend to exhibit variability (Davies and Albrigo, 1998). The solution to this dilemma currently lies in the use of marker assisted breeding, where molecular markers are utilized to select for the desired trait. This eliminates the problems of having to wait for seedlings to go through the juvenile stages, it also partially eliminates the need for making numerous crosses and screening many seedlings in order to identify favourable characteristics (Davies and Albrigo, 1998).

Protoplast fusion (Grosser and Gmitter, 1990) was successfully used to incorporate phytophthora and citrus nematode resistance found in *Severinia buxifolia* (a genus within the citrus subtribe) in to sweet orange varieties. Selection of the regenerated plants was done by use of molecular markers (Grosser and Gmitter, 1990). A more recent and effective way of introducing new and desirable genes into citrus is by the use of genetic transformation.

(Kobayashi and Uchimaya, 1989; Hidaka *et al.,* 1990; Vardi *et al.,* 1990; Moore *et al.,* 1992; Bond and Roose, 1998). This technique was first applied by using GUS and *npt*II as reporter and selection genes, respectively on *Citrus* genetic transformation. However, there are few agronomically important transformations to date; they include the gene that encodes for the *Citrus* tristeza virus coat protein (Gutiérrez-E. *et al.,* 1997; Domínguez *et al.,* 2000; Yang *et al.,* 2000), the *HAL2* gene that confers tolerance to salinity (Cervera *et al.,* 2000), *LEAFY* and *APETALA1* genes that promote early flower initiation (Peña *et al.,* 2001), and *CS-ACS1* gene that controls the ethylene biosynthesis in *Citrus* (Wong *et al.,* 2001).

2.6.5 Citrus tissue culture used to control Huanglongbing disease.

Several tissue culture techniques have been applied for eradication of diseases in seedlings of various plant species. In citrus, nucellar embryo culture, ovule culture and shoot tip grafting (micro-grafting) have been used to produce disease free seedlings (Navarro, 1984). *In vitro* culture of nucellar embryos in polyembryonic and monoembryonic citrus varieties plays a vital role in producing disease free citrus rootstocks, scions and whole plants (Rangan *et al.,* 1969). The success of this technique is based on the fact that most phloem limited citrus viruses and pathogens are not transmitted through seeds and especially not through nucellar embryos since there is no direct vascular connection between the parent and either the zygotic or nucellar embryos (Button and Kochba, 1977). However the size and the developmental stage of the fruit from which the seeds are extracted from is very important in the delivery of clean seedlings. The percentage of ex-plants producing embryos and the percentage of the embryos that bear clean seedlings is largely determined by the age of the fruit at the time of nucellus excision, fertilization and *in vitro* media composition. (Bitters *et al.,* 1970; Button and Kochba, 1977). Diseases like exocortis, veination and tristeza are eliminated in the seedlings developed from

induced embryos in nucellus tissue. The production of these nucellar plants is very prolific and therefore offers great potential for eradication of the greening disease and mass propagation of commercial citrus (rootstocks and scions) varieties (Obukosia *et al.,* 2000).

In vitro ovule culture and *in vitro* shoot-tip-grafting have been shown to be important methods for eliminating pathogens and especially viruses from seedlings (Navarro *et al.,* 1984; Waithaka and Obukosia, 1988; De Lange, 1978; Mukhopadhyay *et al.,* 1997). These procedures have been of immense importance in obtaining clean nucellar plants from seedless polyembryonic varieties. Ovule culture has been used successfully to produce clean seedlings from *Citrus sinensis* L. (Waithaka and Obukosia*,* 1988). The ovules in this case are cultured just before natural abortion takes place. Micrografting (Navarro *et al.,* 1975) is a procedure in which shoots of less than one millimetre are grafted on tissue-cultured rootstocks *in vitro* to produce disease free plants.

2.7 Scientific Techniques

2.7.1 PCR and Optimal conditions.

Karl Mullis discovered the PCR technique in 1985 (Mullis *et al.,* 1986; Mullis and Faloona, 1987; Mullis, 1990). The technique is based on a protocol for the repeated rounds of DNA synthesis and the use of a thermostable DNA polymerase. It is carried out in three relatively simples steps required for any DNA synthesis reaction; (1) denaturation of the template into single strands; (2) annealing of primers to each original strand for new strand synthesis and (3) extension of the new DNA strands from the primers (Delidow *et al.,* 1993). Due to the high temperatures the heat stable DNA polymerase from a thermophyllus bacterium, *Thermus aquaticus* (*Taq*) makes the PCR reaction more effective (Saiki *et al.,* 1988).

The selection of the primers in any PCR amplification procedure is very vital to the efficacy of the reaction. The primers should have the ability to form a stable duplex with the specific site on the target DNA, and no duplex formation with another primer molecule or no hybridisation at any other target site (Rychlik *et al.,* 1991). The primers should be free of significant complimentarity at their 3' termini as this promotes the formation of primer-dimer artefacts that reduce PCR product yield (Saiki *et al.,* 1988). The PCR primers should have above 50% GC/AT ratio, this is because most templates might have a similar or lower GC/AT ratio. The ratio is important since it influences the melting temperature. Primers that are stable at their 5' terminus but somewhat unstable on their 3' ends perform best in sequencing and PCR as well. A primer with low stability on its 3' end will function well in PCR because the base pairing near the 3' end are not sufficiently stable to initiate synthesis. It prevents false priming during lower than optimal annealing temperatures (Saiki *et al.,* 1988).

2.7.2 DNA Cloning

DNA fragments were first cloned into plasmids following the realization that restriction nucleases for instance *EcoRI* generates specific cohesive ends that can later be sealed up by DNA ligase (Watson *et al.,* 1993). Currently DNA cloning is used widely to improve the DNA sequencing product especially while using Sangers enzymatic DNA sequencing method (Berget *et al.,* 1977). Cloning vectors were developed based on the single stranded filamentous DNA bacteriophage M13 (Yanisch and Messing, 1985). The M13 cloning vectors produce single stranded template DNA, the optimal form of DNA for Sanger-dideoxy sequence analysis. The DNA fragment to be sequenced is ligated into MI3 vectors at the polylinker site.

An oligonucleotide primer (universal sequencing primer) that anneals adjacent to this polylinker region is used to sequence the inserted DNA fragment.

In other procedures the DNA cloning step is by passed and the amplified DNA is sequenced directly (Gyllensten and Erlich, 1988) Double stranded PCR products are also sequenced directly (Gyllensten, 1989). Single stranded DNA is the best template for Sangers-dideoxy chain termination method, therefore a technique called asymmetric PCR has been devised to produce single-stranded DNA (Gyllensten and Erlich, 1988). A standard PCR is set up except that the concentration of the two primers (forward and reverse) differ by a factor of 100.

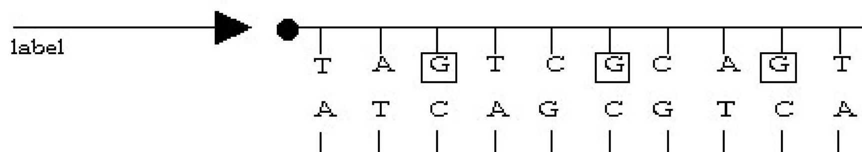
2.7.3 DNA sequencing

The DNA molecule that comprises of several nucleotides in its helical structure can only be deciphered if the exact nucleotide sequence is revealed. The dire need for deeper insights about the organization of DNA in the 1960's culminated in the sequencing of relatively small tRNA molecules that contain 75-80 nucleotides (Watson *et al.,* 1992). In 1975 Sanger devised the plus-minus method of DNA sequencing. It was based on using enzymatic restriction and the elongation of DNA chains with DNA polymerase. In 1977 Maxam and Gilbert developed an equally powerful but currently relegated DNA chemical degradation method.

Maxam and Gilbert Chemical Degradation Method of DNA Sequencing

This method of DNA sequencing was developed in 1977 (Maxam and Gilbert 1977; Maxam and Gilbert 1980). In this procedure a segment of DNA is labelled at one end with ^{32}P. The labelled DNA single strand is divided into four samples and each sample is treated with a chemical that specifically destroys one or two of the four bases in the DNA generating a series

of labelled fragments, the lengths of which depend on the distance of the destroyed base from the labelled end of the molecule. For example if there are G residues 3,6 and 9 bases away from the labelled end then treatment of the DNA strand with chemicals that cleave G will generate labelled fragments 2, 5 and 8 bases in length.



The sets of labelled fragments obtained from each of the four reactions are run side by side on an acrylamide gel that separates DNA fragments according to size, and the gel is autoradiographed. The pattern of bands on the x-ray film is read to determine the DNA sequence (Maxam and Gilbert, 1977).

Sanger-Dideoxy nucleoside triphosphate chain termination sequencing

This method of DNA sequencing was developed in 1977 (Sanger *et al.,* 1977). As opposed to the Maxam and Gilbert method, the Sanger-dideoxy nucleoside method uses enzymatic rather than chemical techniques. First 2', 3'- Dideoxyribonucleoside triphosphates (ddNTP) of each of the four based are prepared. The ddNTPs are incorporated into a growing DNA strand. Since the ddNTP cannot form a phosphodiester bond with the next incoming dNTP, growth of that particular DNA chains stops. If the correct ratio of ddNTP: dNTP is chosen, a series of labelled strands will result. The lengths of which are dependent on the location of a particular base relative to the end of the DNA. Four DNA polymerase reactions are set and the resultant labelled fragments are separated by size on an acrylamide gel and auto-radiographed.

2.7.4 Automated fluorescent DNA sequencing

Manual sequencing by use of radioactive labelling is currently being replaced with the use of non-radioactive sequencing due to cost efficiency and higher sequence read length and high sequence turnover (Chen, 1994). The process has been greatly improved and many of the limiting steps in DNA sequencing have been bypassed. A merit of this method is that the extra cost of building structures and acquiring equipment for control of the dangerous radioactive rays is avoided. The risk of researchers coming in contact with strong radioactive contamination is also avoided (Kelly , 1994).

The radioactive labels are replaced with four different dyes which when excited by use of laser beam they emit light at different wavelengths, which are detected by a photo-multiplier connected to the computer with a relevant programme. These dyes can be used to label the M13 universal sequencing primers or each of the four dideoxy chain terminators. The fluorescent-tagged dideoxy fragments migrate down the denaturing polyacrylamide gel and pass through a laser beam. As they do so the beam excites the fluorochromes and the light they emit is detected by Photo-multiplier. The emission peaks overlap and a computer analysis is necessary to resolve them (Watson *et al.,* 1992; Davis *et al.,* 1991;Smith *et al.,* 1986).

The Sequenase (T7) polymerase and Taq DNA polymerase are the choice enzymes in the sequencing reaction. However, the advantage of using Taq over sequenase is that multiple rounds of sequencing can be performed without the need to add fresh enzyme. This allows the use of much less template DNA, making this the method of choice in many circumstances (Holmberg *et al.,* 1994).

Modifications to the Taq DNA polymerase that enable it to incorporate the dye-labelled terminators more evenly, resulting in less pronounced peak height variability and consistently high accuracy have made this the most commonly used automated sequencing method. Other innovations such as the introduction of fluorescence energy transfer (FRET) have improved signal intensity and spectral separation to a level where very low amounts of templates can be used. This has also allowed sequencing of large templates such as bacterial artificial chromosomes (BACS) (Mc Combie, 1994).

2.7.5 Primer design and preparation.

The primer sequences used in PCR and fluorescent cycle sequencing can have a major influence on the specificity and sensitivity of the reaction (Mullis *et al.,* 1986). Primers can be designed using a few simple guidelines, although primer design programs, now readily available, can make good primer design more reproducible (Dieffenbach *et al.,* 1995). Primers should be 20-30 bp long and have melting temperatures (Tm) of 55-65^0 C. Where possible, primers should be made with a GC content of 50-55%. For primers with a much lower GC content, the primer sequence may need to be extended to more than 20 bases to keep the Tm above the recommended lower limit of 55^0 C (Saiki *et al.,* 1988). Wherever possible there should be at least one G or C at the 3' end to stabilise this end of the primer (Innis and Gelfand, 1990). Primers that show secondary structure or that can hybridise to form dimers or oligomers should be avoided. These internal relationships can be most easily predicted if a primer design program is used. Primers should be resuspended in sterile, distilled water (Innis and Gelfand, 1990).

2.7.6 Computational analysis of biological sequences

Sequence homology or similarity search is an important feature in describing sequences (DNA or protein) in relation to already published sequences in the available databases. A good homology search will provide sequence similarities that correlate with biologically significant properties. To do a homology search computational techniques for sequence alignment are necessary (Sutton and Kerlavage, 1994; Tripathi, 2000).

Sequence alignment can be seen as evolutionary or structural (States and Boguski, 1992). Evolutionary sequence alignment enables the researcher to determine if two sequences display sufficient similarity to justify the inference of homology. In this case similarity is an observable quantity that may be expressed as percent identity or some other measure, while homology is a conclusion drawn, that two or more genes share a common evolutionary history (States and Boguski, 1992). Homologous sequences suggest divergence from a common ancestral sequence through iterative molecular changes but the ancestral sequence in not known (Liz Frank, 1996). In such alignments residues that have aligned and are not identical represent substitutions. Regions in which the residues of one sequence correspond to nothing in the other are interpreted as either an insertion or deletion (Liz Frank, 1996). These regions are represented in an alignment as Gaps. On the other hand, structural basis for alignment assists the researcher decipher the structure and corresponding function of protein sequences. If two proteins with identical alignment of residues will fold in similar positions and have similar functions. Thus, protein sequence alignment is often an approximate predictor of the underlying 3-D structural alignment (States and Boguski, 1992).

Sequences are aligned either by pairwise or by multiple sequence alignment methods (Orengo *et al.,* 1996; Altschul *et al.,* 1990; Altschul *et al.,* 1997; Gerstein *et al.,* 1996; Krogh *et al.,* 1994; Lipman *et al.,* 1989; Subbiah *et al.,* 1993). Both methods have specific applications in computational analysis. A pairwise alignment can be used for both protein and DNA sequences, and its main functions are to provide a basis for other analyses, infer protein function, predict the structure, outline homology modelling and reveal conserved positions and functionally critical residues (Gerstein *et al.,* 1996). In addition pairwise alignments are used for phylogeny, PCR primer design, mutagenesis studies and detection of previously known motifs (Subbiah *et al.,* 1993). This alignment can be done by the Classic Needleman-Wunsch algorithm or by other algorithms like Align (Dayhoff, early 80's), Gap (GCG package), Smith-Waterman (Smith, Waterman), Bestfit (GCG Package), FASTA (Pearson, Lipman), BLAST (Altschul, Lipman), PSI-BLAST, PHI BLAST, HMM methods (Haussler, Eddy) among others (Subbiah *et al.,* 1993). BLAST is the principle method used in the National Centre for Biotechnology Information (NCBI) Genbank.

The multiple sequence alignment methods uses fairly complex algorithms and it fulfils all the functions previously discussed for pairwise methods but with more subtle information, the method creates sequence profiles, deduces sequence motifs, analyses protein family relationships and it serves as a convenient backdrop for annotation and summarizing information results of various sequence analysis (Altschul *et al.,* 1997; Lipman *et al.,* 1989; Subbiah and Harrison, 1986). Multiple sequence alignments are done by among many programmes, progressive alignment algorithms like TULLA (Subbiah and Harrison, 1986), Clustal, ClustalW, PileUp and MultiPipMaker (Schwartz *et al.,* 2003) available at http://bio.cse.psu.edu/.

There are three major DNA sequence databases available. (i) The DNA Databank Of Japan DDBJ (ii) The European Molecular Biology Laboratory Nucleotide Sequence Data Library (EMBL) and (iii) The Gen Bank Genetic Sequence Date Banks (Gen Bank) most of the important sequence are stored in these databases with accession numbers. Retrieval can be done and sequence comparison performed.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Site selection

3.1.1 Machakos district site description.

Machakos district covers an area of approximately 6,165 km². The district borders Kitui to the East, Makueni to the South, Kajiado and Nairobi to the West, Murang'a and Embu to the North and Mwingi to the Northeast. It has a bimodal rainfall regime (March to May – long rains and October to December – short rains). Most citrus trees are planted during the short rains. Total rainfall averages between 500 mm to 1,000 mm. The altitude ranges from lowland areas 700 – 1,700 m ASL. The most important agro-ecological zones (AEZs) for citriculture in Machakos district are zone II, III, IV and V. Zone II covers the upper slopes of the hill masses of Iveti, Mua and Kangundo; Zone III covers the lower slopes of Iveti, Mua and Kangundo hills, Matungulu and Mitamboni areas. Zone IV covers the largest part of the district including most parts of Mwala, Ndalani, Kinyatta, Yatta, Kangonde and Ndithini in Masinga, Matungulu and Donyo Sabuk in Kangundo. In regions covered by zones IV and V, in Yatta and Matuu most of the horticultural activity is concentrated along the Yatta canal. Three major agroecological zones are present in Machakos as presented below (Table 3.1). Machakos district was sampled purposively since it lies within the low altitude areas.

Table 3.1. Agro ecological zone characteristics of Machakos district.

Agro-ecological Zone	Altitude	Mean Temp	Rainfall	Others
Lower Highlands (LH)	1800/1900-2200/2400m	15°C-18°C	1000-1800mm	Low evaporation < 1400mm/yr
Upper Midlands (UM)	1300/1500-1800/1900m	18°C- 21°C	700- 1800mm	
Lower Midlands (LM)	800-1300/1500m	21°C- 24°C	700-1800mm	High evaporation 1800-2000mm/yr

Figure 3.1 Map showing the agroecological zones in the wider Machakos district.

3.1.2 Kakamega district site description.

Kakamega district is located in Western province. It has a total district area of 2,963 square kilometres of which 327 km² is forest, 2,481 km² being arable and cultivable land. The district is within the highland regions about 1,250 – 2,000 m above sea level. The district has a bimodal rainfall regime with an average annual rainfall of 1,968.8 mm. The average temperatures range at 22 – 28°C mean maximum and 14 – 18°C mean minimum. The district lies within the Agro-Ecological Zones of UM0 – LM2 (Table 3.2). Zone UM0 covers Shinyalu and Malava, forest covers massive parts of this zone. Zone UM1 covers Shinyalu and Ikolomani. Zone UM4 (maize/sunflower) covers Matere, Lugali and Likuyani, Zone LM1 (sugarcane zone) covers Mumias and Butere while Zone LM2 (marginal sugar cane zone) covers Navakholo, Kabras, Lurambi and Khwisero. The land use pattern can be distinctly associated with the AEZ and the soil type of a given region. Towards the North, South and Central Kakamega we find dark brown sandy loam's, this region is characterised with the cultivation of maize, beans, horticultural crops, sunflower, pasture and forage. The South and East divisions have dark-red soils covered with Lumic. Crops grown in this region include maize, beans, millet, sorghum, tea, coffee, bananas, forest, pasture and forage. The West and North regions have yellow-read loamy sands typically used for the cultivation of maize, beans, millet, and sugarcane. Kakamega district was sampled purposively since it lies within the high altitude areas.

Table 3.2. Agro ecological zone characteristics of Kakamega district.

Agro-ecological Zone	Altitude	Mean Temp	Rainfall	Others
Upper Midlands (UM)	1500/1700-1500/1950m	18°C-20.6^{o}C	1200-2000mm	Low evaporation < 1400mm/yr
Lower Midlands (LM)	1200/1400-1300/1500m	20°C- 23°C	1200-1800mm	High evaporation 1600-1800mm/yr

Figure 3.2 Map showing the agroecological zones in the wider Kakamega district.

3.2 Sampling of survey sites

Both districts were clustered according to the Agro-ecological zones (AEZs) and the most important zones for citrus production were identified. Three farms were randomly sampled from each AEZ. There were 10 AEZs in Machakos district, of which LM5, LM4, LM3, UM4, UM3, LH5 and LH4 AEZs show most horticultural activity. In Kakamega the AEZs, LM2, LM1, UM4, UM1 and UM0 were purposely sampled due to the presence of most citrus orchards. In Machakos, four samples were picked from each farm, each sample obtained from a different citrus variety. On the other hand, only two varieties were consistently present in Kakamega orchards, therefore two samples from only two varieties were picked from every farm. A total of one hundred and fourteen diseased leaf samples were picked from the two districts. (Table. 3.3).

Table 3.3 Sampling Procedure in Machakos and Kakamega district.

District	AEZ	Farms	Varieties	Total
Machakos	LM5	3	Valencia; Washington navel Rough lemon; Tangerine	12
	LM4	3	Val; W.N.; R. L.; Tangerine	12
	LM3	3	Val; W.N.; R. L.; Tangerine	12
	UM4	3	Val; W.N.; R. L.; Tangerine	12
	UM3	3	Val; W.N.; R. L.; Tangerine	12
	LH5	3	Val; W.N.; R. L.; Tangerine	12
	LH4	3	Val, W.N.; R. L.; Tangerine	12
Kakamega	LM2	3	W. Navel; R. Lemon	6
	LM1	3	W. Navel; R. Lemon	6
	UM4	3	W. Navel; R. Lemon	6
	UM1	3	W. Navel; R. Lemon	6
	UM0	3	W. Navel; R. Lemon	6
	TOTAL			114

3.3 Survey Scoring

Scores were used to measure the following parameters; (i) distribution of the HLB disease across agroecological zones and citrus varieties, determined by visual scores (ii) distribution of the psyllid vectors across agroecological zones and citrus varieties, determined by visual scores and (iii) distribution of the HLB disease across agroecological zones and citrus varieties, determined by PCR scores. During the survey questionnaires and field visual score forms (Appendix 4) were used to obtain the data. These were recorded by visual examination of the citrus bushes. Visual disease scores were based on symptoms found on citrus at different stages of infection. A visual score of 1 was awarded when the plant sampled had interveinal chlorosis on less than 50% of the leaves on the plant. A score of 2 was awarded to plants that had more than 50% interveinal chlorosis with blotchy mottles and /or with lopsided fruit. A score of 3 was given for trees that had small-upright-leathery leaves, massive fruit and leaf drop and dieback. PCR scores were based on the presence or absence of a 716 bp DNA band on agarose gels after PCR amplification with the L10/L12 ribosomal protein DNA PCR primers. A score of 1 was given on positive amplification while a score of 0 was awarded for negative amplification. The vector scores were based on the presence or absence of psyllid vectors. A score of 1 was awarded if psyllids were present while a 0 score was awarded when they were absent.

3.4 Plant sample collection and preparation

Leaves were collected from citrus plants showing both secondary and primary symptoms of the HLB disease from three citrus species; *Citrus sinensis* (washington navel and valencia orange), *Citrus reticulata* (tangerines) and *Citrus limon* (rough lemon). Plants showing multiple or co-infection with other citrus diseases (both bacterial and viral e.g. tristeza) were

also picked. Leaves from healthy plants especially from the University of Nairobi greenhouses were collected and used as negative controls.

Samples of HLB infected citrus were collected from Machakos district (representative of the lowland areas 700 – 1,700 m ASL). The material was transported in an icebox to the Faculty of Agriculture, University of Nairobi, where they were immediately frozen at –20ºC awaiting DNA extraction. In addition to leaf samples, a survey questionnaire was administered to farmers in whose farms samples were taken (Appendix 5). This questionnaire provided information on the field disease visual scores, the distribution of the disease across AEZs and varieties and the psyllid vector score.

3.5 DNA extraction and partial purification

DNA was extracted using a modification of the protocol by Jagoueix *et al.* (1996). Leaf midribs (0.1 to 0.3 grams) were chopped to a fine mince with a razor blade in a disposable petri-dish containing 1 ml of TE buffer (10 mM Tris pH 8.0, 400 mM EDTA, 1% SDS and 0.25 mg of proteinase K). The homogenate was transferred to an eppendorf tube and incubated for two hours at 65ºC. The suspension was centrifuged for 15 minutes at 12,000xg and the supernatant mixed with 1 ml of wizard min prep DNA purification resin (promega). The resin was transferred to a mini-column and washed twice with 2 ml of 80% isopropanol. Thereafter, 50 µl of hot water (80ºC) was added, this was incubated for one minute then the column was centrifuged for 30 seconds at 16,000xg in an eppendorf tube. This step was repeated to have a total yield of 100 µl of extract. This was stored at -20°C.

3.6 Amplification of the L10-L12 ribosomal protein rDNA

Two primers were used to specifically amplify the L10-L12 intergenic ribosomal protein DNA, L10 ribosomal protein DNA and a part of the L12 ribosomal protein DNA. These primers were developed from the published sequence information of the L10-L12 (Garnier *et al.,* 2000; Villechanoux *et al.,* 1993; Planet *et al.,* 1995) and prepared by PRIMEGENS (Primer Design Using Gene Specific Fragments) software tool. This programme automates the selection of PCR primers in a DNA sequence for the purpose of amplifying a unique site in the genome, and selects gene-specific fragments and then design primer pairs for PCR amplifications.

Forward 5' (CATCGGGAGATGAAAGTTGAATA)

Reverse 5' (TTCCCCTGCCGCAGACGCAACA)

These primers were designed from the complete rplKAJL-rpoBC operon sequence reported at Laboratoire de Biologie Cellulaire et Moléculaire (I.N.R.A) (Planet *et al.,* 1995) also available on the NCBI Gen Bank website. http://www.ncbi.nlm.nih.gov/. New primers were made because the rplA2 and rplJ5 primers developed at I.N.R.A were limited to the L10 protein and could not amplify the intergenic region.

Amplification was done by PCR (Hybrid OMN-E Thermal Cycler). The PCR reaction was performed in 50 µl of reaction mixture containing 1µm of each of the primers, Gibco buffer, 200 µm of each of the four dNTP, 2 mM $MgCl_2$, 0.05% W1 detergent (Gibco BRL), 100 µg Ml-1 BSA and 2.5 U of Taq polymerase (Gibco BRL) (Jagoueix *et al.,* 1996). The reaction

mix was amplified for 35 cycles with the thermocycler at: 92ºC for 20 seconds (denaturation of template into single strand); 62ºC for 20 seconds (annealing of primers to each original strand, high temperatures are used due to the specificity of the primers); and 72ºC for 45 seconds (elongation and new strand synthesis). PCR products were analyzed by electrophoresis of 20µl of each sample (Jagoueix *et al.,* 1996).

3.7 Amplification of the 16S/23S rDNA

Two primers were used to amplify the 16S/23S intergenic region, which is useful for differentiating Liberobacter species. Forward primer 012C with the reverse primer 23SI.

Forward Primer 012C: 5'ATG GGT TGC GAA GTC GGG AGGC 3'
Reverse Primer 23SI: 5' CGC CCT TCT CGC GCT TGA 3'

The 16S/23S intergenic region of each isolate was amplified by PCR (Hybrid OMN-E Thermal Cycler) with the above primers (Jagoueix *et al.,* 1997). The PCR was performed as elaborated by Jagoueix *et al* (1997). The PCR reaction was performed in 50 µl of reaction mixture containing 0.5 µm of each of the primers, 200 µm of each of the four dNTP, 78 mM Tris-HCL pH 8.8, 2 mM $MgCl_2$, 17 mM $(NH_4)_2SO_4$, 10 mM β mercaptoethanol, 0.05% W1 detergent (Gibco BRL), 200 µg Ml-1 BSA and 2.5 U of Taq polymerase (Gibco BRL). This was amplified with 35 cycles with the thermocycler using the following program: 92ºC for 20 seconds (denaturation); 62ºC for 20 seconds (annealing of primers) and 72ºC for 45 seconds (new strand synthesis). The reaction mixture was analysed by electrophoresis.

3.8 Agarose gel electrophoresis

Electrophoresis on 1% agarose gels was used to analyse all PCR products. Tris-borate (TBE) was prepared at 5X concentration (54g Tris base, 27.5g boric acid, 20ml 0.5M EDTA pH 8.0)

Sambrook *et al.* (1989). The electrophoresis plates were put in place and appropriate amounts of electrophoresis buffer was prepared. One gram of agarose was weighed and 100ml of 1X TBE was added. This was boiled in a microwave and incubated at room temperature to cool. After cooling to 60°C, 5µl of ethidium bromide was added from a stock solution of 10mg/ml to attain a final concentration of 0.5µg/ml. This was mixed carefully to avoid bubble. The solution was then poured on to the gel mould to make a 5mm thick gel. TBE (1X) buffer was added to completely cover the gel. Thereafter, 20µl of the PCR products were mixed with 1µl of gel loading dye and loaded in to the ready gel. The lid was closed and the electrical leads fixed to link the gel tank to the power pack. A voltage of 4 V/cm was applied and the loading dye was used to track the movement on the gel. After the run the gel was photographed under ultraviolet light.

3.9 Extraction of DNA from Agarose Gels

After electrophoresis and photography, DNA was extracted from the gel by use of a GIBCO Gel extraction Kit (Life Technologies). The kit uses spin cartridges containing silica membranes to capture and purify the DNA fragment. The agarose gel was dissolved in sodium perchlorate and the DNA was adsorbed on the silica support. This adsorption was influenced by buffer composition and temperature. Agarose and electrophoresis buffers were removed with alcohol containing wash buffers. The silica support adhered DNA was eluted by and reconstituted in 50µl of warm double distilled water and frozen until DNA sequencing.

In the protocol (GIBCO Gel extraction Kit manual), a gel slice containing the DNA was cut with a sharp razor under UV Light. Surrounding agarose was minimized. The gel slice was weighed and 400mg of the 1% gel was placed into a 1.5 ml propylene tube. Thereafter, 1.2 ml

of gel solubilization buffer (concentrated sodium percholate, sodium acetate, and TBE solubilizer) was added. This was incubated at 50°C for 15 minute, during which the solution was mixed every 3 minutes to ensure compete gel dissolution. A cartridge was then placed in a 2ml wash tube and the mixture from the incubation step was pipetted into the spin cartridge. The mixture was then centrifuged at ≥12000x g for 1 minute and the flow through was discarded. The cartridge was then placed back into the 2ml wash tube and 700µl of wash buffer containing ethanol was added, incubated for 5 minutes at room temperature then centrifuged at ≥12000x g for 1 minute and the flow through was discarded. This was centrifuged again for 1 minute to remove residual wash buffer. The DNA was recovered with 50µl of warm water (65°C) with centrifugation at ≥12000x g for 2 minutes.

3.10 DNA sequencing

Automated fluorescent DNA sequencing/Sanger-Dideoxy Nucleoside Triphosphate Chain Termination DNA Sequencing method was used to determine the DNA sequence of extracted PCR fragment. This was done at International Livestock Research Institute (ILRI). Big Dye chemistry with ABI PRISM® BigDye™ Terminators v 3.0 and AmpliTaq® DNA polymerase Dyes attached to the four di-deoxynucleotide terminators were used for sequencing. The cycling reaction was done on a GeneAmp® PCR System with a MicroAmp® 96-Well Reaction Plate. The PCR fragments obtained were then separated on a denaturing polyacrylamide gel on Perkin Elmer ABI PRISM® 377 DNA sequencer. The dyes were detected by fluorescence after excitation. The sequence information was decoded by a 3100-Avant Genetic Analyzer software. The PCR product was sequenced directly without cloning it in vectors. The sequence was then compared with other published sequences in the GenBank, EMBL, DDBJ, PDB Database using the BLAST (Basic Logical Alignment Sequencing Tool)

(Altschul *et al.,* 1997), on the National Center for Biotechnology Information (NCBI) Genbank website (http://www.ncbi.nlm.nih.gov/).

3.10.1 Purification of the sequencing template.

This procedure was performed to remove primers and dNTPs, which interfere with the reaction (Applied Biosystems, 2002; The QIAGEN Guide, 1998). Using G-50 w/spin column. In the protocol ~300µl of H_2O was added to the spin column and rinsed by quick spin in a microcentrifuge, then 600µl Sephadex G-50 slurry (1g/15mL in dd$H_2$0) was added to spin column and centrifuged at 4900 RPM for exactly 60 seconds. Thereafter, 20µl of PCR product was loaded to the center of the Sephadex and centrifuged at 4900 RPM for 60 seconds into a fresh 1.5 mL eppendorf tube.

3.10.2 Extension/termination reactions

The Extension/termination reactions were done by first mixing the reaction reagents template DNA 30-90 ng, Primer 2.0 –3.0 pmol, Buffer 4.0µl, Terminator Ready Reaction mix 2.0µl, Sterile H_2O to final volume 20.0µl in a 0.5 ml microfuge tube (Applied Biosystems, 2002; The Perkin-Elmer Corporation, 1997). This was mixed briefly by pipetting and it was overlayed with 2 drops of mineral oil and centrifuged. For the reaction, the tubes were placed in a thermalcycler that had been preheated to 95^oC and the cycling program was started. It was important to preheat the thermalcycler to 95^oC to denature all dsDNA and thereby prevent nonspecifically annealed primers from being elongated by Taq DNA polymerase. The programme was as follows; 95^oC for 1 min 30 sec preheating, then 95^oC for 30 sec; 59^oC for 15 sec (sequence-specific annealing temperature); and 60^oC for 4 min. The reaction was done for a total of 25 cycles then the tubes were stored at 4^oC.

3.10.3 Purification of extension products using Centri-sep columns

Excess dye terminators interfere with the read length obtained form a sequencing Gel. These impurities were removed by running the reactions through a Centri-sep spin column before analyzing the reactions on a gel. This was done in a series of steps (Applied Biosystems, 2002; The Perkin-Elmer Corporation, 1997). The column was gently tapped to cause the gel material to settle to the bottom of the column, then the upper end cap was removed and 0.8 ml of deionized water was added. The cap replaced and the column invert a few times to mix the water and the gel material. At that moment the gel was allowed to hydrate at room temperature for at least 2 hours. This was necessary because rehydrated columns can be stored for a few days at 2-6°C but must be warmed to room temperature before use. After rehydration air bubbles were removed by inverting or tapping the column and allowing the gel to settle. The upper end cap was removed before the bottom cap and the column was allowed to drain completely by gravity. Gentle pressure was applied to some columns with a pipette bulb if the flow did not begin immediately. The column was inserted into a wash tube and centrifuged at 730 x g for 2 minutes to remove the interstitial fluid. At this stage it was crucial to spin at speeds not exceeding 730 x g and for a period not longer than 2 minutes. The column was removed from the wash tube and inserted into a sample collection tube. The extension reaction mixture was removed from its tube and loaded carefully on top of the gel material. The column was then spun in a microfuge at 730 x g for 2 minutes. The column was discarded and the sample was retained in the collection tube. The sample was dried in a vacuum centrifuge. The sample was resuspended in 3 µl of loading dye and 1.5 µl was loaded on a 4.75% Long Ranger, 6M urea gel for sequencing.

3.10.4 Running a 48cm ABI sequencing gel (5.0% long ranger acrylamide, 6m urea)

A gel solution with the following reagents was prepared; 18.0 g urea, 5.0 ml 10X TBE, 5.0 ml Long Ranger, 25.0 µl TEMED, 250.0 µl 10% APS and 27 mls ddH_2O added up to 50 ml (Applied Biosystems, 2002; The Perkin-Elmer Corporation, 1997). This solution was filtered before use. The gel was allowed to polymerize for 10-15 minutes and the bottom of the gel was wrapped in saran wrap. A small amount of 1X TBE was squirted between the bottom of the gel and the saran wrap. This kept the gel moist as it polymerized. This precaution is necessary because if the gel dries out, air bubbles will form between the gel and the glass plates and as the sequencing run progresses these bubbles creep up the gel, eventually reaching the scanning area and interfering with the scanning. After gel polymerization a plate check was performed then the bottom buffer chamber was filled with 715 mls of 1X TBE. The top buffer chamber was filled with 540 mls ddH_2O and all air bubble wells were flushed out.

After setting up the gel the equipment was switched on by pressing the RUN key followed by SAVE key to save the run file. Immediately after then machine started up the PAUSE key was pressed. This was done to let the gel heat to 51°C in about 15 minutes. STATUS was selected from the windows menu and the gel was heat to 95°C for 2 minutes and quenched on ice before loading samples. The wells were flushed to remove urea and 1-1.5 µl of the sample was loaded in each well. Buffer was loaded into any empty wells to the right and left of the samples. The RESUME function was pressed for 1.5 to 2 minutes then 60 mls of 10X TBE was added into the upper chamber and well mixed to make final concentration to 1X. The lid was placed on the on buffer chamber then operation was resumed.

The “Run File” settings and the “Sample Sheet” settings were set as follows:

>Run Module “Seq Run 48E-1200”.

>Dye Set/Primer File “DT {BD Set Any-Primer}”

>Instrument File “Big Dye”. Instrument File “Big Dye”.

>Lanes “48”.

>Run Module “XL Scan”.

>Collect Time “10 hours”.

>Well-to-Read length “48 cm”.

>Use ABI 150 basecaller to analyze the data.

The results of ABI base-caller were received in ASCII format and were subjected to phylogenetic analysis.

3.11 Data analysis

Data was obtained in three forms field visual scores, PCR scores and sequences. The field visual scores and PCR scores generated data on disease distribution, varietal susceptibility and psyllid vector scores. The ribosomal proteins L10-L12 DNA sequences provided information for bacterial characterization.

The visual scores and PCR scores were analysed by ANOVA in the Genstat statistical programme to show the relationships between disease presence and vector presence. The analysis showed the relationship between disease and the agro-ecological zones and lastly it showed the relationship between disease and varieties. The visual scores were then correlated with the PCR scores using the same programme.

In characterizing the bacterium by amplifying the ribosomal proteins L10-L12 the analysis was done by comparing the sequence information obtained from the different AEZs and districts. These sequences were tested for homology and similarity using the BLAST (basic logical alignment sequencing tool (Altschul *et al.,* 1997), on the National Center for biotechnology information (NCBI) Genbank website (http://www.ncbi.nlm.nih.gov/). The sequences were aligned and compared with rDNA sequences obtained from the Gene Bank database (GenBank, EMBL, DDBJ, PDB) (accession numbers U61359, U61360 among others). This comparison gave indications of the closest relatives of the Kenyan bacteria and therefore the origin of the disease. The MultiPipMaker analysis tool was used to compute alignments and similarity scores for of more than two sequences over the length of the reference sequence (Schwartz *et al.,* 2003). The tool is available at http://bio.cse.psu.edu/.

The DNA sequence of the L12 protein from Kenyan samples was translated and compared to the L12 proteins of the bacteria from South Africa and Asia using the BLAST software (Altschul *et al.,* 1997).

CHAPTER 4

4.0 RESULTS

4.1 Survey of HLB disease in Machakos and Kakamega districts.

The Huanglongbing disease was encountered in all agro-ecological zones (AEZs) in Machakos and Kakamega districts. The disease was also found on all citrus varieties in the two districts and the morphological symptoms of the disease were vivid on all affected citrus trees (Figure 4.2 a, b).The psyllid pests/vectors (*Trioza erytreae* Del guercio) were encountered in both Machakos and Kakamega districts. They were also recorded on all the varieties in both districts but at differing rates (Figure 4.1). Some AEZs and some citrus varieties had high disease and vector scores while others had low scores. The findings on the diseases and pests are presented below.

4.1.1 Distribution of the HLB disease across varieties and AEZs in Machakos (visual scores).

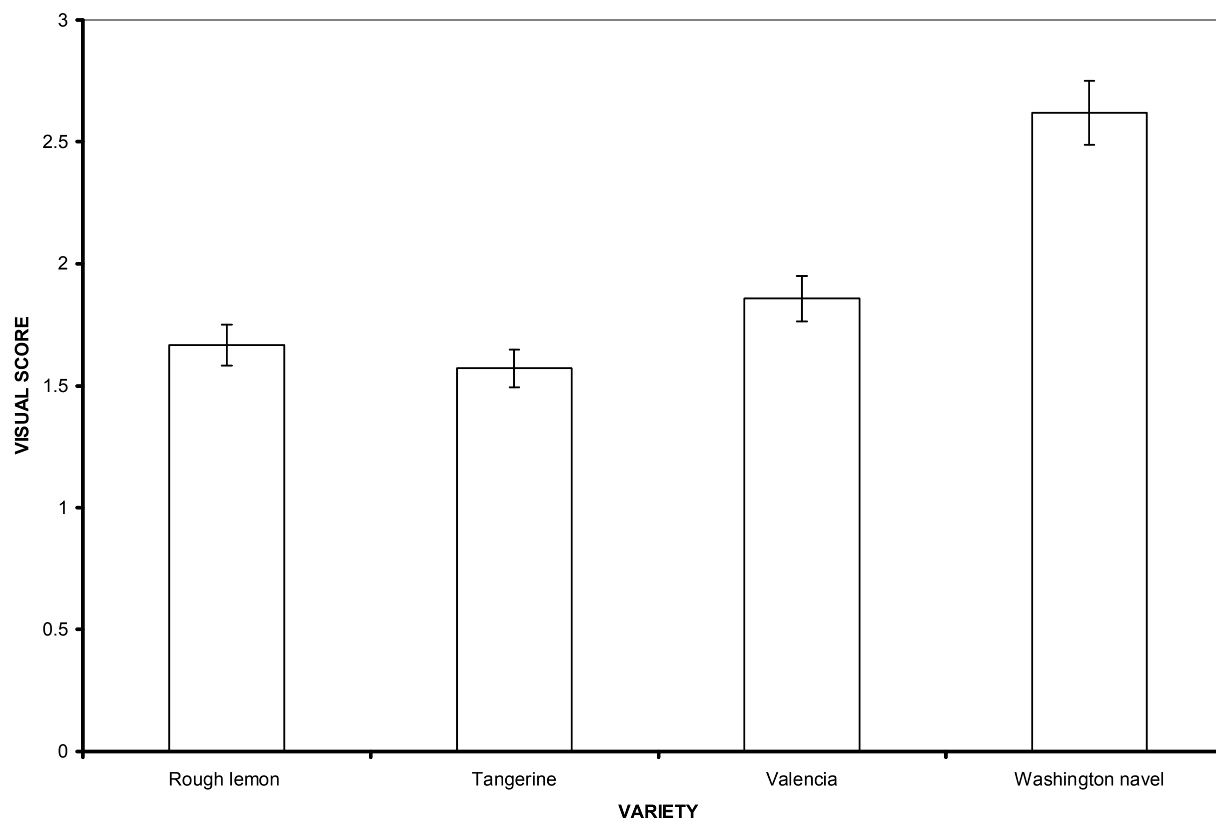
There were significant ($P<0.05$) differences in the distribution of the HLB disease among the four citrus varieties in Machakos district (Appendix 1). The washington navel oranges had the highest mean infection with a mean visual score of 2.62 (Figure 4.3), while the tangerines showed the lowest mean visual score of 1.57. The rough lemons and the valencia oranges had a mean visual score of 1.66 and 1.86 respectively. Washington navel oranges had a high mean visual score (greater than 2) in six of the seven sampled AEZs in Machakos district, except in LM5 where it scored 1.67 (Table 4.1). Washington navel orange had the highest mean HLB disease visual score in the district, irrespective of the agro-ecological conditions and the altitude. Valencia oranges had a mean visual score of more than 2 in four of the seven AEZs. These four AEZs were located within the lower highlands and the upper midlands.

Figure. 4.1 Psyllids activity on citrus leaves.
(a)*Trioza erytreae* ;Del Guercio psyllids eggs hatching and adults feeding on the under side of young valencia leaves
(b)*Trioza erytreae* ; Del Guercio psyllids adults feeding on the under side of mature washington navel leaves
(c)*Diaphorina citri* Kuwayama psyllids feeding on both sides of tangerine leaves (Courtesy; Coelho and Marques, 2002)
(d)Rough lemon leaf deformed due to the activity of psyllids.

Figure 4.2a Huanglongbing diseased washington navel leaves showing interveinal chlorosis and blotchy mottles.

Figure 4.2b Huanglongbing diseased valencia trees exhibiting massive fruit and leaf fall.

Figure 4.3. Mean HLB symptoms visual score on different citrus varieties in Machakos district.



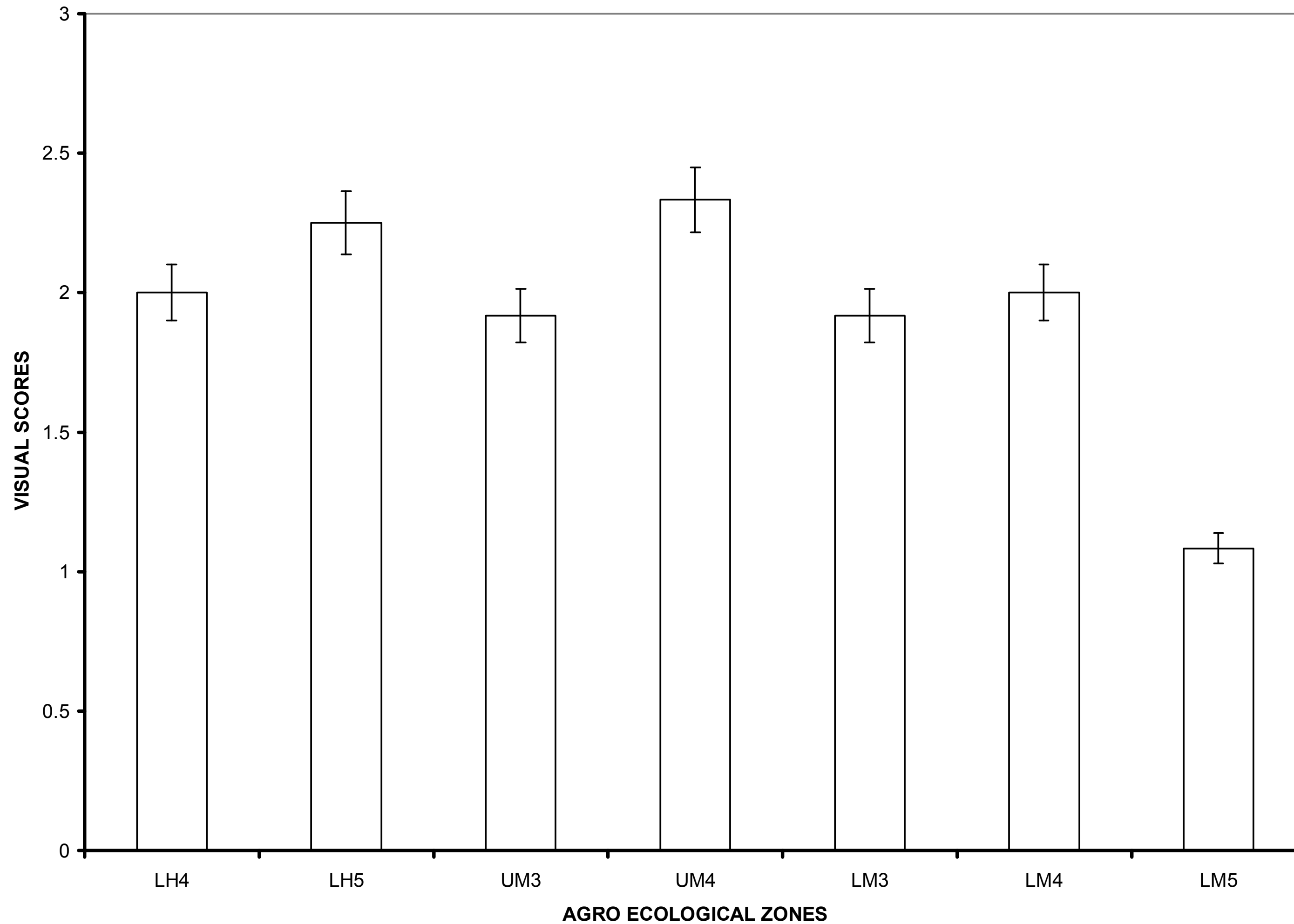
Error bars represent standard errors. Data are means ± s.e of 21 plants (n=84). Varieties were compared by single factor ANOVA and means separated at p=0.05 level. The numerical visual scores represent the following; 1- interveinal chlorosis; 2- blotchy mottles with lopsided fruit; 3- small upright leathery leaves, massive fruit drop and dieback.

A mean visual score of more than 2 was recorded on tangerines growing only in the higher altitudes found in LH4, LH5 and UM4 (Table 4.1). Lastly, the rough lemon had the highest mean visual score of 2.67 in the lower highland 5 of Machakos district (Table 4.1). Rough lemon and tangerines had low HLB disease mean visual scores in the lower altitude areas, however the two varieties had high mean visual scores in the high altitude areas.

There were significant ($P<0.05$) differences in the distribution of HLB disease among the agro-ecological zones of Machakos district (Appendix 1). The upper midland 4 AEZ had the highest visual mean scores of 2.33 (Figure 4.4), followed by lower highland 5, Lower highland 4, Lower midland 4, Upper midland 3, Lower midland 3 and lower midland 5, with mean HLB disease visual scores of 2.25, 2.00, 2.00, 1.92, 1.92, and 1.08 respectively. A disease trend was such that the higher altitudes had higher disease visual mean scores than the lower altitudes in Machakos district. In lower midland 5, rough lemons and valencia orange recorded the lowest visual mean scores of 0.3 (Table 4.1), while the highest mean visual scores (3.0) were recorded in the lower highland 3. Washington navel oranges had the highest mean visual score in the lower highlands (3.0) while their lowest score was in lower midland 5 (1.7) (Table 4.1).

In Machakos district the variety and the agro-ecological zones significantly ($P<0.05$) influenced the distribution of the HLB disease (Appendix 1). Rough lemon and tangerines had the lowest mean visual scores in the lowest altitudes in LM5 (< 2) (Table 4.1), while washington navel oranges had the highest HLB disease mean visual scores in LH4 and LH5 (>2) (Table 4.1).

Figure 4.4. Mean HLB symptoms visual score on citrus trees in different AEZs in Machakos district.



Error bars represent standard errors. Data are means ± s.e of 12 plants (n=84) AEZs were compared by single factor ANOVA and means separated at p=0.05 level. The numerical visual scores represent the following; 1- interveinal chlorosis; 2- blotchy mottles with lopsided fruit; 3- small upright leathery leaves, massive fruit drop and dieback.

Table 4.1. Mean HLB visual scores on different citrus varieties across AEZs in Machakos district

VARIETY / AEZ s	Rough lemon	Tangerine	Valencia	Washington navel
LH4	1.667	1.667	1.667	3.000
LH5	2.667	0.667	2.667	3.000
UM3	2.333	1.000	2.000	2.333
UM4	1.333	2.333	3.000	2.667
LM3	1.000	1.333	2.333	3.000
LM4	2.333	2.000	1.000	2.667
LM5	0.333	2.000	0.333	1.667

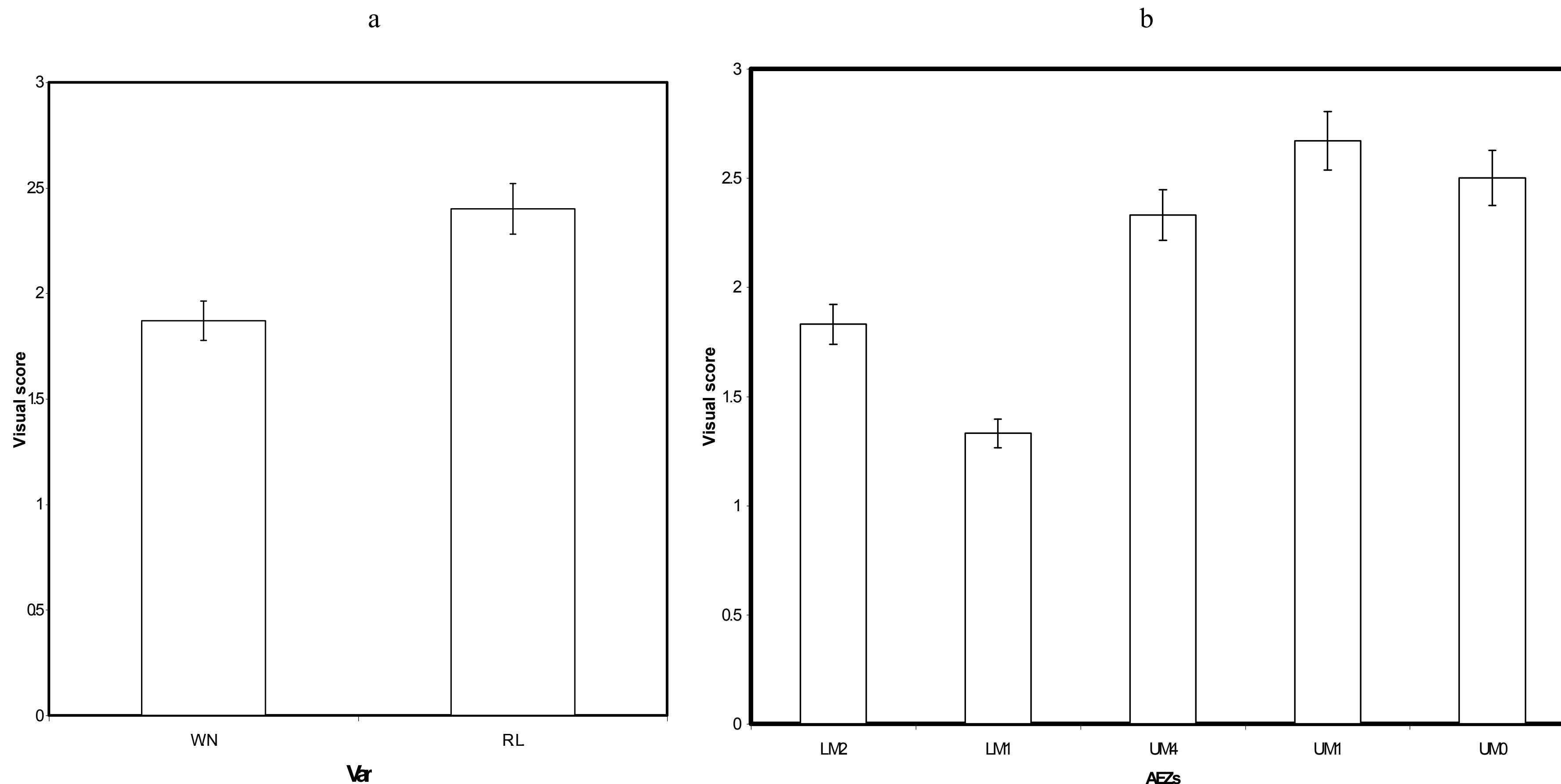
4.1.2 Distribution of the HLB disease across varieties and agro-ecological zone in Kakamega district (visual scores).

There were significant (P<0.05) differences in the distribution of the HLB disease among citrus varieties in Kakamega district (Appendix 2). Rough lemon had the highest mean visual score at 2.40 while washington navel oranges had the least mean visual score of 1.87 (Figure 4.5a). The rough lemon scored 3.0 in upper midland 0 and upper midland 1, while it had the least mean score in upper midland 4 with 1.67 (Table 4.2). Washington navel oranges had a high mean visual score in upper midland 4 (3.0) while it had a low mean score in lower midland 2 (Table 4.2).

There were significant (P<0.05) differences in the distribution of HLB disease among the agro-ecological zones of Kakamega district (Appendix 2). The Upper midland 1 AEZ had the highest visual mean scores of 2.67 (Figure 4.5b), followed by upper midland 0, Upper midland 4, Lower midland 2, and lower midland 1, with mean HLB disease visual scores of 2.50, 2.33, 1.83 and 1.33 respectively (Table 4.2). The results show that the HLB disease visual mean scores were higher in the upper midlands than in the lower midlands of Kakamega district.

There was significant (P<0.05) interaction between the agroecological zones and the citrus varieties in influencing the mean visual disease scores in Kakamega district (Appendix 2). Citrus varieties (washington navel orange and rough lemon) located in the high altitude AEZs like upper midland 0 and upper midland 1 had higher HLB disease scores than those located in lower AEZs (lower midland 2, lower midland 1) (Table 4.2).

Figure 4.5. Mean HLB symptoms visual score on two citrus varieties (a) in different AEZs (b) of Kakamega district.



Error bars represent standard errors. Data are means ± s.e of 6 plants per AEZ and 15 plants per variety (n=30) varieties and AEZs were compared by single factor ANOVA and means separated at p=0.05 level. The numerical visual scores represent the following; 1- interveinal chlorosis; 2- blotchy mottles with lopsided fruit; 3- small upright leathery leaves, massive fruit drop and dieback.

Table 4.2. Mean HLB visual scores on different citrus varieties across AEZs in Kakamega district

AEZ s \ VARIETY	Rough lemon	Washington navel
LM1	2.33	0.33
LM2	2.00	1.67
UM0	3.00	2.00
UM1	3.00	2.33
UM4	1.67	3.00

4.2 Survey of psyllid distribution across AEZs and varieties in Machakos district.

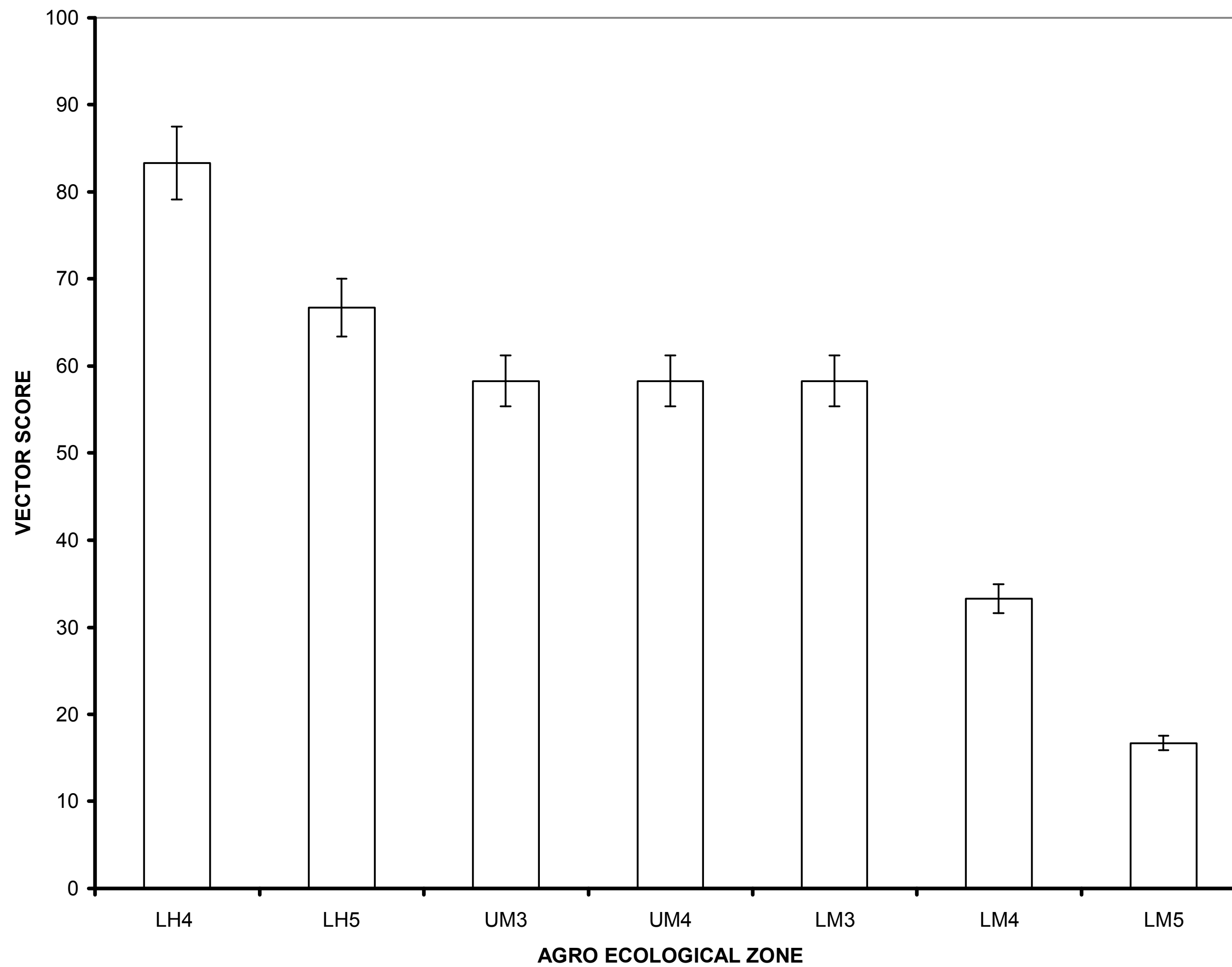
Results on the distribution of the psyllid (*Trioza erytreae*) are presented below.

4.2.1 Distribution of psyllids across AEZs and varieties in Machakos district

The results show that there were significant ($P<0.05$) differences in the mean psyllid (*T. erytreae)* score among the AEZs in Machakos district (Appendix 1). The high altitude areas in Machakos district (LH4, LH5, UM3 and UM4) had larger mean psyllid scores (0.883, 0.667, 0.583 and 0.583 respectively) while the lower midlands (LM3, LM4 and LM5) had lower means (0.483, 0.333 and 0.167 respectively) (Figure 4.6). The results show a gradual decrease in the mean vector score with decrease in altitude. The highest mean vector score (1.0) was found in LH4 on rough lemon and washington navel orange. The high score was also found in LH5 on washington navel and valencia. The least mean psyllid score of (0.0) was found on tangerine (LM5, UM3, UM4), washington navel (LM5, LM5) and valencia (LM5) (Table 4.3).

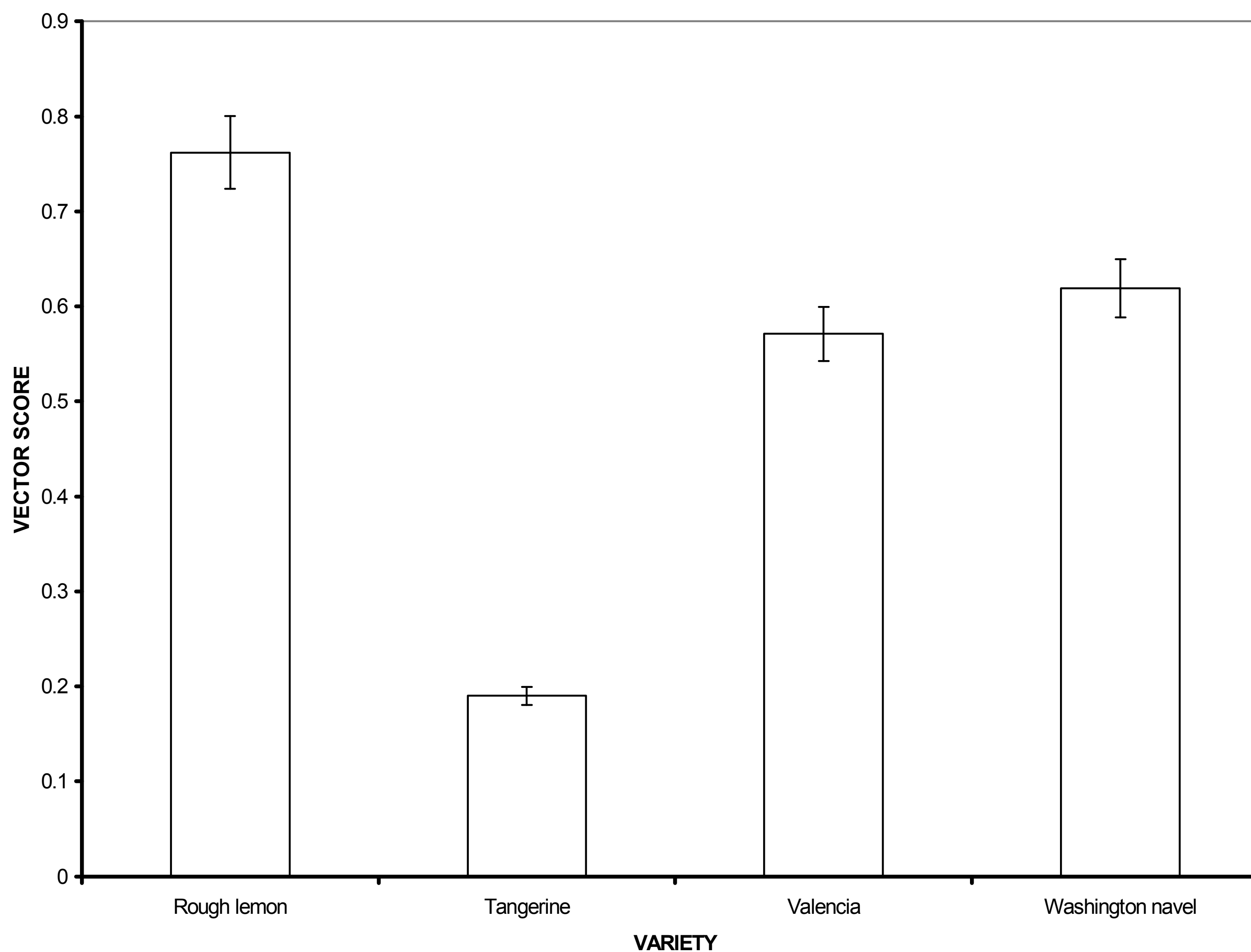
The four citrus varieties significantly ($P<0.05$) differed in their mean psyllids score in Machakos district (Appendix 1). Rough lemon had the highest mean psyllid score of 0.762 followed by washington navel, valencia and tangerine at a mean vector score of 0.619, 0.571 and 0.190 respectively (Figure 4.7). The rough lemons had high mean psyllid scores, both in high and low altitude areas (>0.667) (Table 4.3). The lemons had a maximum mean score (1.0) in LH4 and UM4; they had an equally high score (0.667) in LH5, LM3, LM4, LM5 and UM3. Tangerines had low mean scores in all agroecological zones except in LH4 where they had a score of 0.667 (Table 4.3). The tangerines had a 0.0 mean psyllid score in UM4, UM3, LM5 and LH5. This score influenced tangerines to have the least scores and rough lemons to be on the other extreme. Valencia and washington navels oranges had average scores (Figure 4.7).

Figure 4.6. Mean psyllid score on citrus in different AEZS in Machakos district.



Error bars represent standard errors. Data are means ± s.e of 12 plants (n=84). AEZS were compared by single factor ANOVA and means separated at p=0.05 level.

Figure 4.7. Mean psyllid score on different citrus varieties in Machakos district.



Error bars represent standard errors. Data are means ± s.e of 21 plants (n=84). Varieties were compared by single factor ANOVA and means separated at p=0.05 level.

Table 4.3. Mean psyllid scores on different citrus varieties across AEZs in Machakos district

VARIETY / AEZ s	Rough lemon	Tangerine	Valencia	Washington navel
LH4	1.000	0.667	0.667	1.000
LH5	0.667	0.000	1.000	1.000
UM3	0.667	0.000	0.667	1.000
UM4	1.000	0.000	0.667	0.667
LM3	0.667	0.333	0.667	0.667
LM4	0.667	0.333	0.333	0.000
LM5	0.667	0.000	0.000	0.000

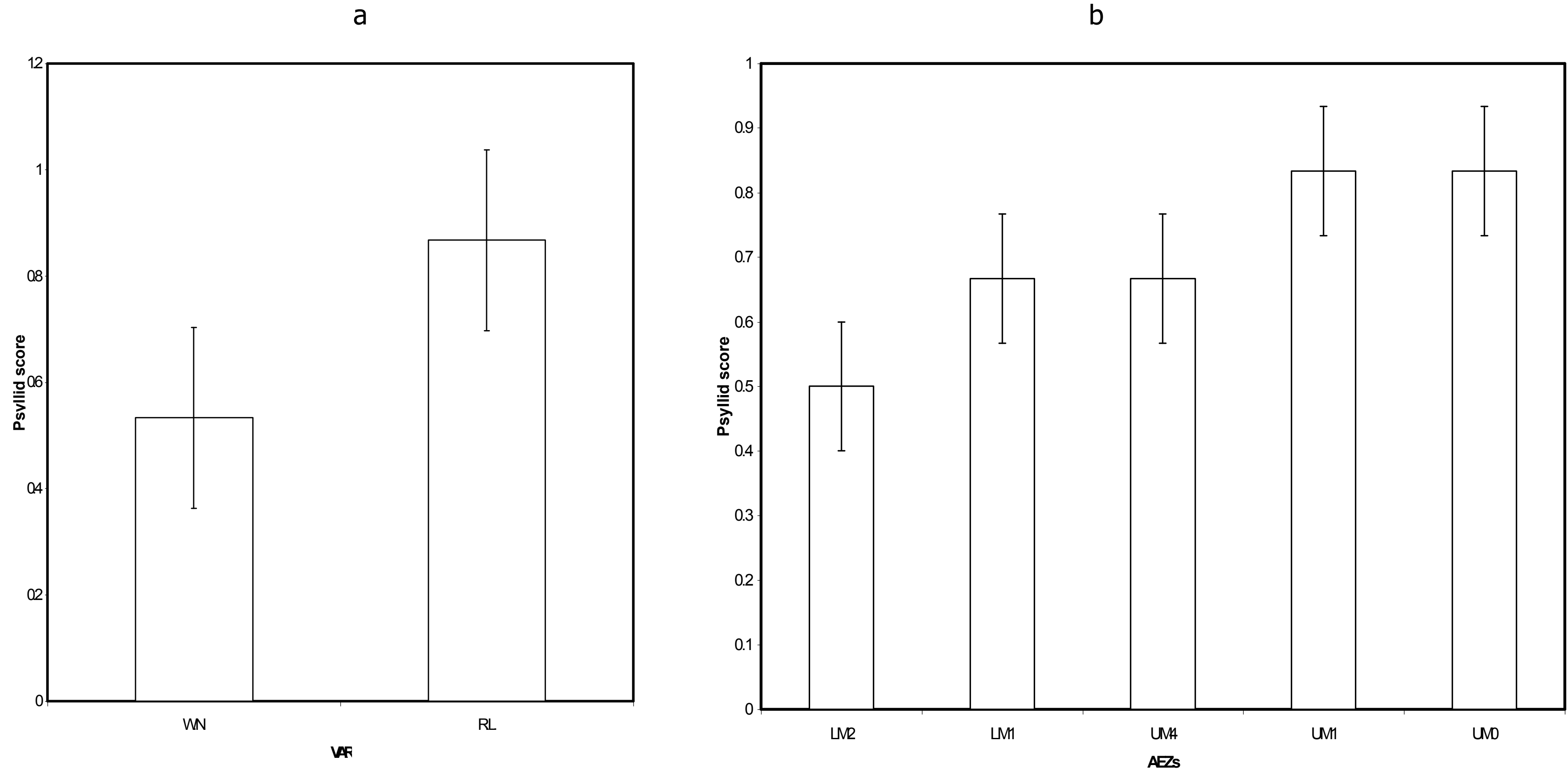
4.2.2 Distribution of psyllids across AEZs and varieties in Kakamega district

Citrus varieties in Kakamega district did not significantly (P<0.05) differ in their mean psyllids scores (Appendix 1). Nevertheless, rough lemon had very high mean scores of 0.867, while the washington navel oranges had an average mean score of 0.533 (Figure 4.8a). Rough lemon had a maximum score of (1.0) in three AEZs (UM0, UM1 and UM4), which lie within the high altitudes, while it had a mean score of 0.667 in the lower altitudes (LM1 and LM2) (Table 4.4). On the other hand washington navel oranges had very low psyllid mean scores (0.333) in two AEZs (LM2 and UM4), while they had high mean scores (0.667) in three AEZs (LM1, UM0 and UM1) (Table 4.4).

There were insignificant (P<0.05) differences in the mean psyllid score among the AEZs in Kakamega district (Appendix 2). Generally the agroecological zones in the lower altitudes (lower midland 2 and lower midland 1) had lower mean psyllid scores (0.500 and 0.667 respectively) while the agroecological zones in the higher altitudes (upper midland 0, upper midland 1 and upper midland 4) had high mean scores (0.833, 0.833 and 0.667 respectively) (Figure 4.8b). The results show a gradual decrease in the mean vector score with decrease in altitude (Figure 4.8b). The rough lemon showed a high psyllid score (>0.667) in all the sampled AEZs in Kakamega district, however washington navel orange had a low psyllid score (0.333) in UM4 and LM2 (Table 4.4).

The interaction between the mean vector score of different varieties and agroecological zones was not significant (P<0.05) in Kakamega district (Appendix 1). These results show that the distribution of psyllid vectors on citrus bushes in Kakamega district was dependent on neither the citrus varieties present nor agroecological zones in which the bushes were located.

Figure 4.8. Mean psyllid vector score on two citrus varieties (a) and in different AEZs of Kakamega district (b).



Error bars represent standard errors. Data are means ± s.e of 6 plants per AEZ and 15 plants per variety (n=30) varieties and AEZs were compared by single factor ANOVA and means separated at p=0.05 level.

Table 4.4. Mean psyllid vector scores on different citrus varieties across AEZs in Kakamega district.

VARIETY / AEZ s	Rough lemon	Washington navel
LM1	0.667	0.667
LM2	0.667	0.333
UM0	1.000	0.667
UM1	1.000	0.667
UM4	1.000	0.333

4.3 Polymerase chain reaction.

4.3.1 Amplification of the L10/L12 rDNA

PCR amplifications showed successful cloning (in-vitro) of the L10/L12 ribosomal protein rDNA of the alpha proteobacteria that causes citrus greening disease in Machakos and Kakamega districts (Figure 4.9). The PCR reaction using the L10/L12 rDNA primers amplified a fragment of 716-720 bp from different bacterial isolates (Figure 4.9 lanes 2,3,4,5,6). This minute difference of 4 bases was not easy to differentiate with agarose based gel electrophoresis (Figure 4.9 lanes 2,3,4,5,6). A DNA band was amplified from all the citrus diseased materials from Machakos and Kakamega districts showing the greening symptoms. There was no amplification from un-diseased plants, which were propagated through tissue culture and maintained in the greenhouses. The DNA band amplified was similar from all the diseased plants in different AEZs. The band was also similar from all the diseased plants from different varieties (Figure 4.9 lanes 2,3,4,5,6).

4.3.2 PCR scores across AEZs and varieties in Machakos district.

The results showed significant ($P<0.05$) differences in the mean PCR score among the agroecological zones in Machakos district at (Appendix 1). High altitude AEZs (LH4 and LH5) had an exceptionally high mean PCR score of 0.833, while low altitude AEZs like LM4 and LM5 had low mean PCR scores of 0.417 and 0.333 respectively (Figure 4.10). Washington navel had a maximum mean PCR score of 1.0 in five AEZs namely LH4, LH5, LM3, LM4 and UM4 (Table 4.5). The 0.0 mean score was rare and was only found on rough lemon in LM5, valencia in LM4 and washington navel in LM5. These results show a trend, in which the lower highlands have large PCR mean scores, the upper midlands have average PCR mean scores and the lower midlands have low PCR mean scores.

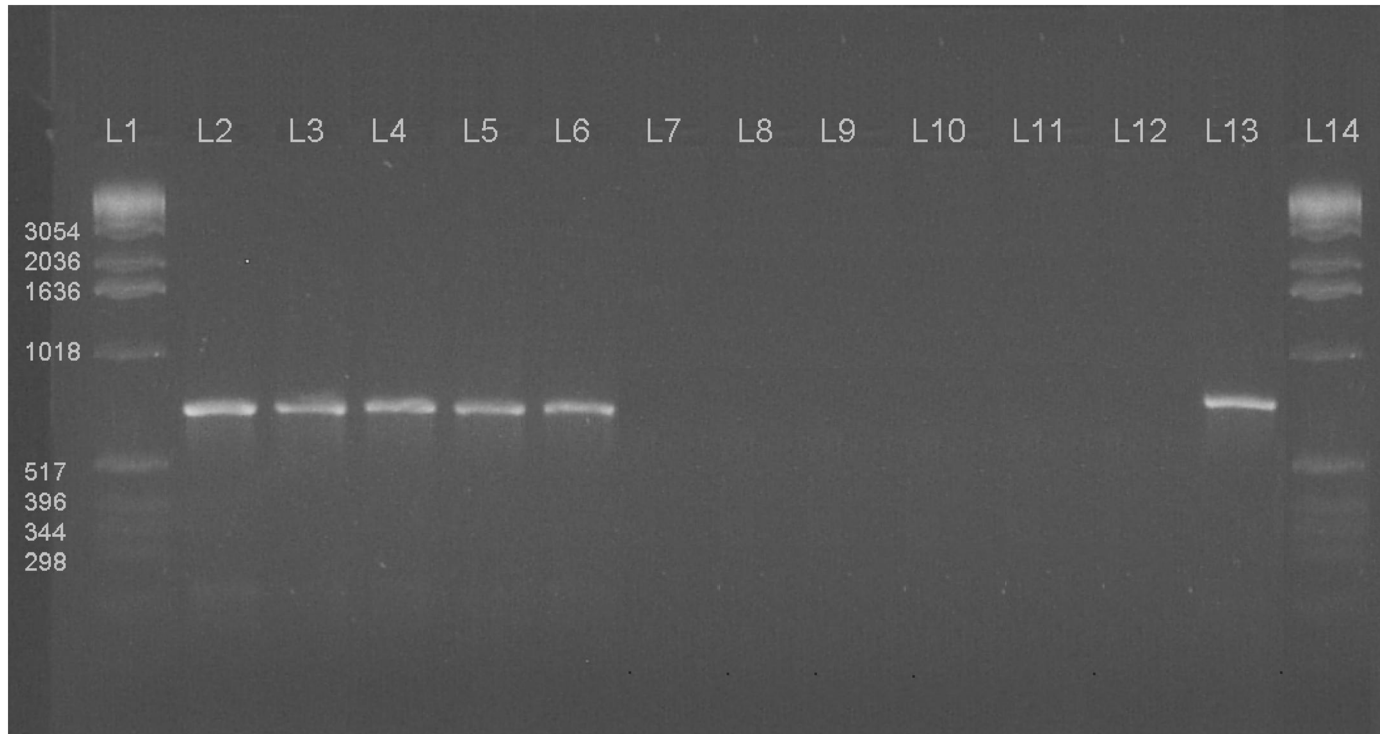
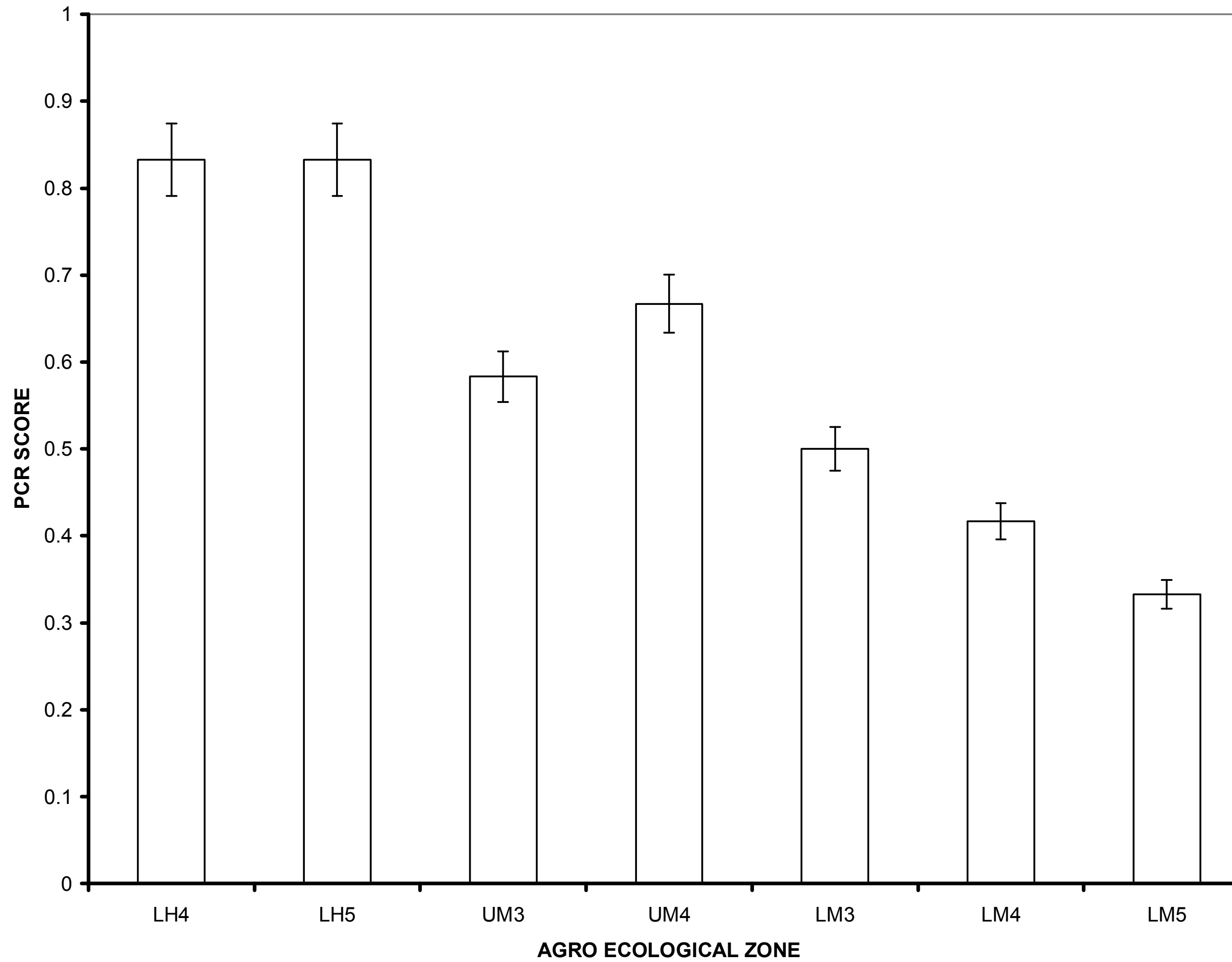


Figure 4.9. 1% agarose electrophoresis gel of L10/L12 ribosomal protein rDNA. Lane12- negative control; Lane 13- positive control; Lanes 7,8,9,10,11- no amplification; Lanes 2,3,4,5,6-positive 716bp amplification from infected samples; Lane 1 & 14-1Kb DNA ladder. In this PCR reaction the bacterial rpl protein intergenic region was amplified from plants that were showing symptoms associated with the disease.

Figure 4.10. Mean PCR score of citrus trees in different AEZs in Machakos district.



Error bars represent standard errors. Data are means ± s.e of 12 plants (n=84) AEZs were compared by single factor ANOVA and means separated at p=0.05 level.

Table 4.5. Mean PCR scores on different citrus varieties across AEZs in Machakos district

VARIETY / AEZ s	Rough lemon	Tangerine	Valencia	Washington navel
LH4	0.667	1.000	0.667	1.000
LH5	1.000	0.333	1.000	1.000
UM3	0.667	0.333	0.667	0.667
UM4	0.333	0.667	0.667	1.000
LM3	0.000	0.333	0.667	1.000
LM4	0.333	0.333	0.000	1.000
LM5	0.000	1.000	0.333	0.000

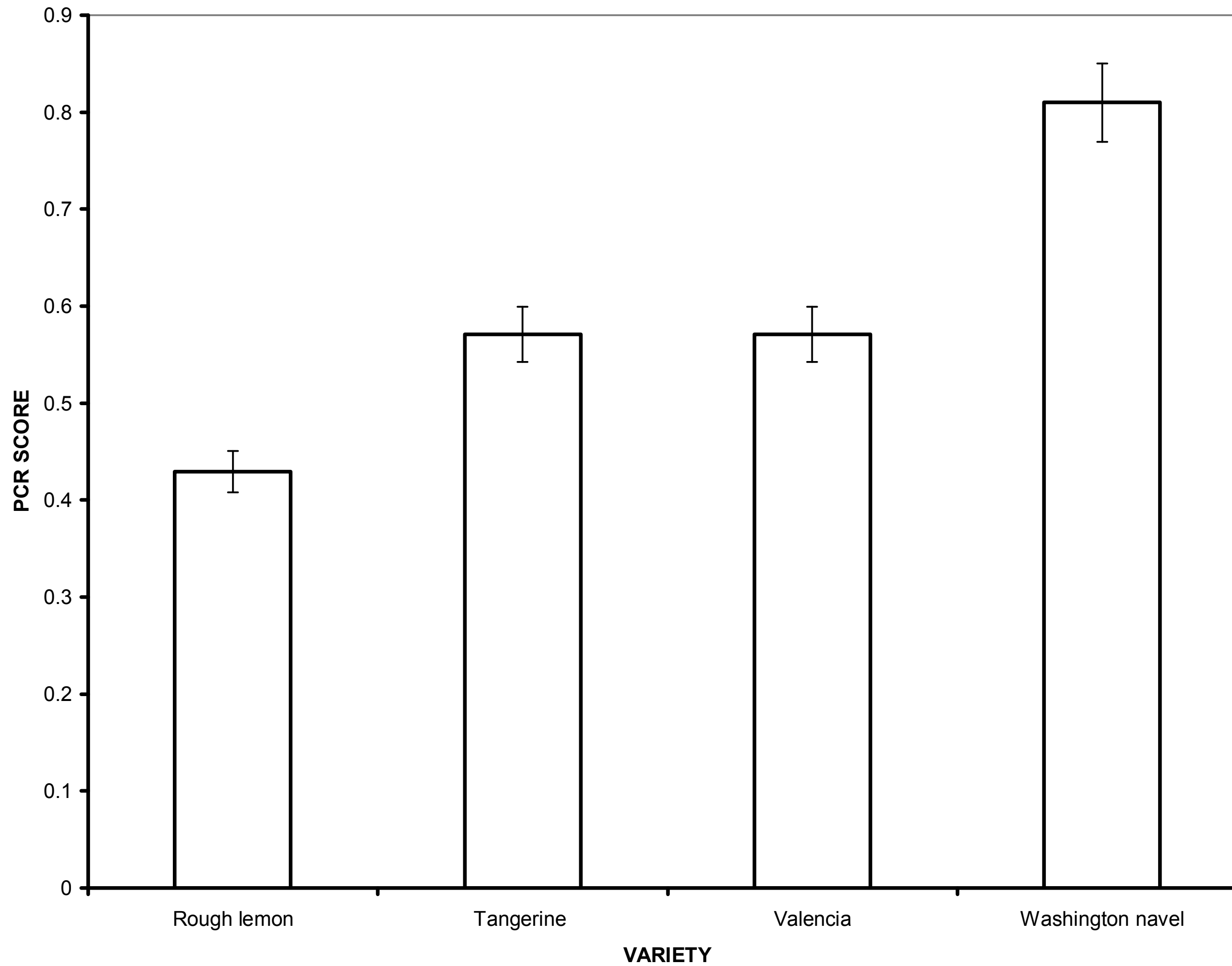
The mean PCR scores differed significantly (P<0.05) among the 4 varieties in Machakos district (Appendix 1). The variety with the highest mean PCR scores was washington navel, which had a score of 0.810. It was followed by valencia, tangerine and rough lemon with mean scores of 0.571, 0.571 and 0.429 respectively (Figure 4.11). The rough lemon had the least mean score (0.0) in lower midland 5 and Lower midland 4, washington navel and valencia had the least mean score (0.0) score in lower midland 5 and lower midland 4 respectively, while the tangerine never had the (0.0) mean score in any of the AEZs in Machakos (Table 4.5).

4.3.3 PCR scores across AEZs and varieties in Kakamega district.

The results show insignificant (P<0.05) differences in the mean PCR score among the agroecological zones in Kakamega district (Appendix 2). However the general trend is that the AEZs found in the high altitudes (upper midland 0, upper midland 1 and upper midland 4) have large mean PCR scores (0.833, 0.667 and 0.667 respectively), while the AEZs in the low altitude regions (lower midland 1 and lower midland 2) have mean PCR scores of 0.333 and 0.667 respectively (Figure 4.12a). Rough lemon has the highest mean score in upper midland 0 (1.0) while it has the lowest score in upper midland 4 (0.333) (Table 4.6).

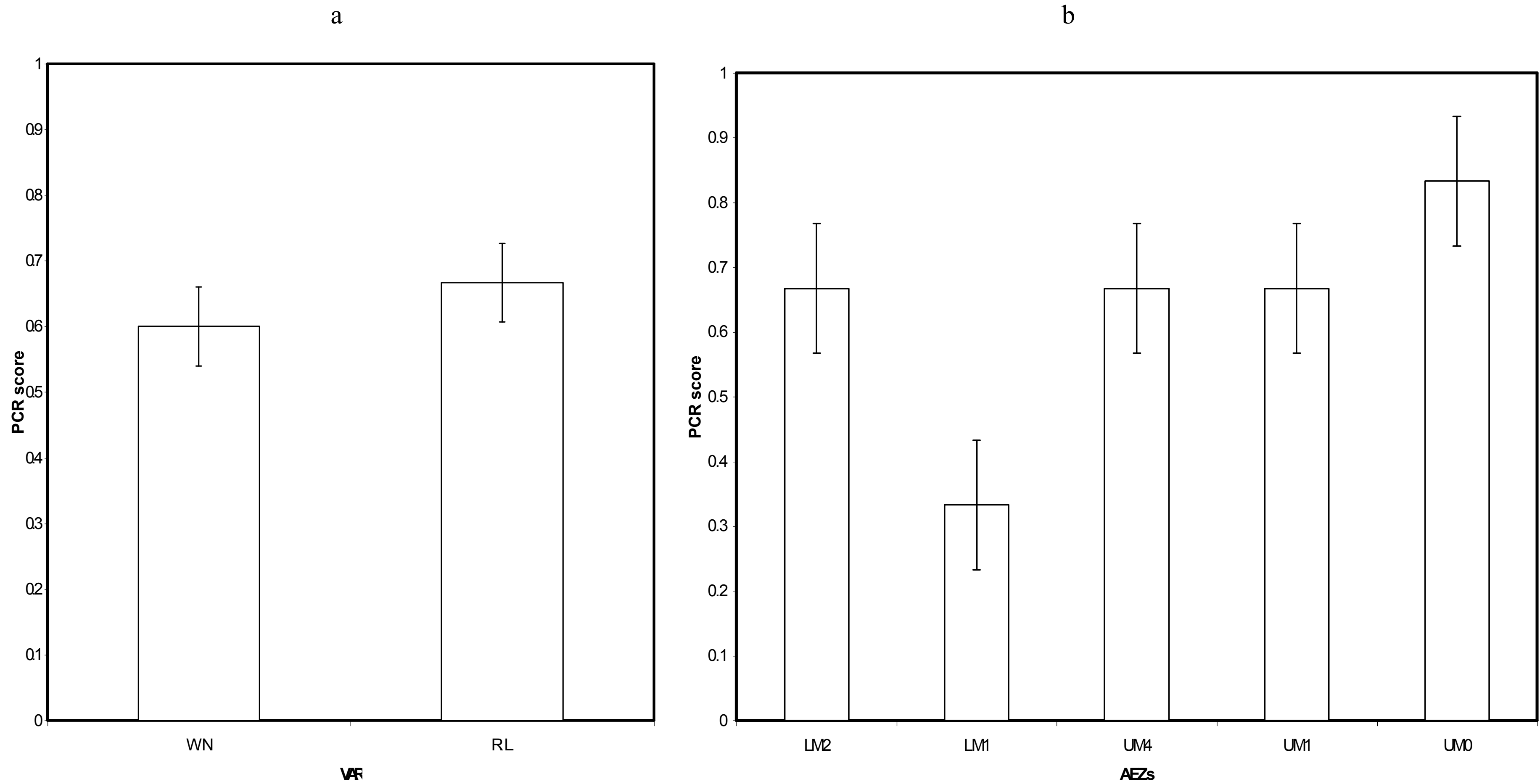
The results show that there are insignificant (P<0.05) mean PCR scores among the varieties in Kakamega district (Appendix 2). However, the two varieties showed high mean PCR scores. Rough lemon had a mean PCR score of 0.667, while washington navel had a score of 0.600 (Figure 4.12b). Washington navel had high mean scores (> 0.667) in all AEZs except in lower midland 1 where it scored 0.0 (Table 4.6). on the other hand rough lemon had a high mean score in all varieties except in upper midland 4 where it score 0.333 (Table 4.6).

Figure 4.11. Mean PCR score on different citrus varieties in Machakos district.



Error bars represent standard errors. Data are means ± s.e of 21 plants (n=84) varieties were compared by single factor ANOVA and means separated at p=0.05 level.

Figure 4.12. Mean PCR score on two citrus varieties (a) in five different AEZs (b) of Kakamega district.



Error bars represent standard errors. Data are means ± s.e of 6 plants per AEZ and 15 plants per variety (n=30) varieties and AEZs were compared by single factor ANOVA and means separated at p=0.05 level.

Table 4.6. Mean PCR scores on different citrus varieties across AEZs in Kakamega district.

VARIETY / AEZ s	Rough lemon	Washington navel
LM1	0.667	0.000
LM2	0.667	0.667
UM0	1.000	0.667
UM1	0.667	0.667
UM4	0.333	1.000

4.3.4 Correlation of the PCR, visual and vector scores.

To ascertain the efficiency of the mean HLB disease PCR score, the mean HLB disease visual score and the mean psyllid vector scores as tools for determining the presence of the HLB disease of citrus, the results from the three tools were correlated. A correlation matrix (Table 4.7 a) for Machakos showed that the mean HLB disease PCR scores and the mean HLB disease visual scores were highly correlated, having a correlation coefficient of 0.703. There was low correlation between the mean psyllid vector score and the mean HLB disease visual score (0.202), similarly, the correlation between the mean psyllid vector score and the mean HLB disease PCR score was low (0.108) (Table 4.7 a).

A correlation matrix for the Kakamega data also exhibited high correlation between the mean HLB disease visual scores and the mean HLB disease PCR scores (0.661) (Table 4.7 b). On the other hand the correlation between the mean HLB disease visual score and the mean psyllid vector score was very low at 0.088, while that between the mean psyllid vector and the mean HLB disease PCR scores was negative (-0.045) (Table 4.7 a). The correlations between the three tools were generally lower in Kakamega than in Machakos district.

The regression line fitted for the visual and vector scores was highly significant ($P<0.05$) for Kakamega and Machakos data alike (Appendix 3). This means that HLB mean visual scores could be sufficiently used in place of PCR scores to detect for the HLB disease in Machakos and Kakamega districts. However, neither the visual nor the PCR scores may be used in place of the vector score.

Table 4.7 a. Correlation matrix of PCR, visual and vector scores in Machakos.

PCR	1.000		
VECTOR	0.108	1.000	
VISUAL	0.703	0.202	1.000
	PCR	VECTOR	VISUAL

Table 4.7 b. Correlation matrix of PCR, visual and vector scores in Kakamega.

PCR	1.000		
VECTOR	-0.045	1.000	
VISUAL	0.661	0.088	1.000
	PCR	VECTOR	VISUAL

4.3.5 Amplification of the 16S rDNA

A DNA fragment, 1100 bp long was amplified from diseased samples from Machakos. This band was not present when disease free samples from the greenhouses were used to provide PCR template. The PCR reaction was used to test and confirm the presence of the disease. The results showed that plants from tissue culture supported nurseries were HLB-disease free (Figure 4.13 lanes 5,6), while plants from low altitude nurseries/fields (1000 m above sea level) and high altitude (2000 m) not supported by tissue culture were infected with the "*Candidatus* L. africanus" giving a PCR fragment of 1160 base pairs (Figure 4.13 lanes2,3,4). However this reaction had no potential of characterizing the bacterium, because the 16S rDNA had limited polymorphism and it was largely conserved than the adjacent 16S/23S rDNA.

4.3.6 Amplification of the 16S/23S rDNA

A band of 800 bp was amplified from diseased samples from Machakos (Figure 4.14, lane 4). This band was not present when disease free samples from the greenhouses were used as the PCR template. The results showed that the 16S/23S rDNA could be used to index for the greening disease in Machakos district (Figure 4.14, lane4). The reaction gave a positive amplification only when samples from Machakos were used. Samples from Kakamega that were positively tested for the presence of the bacteria could not produce the 800bp PCR product, rather they gave low molecular weight products that were thought to be primer dimmer artefacts or a product of mispriming (Figure 4.14, lane 5,6). In this, reaction negative controls consistently gave the expected results but the positive controls did not consistently produce an 800bp PCR product (Figure 4.14, lane 1,2). This situation could have been due to differences in template quality or primer annealing sites.

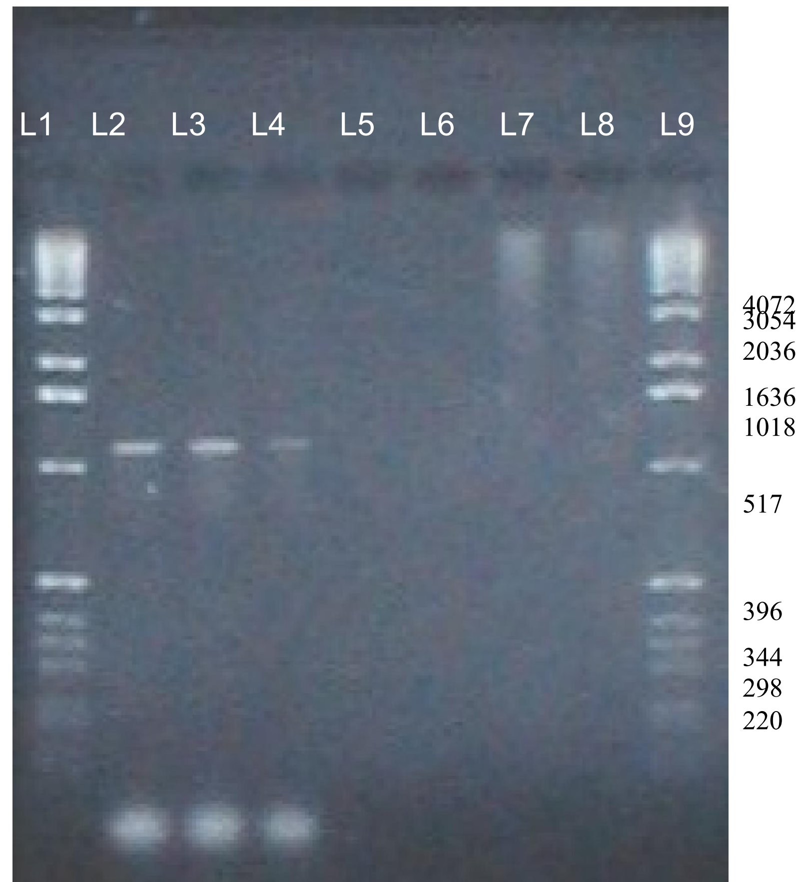


Figure 4.13: 1% agarose electrophoresis gel of 16S rDNA PCR products. Lanes 5&6- undiseased; Lane 2, 3 &4 - positive 1160bp amplification from infected samples; Lane1&9- 1Kb DNA ladder; Lane 7&8- bacterial genomic DNA.

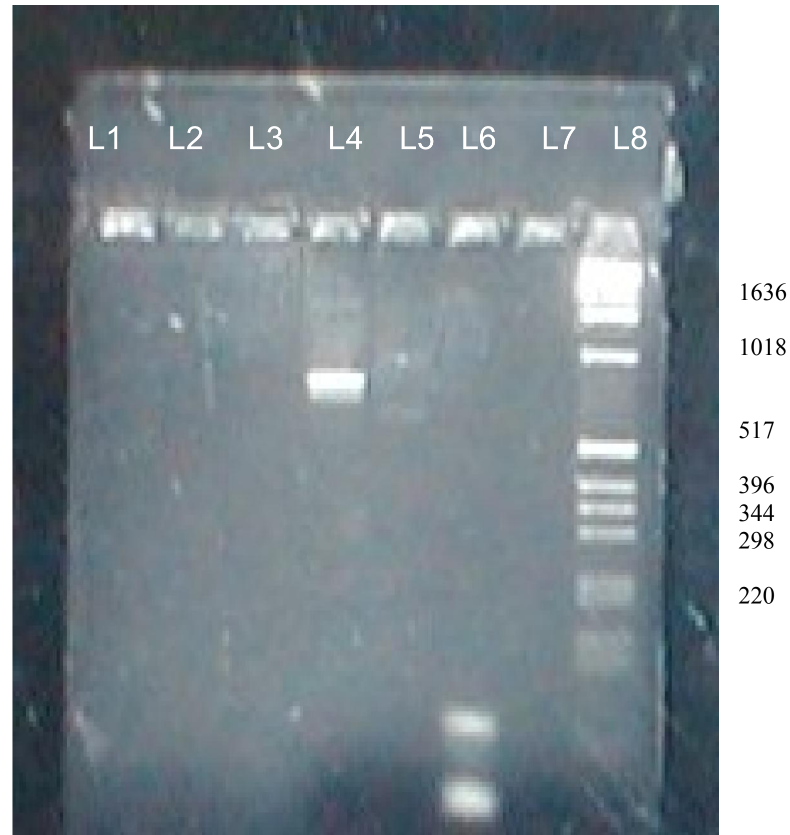


Figure 4.14. 1% agarose electrophoresis gel of 16S/23S rDNA PCR products. A positive amplification band of 800 base pairs was seen in diseased samples from Machakos while it wasn't achieved from diseased samples from Kakamega. L1- Negative control; L2- undiseased tissue culture; L3- undiseased tissue culture; L4-diseased material Machakos; L5- diseased material Machakos; L6-diseased material Kakamega; L7- diseased material Kakamega; L8-1KB DNA ladder.

4.4 Sequences of the L10-L12 ribosomal protein DNA

Sense and antisense strands were sequenced for UM4 and LM4 bacterial isolates while only sense strand sequences were obtained from LH4, UM3, LM3 and LM5 bacterial isolates. In Kakamega two sequences were obtained from upper midland 0 and lower midland 2. These sequences are presented below.

The UM4 sequence had 720 bases with an intergenic sequence of 44 bases between the DNA sequences that code for the L10 and the L12 proteins (Figure 4.15). The sequence that codes for the L10 protein is 516 bases long from base 17 to base 534. The L12 protein starts at base 579 but it is not complete due to the PCR primer position. The intergenic region has 19 adenine bases, 12 thymine bases, 6 cytosine bases and 7 guanine bases. This region has 18 pyrimidines and 26 purines. The UM4 antisense strand was sequenced to confirm the sequence of the sense strand (Figure 4.16). This strand had 720 bases and had complete complimentarity with the sense stand except for those bases that were not read and are denoted by (n). The noted complimentarily established the precision of the DNA sequencer and secondly and more important certified the sense strand.

The UM3 sequence had 720 bases with an intergenic sequence of 44 bases between the DNA sequences that code for the L10 and the L12 proteins (Figure 4.17). The sequence that codes for the L10 protein is 516 bases long from base 17 to base 534. The L12 protein gene starts at base 579 but it is not complete due to the PCR primer position. The intergenic region has 19 adenine bases, 12 thymine bases, 6 cytosine bases and 7 guanine bases. The sequence has a long chain of non-read bases between bases 444 and 479.

Figure 4.15 Upper midland 4 (UM4) ribosomal protein L10/L12 rDNA sense strand.

```
  1 catcggnagatgaaagttgaanaggcaagaaaagagtgtnnaaatttctgaattaagtaa 60
 61 gattttttcttcttcnggatcagttgttgttgcgcactataagggaattagtgtagcgca 120
121 gataaaagatcttcgaaagaaagtgcgagaagctggtggaggtgtaaaggttgcgaaaaa 180
181 tcgtcttgttnagattgccgtcagcgatactagtttgaagggtgtttcagatctttttgt 240
241 tgggcaatcattgattgtttattcggttgaccctattgttgctcctaagatttctgtgag 300
301 ctttgcgaatgataataaacagtttgtggttcttggtggtattttggagaaagatattct 360
361 tgaccaagattctatcaaacggattgcttcgttgcctaatattgatgntattcgttctat 420
421 gatcattagtgctattcaatttaattcgactagattggtaaatcttcttaatgcacctca 480
481 gactaaaattgttcgtgctatttctgcttttgtagataaaaatcaacaaagttagactcc 540
541 aagttattaataccaacaagaataaggaaaatgtgtgatatgtccaatattgaatcaatt 600
601 gttnaaaaattatcgtctcttacgctccttcaagcggcagaactttctaaaagattagaa 660
661 gaagaatggggagtttctgctgctgctcctgtagctgttgttgcgtctgcggcaggggaa 720
```

Figure 4.16 Upper midland 4 (UM4) ribosomal protein L10/L12 rDNA antisense strand

```
  1 ttcccctgcagcagacgcaacaacagctacaggagcagcagcagaaactccncattcttc 60
 61 ttctaatcttttagaaagttctgccgcttgaaggagcgtaagagacgataatttttcaac 120
121 aattgattcaatattggacatatcacacattttccttattcttgttggtattaataactt 180
181 ggagtctaactttgttgatttttatctacaaaagcagaaatagcacgaacaattttagtc 240
241 tgaggtgcattaagaagatttaccaatctagtcgaattaaattgaatagcactaatgatc 300
301 atagaacgaataccatcaatattaggcaacgaagcaatccgtttgatagaatcttggtca 360
361 agaatatctttctccaaaataccaccaagaaccacaaactgtttattatcattcgcaaag 420
421 ctcacagaaatcttaggagcaacaatagggtcaaccgaataaacaatcaatgattgccca 480
481 acaaaaagatctgaaacacncttcaaactagtatcgctgacggcaatcttaacaagacga 540
541 tttttcgcaacctttacacctccaccagcttctcgcactttctttcgaagatcttttatc 600
601 tgcgctacactaattcnnttatagtgcgcaacaacaactgatccagaagaagaaaaaatc 660
661 ttacttaattcagaaattaccacgctcttttcttgnctattcaactttcatctcccgatg 720
```

Figure 4.17 Upper midland 3 (UM3) ribosomal protein L10/L12 rDNA sense strand.

```
  1 catcgggagatgaaagttgaataggcaagaaaagagtgtnnaaatttctgaattaantaa 60
 61 gatttnttcttcttctgnntcagttgttgttgcgcactataagnnaattagtgtagcgca 120
121 gataaaagatcttcgaaagaaagtgcgagaagctggtggnggtgtaaaggttgcgaaaaa 180
181 tcgtcttgttaagattgccgtcagcgatactagtttgaagggtgtttnngatctttttgt 240
241 tgggcaatcattgattgtttattcggttgaccctattgttgctcctaagatttctgtgag 240
301 ctttgcnaatgataataaacagtttgtggttcttggtggtattttggagaaagatattct 360
361 tgaccaagattctatcaaacnnattgcttcgttgcctaatattgatngtattcgttctat 420
421 gatcattagtgctattcaatttannnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnna 480
481 gactaaanttgttcgtgctatttctgcttttgtagataaaaatcaacaaagttagactcc 540
541 aagttattaataccaacaagaataaggaaaatgtgtgatatgtccaatattgaatcaatt 600
601 gttgaaaaattatcgtctcttacgctccttcaagcnncagaactttctaaaagattagaa 660
661 gaagaatggggagtttctgctgctgctcctgtagctgttgttgcgtctgctgcaggggaa 720
```

The LM3 sequence had 720 bases with an intergenic sequence of 44 bases between the DNA sequences that code for the L10 and the L12 proteins (534-579) (Figure 4.18). The sequence that codes for the L10 protein is 516 bases long from base 17 to base 534. The L12 protein starts at base 579. The intergenic region has 19 adenine bases, 12 thymine bases, 6 cytosine bases and 7 guanine bases. This region has 18 pyrimidines and 26 purines.

The LM4 sequence had 720 bases with an intergenic sequence of 44 bases between the DNA sequences that code for the L10 and the L12 proteins (Figure 4.19). The sequence that coded for the L10 protein was 516 bases long from base 17 to base 534. The L12 protein started at base 579 but it was not complete due to the PCR primer position. The intergenic region was 19 adenine bases, 12 thymine bases, 6 cytosine bases and 7 guanine bases. This region had 18 pyrimidines and 26 purines.

The UM4 antisense strand was sequenced and found to have 720 bases just as its sense strand (Figure 4. 20). The sequence had an intergenic sequence of 44 bases between the DNA sequences that code for the L10 and the L12 proteins. The L12 protein started at base 579 but it was not complete due to the PCR primer position. The intergenic region was 19 adenine bases, 12 thymine bases, 6 cytosine bases and 7 guanine bases. This region had 18 pyrimidines and 26 purines. This sequence showed complete complimentarily with the sense stand except for those bases that were not read and denoted by (n). The noted complimentarily established the precision of the DNA sequencer and secondly and more important certifies the sense strand.

Figure 4.18 Lower midland 3 (LM3) ribosomal protein L10/L12 rDNA sense strand.

```
  1 catcgngagatgaaagttgaataggcaagaaaagagtgtnnaaatttctnaattaagtaa 60
 61 gatttttctncttctggatcaattgttgttgcgcactataagggaattagtgtagcgca 120
121 gataaaagatcttcgaaagaaagtgcgagaagctggtggaggtgtaaaggttgngaaaaa 180
181 tcgtcttgttaagattgccgtcagcgatactagtttgaagggtgtttcagatctttttnt 240
241 tgggcaatcattgattgtttattcggttgaccctattgttgctcctaagatttctgtgag 300
301 ctttgcgaatgataataaacagtttgtggttcttggtggtattttggagaaagatattct 360
361 tgaccaagattctatcaaacggattgcttcgttgcctaatattgatggtattcgttctat 420
421 gatcattagtgctattcaatttaattcgactagattggtaaatcttcttaatgcacctca 480
481 gactaaaattgttcgtgctatttctgcttttgtagataaaaatcaacaaagttagactcc 540
541 aagttattaataccaacaagaataaggaaaatgtgtgatatgtccaatattgaatcaatt 600
601 gttnaaaaattatcgtctcttacgctccttcaagcggcagaactttctaaaagattagaa 660
661 gaagaatggggagtttctgctgctgctcctgtagctgttgttgcgtctgctgcaggggaa 720
```

Figure 4.19 Lower midland 4 (LM4) ribosomal protein L10/L12 rDNA sense strand

```
  1 catcgggagatgaaagttgaataggcaagaaaagagtgtnnaaatttctgaattaagtaa 60
 61 gatttttcttcttctggatcagttgttgttgcgcactataagggaattagtgtagcgca 120
121 gataaaagatcttcgaaagaaagtgcgagaagctggtggaggtgtaaaggttgcgaaaaa 180
181 tcgtcttgttaagattgccgtcagcgatactagtttgaagggtgtttcagatctttttgt 240
241 tgggcaatcattgattgtttattcggttgaccctattgttgctnnnaagatttctgtgag 300
301 ctttgcgaatgataataaacagtttgtggttcttggtggtattttggagaaagatattct 360
361 tgaccaagattctatcaaacggattgcttcgttgcctaatattgatnntattcgttctat 420
421 gatcattagtgctattcaatttaattcgactagattggtaaatcttcttaatgcacctca 480
481 gactaaaattgttcgtgctatttctgcttttgtagataaaaatcaacaaagttagactcc 540
541 aagttattaataccaacaagaataaggaaaatgtgtgatatgtccaatattgaatcaatt 600
601 gttgaaaaattatcgtctcttacgctccttcaagcggcagaactttctaaaagattagaa 660
661 gaagaatggggagtttctgctgctgctcctgtagctgttgttgcgtctgctgcaggggaa 720
```

Figure 4.20 Lower midland 4 (LM4) ribosomal protein L10/L12 rDNA antisense strand

```
  1 ttcccctgcagcagacgcaacaacagctacaggagcagcagcagaaactccgcattcttc 60
 61 ttctaatcttttagaaagttctgccgcttgaagnagcgtaagagacgataatttttcaac 120
121 aattgattcaatattggacatatcacacattttccttattcttgttggtattaataactt 180
181 ggagtctaactttgttgatttttatctacaaaagcagaaatagcacgaacaattttagtc 240
241 tgaggtgcattaagaagatttaccaatctagtcgaattaaattgaatagcactaatgatc 300
301 atagaacgaataccatcaatattaggcaacgaagcaatccgtttgatagaatcttggtca 360
361 agaatatctttctccaaaataccaccaagaaccacaaactgtttattatcattcgcaaag 420
421 ctcacagaaatcttaggagcaacaatagggtcaaccgaataaacaatcaatgattgccca 480
481 acaaaaagatctgaaacacccttcaaactagtatcgctgacngcaatcttaacaagacga 540
541 tttttcgcaacctttacacctccaccagcttctcgcactttctttcgaagatcttttatc 600
601 tgcgctacactaattcccttatagtgcgcaacaacaactgatccagaagaagaaaaaatc 660
661 ttacttaattcagaaattaccacgctcttttcttgnctattcaactttcatctcccgatg 720
```

The LM5 sequence had 720 bases (Figure 4.21) with an intergenic sequence of 44 bases between the DNA sequences that code for the L10 and the L12 proteins. The sequence that codes for the L10 protein is 516 bases long from base 17 to base 534. The L12 protein starts at base 579 but it is not complete due to the PCR primer position. The intergenic region has 19 adenine bases, 12 thymine bases, 6 cytosine bases and 7 guanine bases. This region has 18 pyrimidines and 26 purines.

The sequence from upper midland 0 (UM0) from Kakamega had 715 bases (Figure 4.22). Within this sequence there was an intergenic sequence of 40 bases between the DNA sequences that code for the L10 and the L12 proteins. This sequence was shorter than those from Machakos. The sequence that codes for the L10 protein is 516 bases long from base 17 to base 534. The L12 protein starts at base 574. The intergenic region has 16 adenine bases, 11 thymine bases, 6 cytosine bases and 7 guanine bases. This region has 17 pyrimidines and 23 purines.

The sequence from Lower midland 2 (LM2) from Kakamega had 715 bases (Figure 4.23). Within this sequence there was an intergenic sequence of 40 bases between the DNA sequences that code for the L10 and the L12 proteins. This sequence was shorter than those from Machakos. The sequence that codes for the L10 protein is 516 bases long from base 17 to base 534. The L12 protein starts at base 574. The intergenic region has 16 adenine bases, 11 thymine bases, 6 cytosine bases and 7 guanine bases. This region has 17 pyrimidines and 23 purines.

Figure 4.21 Lower midland 5 (LM5) ribosomal protein L10/L12 rDNA sense strand.

```
  1 catcgggagatgaaacttgaataggcaagaaaagagtgtnnaaannnctgaattaagtaa 60
 61 gatttttcttcttctggatcagttgttgttgcgcactataagggaatnagtgtagcgca 120
121 gataaaagatcttcgaaagaaagtnngagaagctggtggaggtgtaaaggttgcgaaaaa 180
181 tcgtcttgttaagattgccgtcagcgatactagtttgaagggtgtttcagatctttttgt 240
241 tgggcaatcattgattgtttattcggttgaccctattgttgctcntaagatttctgtgag 300
301 ctttgcgaatgataataaacagtttgtggttcttggtggtattttggagaaagatattct 360
361 tgaccaagattctatcaaacggattgcttcgttgcctaatattgatggtattcgttctat 420
421 gatcattagtgctattcaatttaattcgactagattggtaaatcttcttaatgcacctca 480
481 gactaaaattgttcgtgctatttctgcttttgtagataaaaatcaacaaagttagactcc 540
541 aagttattaataccaacaagaataaggaaaatgtgtgatatgtccaatattgaatcaatt 600
501 gttgaaaaattatcgtctcttacgctccttcaagcngcagaactttctaaaagattagaa 660
661 gaagaatgnggagtttctgctgctgctcctgtagctgttgttgcgtctgctgcaggggaa 720
```

Figure 4.22 Upper midland 0 (KUM) ribosomal protein L10/L12 rDNA sense strand from Kakamega district

```
  1 catcgggagatgaaagttgaataggcaagaaaagagtgtnnaaatttctgaattaagtaa 60
 61 gatttttcttcttctggatcagttgttgttgcgcactataagggaattnnngtagcgca 120
121 gataaaagatcttcgaaagaaagtgcgagaagctggtggaggtgtaaaggttgcgaaaaa 180
181 tcgtcttgttaagattgccgtcagcgatactagtttgaagggtgtttcagatctttttgt 240
241 tgggcaatcattgattgtttattcggttgaccctattgttgctcctaagatttctgtgag 300
301 ctttgcgaatgataataaacagtttgtggttcttggtggtattttggagaaagatattct 360
361 tgaccaagattctatcaaacggattgcttcgttgcctaatattgatggtattcgttctat 420
421 gatcattagtgctattcaatttaattcgactagattggtaaatcttcttaatgcacctca 480
481 gactaaaattgttcgtgctatttctgcttttgtagataaaaatcaacaaagttagactcc 540
541 aagttattccaacaagaataaggaaaatgtgtnatatgtccaatattgaatcaattgttg 600
601 aaaaattatcgtctcttacgctccttcaagcggcagaactttctaaaagattagaagaag 660
661 aatggggagtttctgctgctgctcctgtagctgttgttgcgtctgctgcaggggaa 715
```

Figure 4.23 Lower midland 2 (KLM) ribosomal protein L10/L12 rDNA sense strand from Kakamega

```
  1 catcgggagatgaaagttgaatnngcaagaaaagagtgtnnaaatttctgaattaagtaa 60
 61 gatttttcttcttctggatcagttgttgttgcgcactataagggaattagtgtagcgca 120
121 gataaaagatcttcgaaagaaagtgcgagaagctggtggaggtgtaaaggttgcgaaaaa 180
181 tcgtcttgttaagattgccgtcagcgatactagtttgaagggtgtttcagatctttttgt 240
241 tgggcaatcattgattgtttattcggttgaccctattgttgctcctaagatttctgtgag 300
301 ctttgcgaatgataataaacagtttgtggttcttggtggtattttggagaaagatattct 360
361 tgaccaagattctatcaaacggattgcttcgttgcctaatattgatggtattcgttctat 420
421 gatcattagtgctattcaatttaattcgactagattggtaaatcttcttaatgcacctca 480
481 gactaaaattgttcgtgctatttctgcttttgtagataaaaatcaacaaagttagactcc 540
541 aagttattccaacaagaataaggaaaatgtgtgatatgtccaatattgaatcaattgttg 600
601 aaaaattatcgtctcttacgctccttcaagcggcagaactttctaaaagattagaagaag 660
661 aatggggagtttctgctgctgctcctgtagctgttgttgcgtctgctgcaggggaa 715
```

4.4.1 *DNA sequences of L10 ribosomal protein genes*

DNA fragment of 7I6 basepairs was amplified and sequenced, producing 535 basepairs of DNA encoding the L10 protein, 44 basepairs of DNA intergenic region and 136 basepairs of DNA that partially encodes the L12 protein. The L10 protein gene from both Machakos and Kakamega was highly conserved. Comparison of the DNA sequence of the rpL10 protein gene showed that the Kenyan strain isolates from different agro-ecological zones were100% homologous to one another. This was true for sequences from both study districts except the sequences from LM5 and LM3 (Figure 4.24). The sequence from LM5 had a single base substitution towards the 5' end of the L10 protein gene. The C-G substitution was on the 16th base. The LM3 sequence had a mononucleotide substitution (A-G) on the 83rd base. In contrast to the conserved nature of the L10 protein DNA sequences from Kenyan strains, there was variation when the Kenyan L10 DNA sequence was compared to other published sequences. The Kenyan strains were 99%, 83% and 83.18% similar to "*Candidatus* L. africanus" strain Nelspruit, "*Candidatus* L. africanus" subspecies Capensis and "*Candidatus* L. asiaticus" strains, respectively (Figure 4.24). An interesting finding in the results is that there are almost similar variations (17%) between the Kenyan and the "*Candidatus* L. africanus" subspecies Capensis and "*Candidatus* L. asiaticus".

4.4.2 *DNA sequences of L12 ribosomal protein genes*

Comparisons of the partial rpL12 gene sequence (136 bases) showed 100% homology among isolates from agro-ecological zones in both Machakos and Kakamega districts except in the sequence from UM4 that had a mononucleotide substitution (G-T) at the 712th base towards the 3' of the partial rpL12 sequence. When the Kenyan sequences were compared to other published sequences it was found that it had 99.26%, 91.9% and 84.56% similarity to the

"*Candidatus* L. africanus" strain Nelspruit, "*Candidatus* L. asiaticus" and "*Candidatus* L. africanus subspecies Capensis" strains, respectively (Figure 4.24). The Asian sequence showed the expected, enormous difference of up to 8.1% in only 136 bases of a vital ribosomal protein gene suggesting differences in the structure and function of the protein. However, the large difference of 15.44%, between the Kenyan strains and the "*Candidatus* L. africanus subspecies Capensis" was not expected.

4.4.3 Intergenic region of L10-L12 ribosomal protein genes

A 44 DNA base sequence comprising of the intergenic region of "*Candidatus* L. africanus" strains from Kenya was obtained from UM3, UM4, LM5, LM3, and LM4 agro-ecological zones which were five bases shorter than the sequences obtained from *"Candidatus* L. asiaticus" and *"Candidatus* L. africanus subspecies Capensis" and "*Candidatus* L. africanus strain Nelspruit*"* (Figure 4.24). However, intergenic sequences of Kenyan strain LH4 had an 11-basepair deletion compared to "*Candidatus* L. asiaticus*"* and *"Candidatus* L. africanus*"* strains. The intergenic regions between the L10 and L12 ribosomal protein genes from isolates from UM3, UM4, LH4, LM5, LM3, KLM, KUM, *"Candidatus* L. africanus strain Nelspruit", *"Candidatus* L. asiaticus*"* and *"Candidatus* L. africanus subspecies Capensis" strains were 100%, 100%, 84.09%, 100%, 100%, 90.9%, 90.9%, 81.8%, 43.2% and 61.4%, homologous to isolates from LM4, respectively. Up to 80% of the variation within the intergenic region was located on adenine and thymine bases in the form of deletions/additions and substitutions (Figure 4.26).

Isolates from UM3, UM4, LM5, LM3 and LM4 had five base deletions within their intergenic regions when compared to other published sequences (Figure 4.24). These deletions were

adjacent to adenine residues except for one thymine residue on the 5' side of the deletion when aligned with *"Candidatus* L. asiaticus". Isolates from KLM and KUM had nine base pair deletions with extra 4 base deletions extending towards the 3' end of the five base deletions found in LM4 isolates. However isolates from LH4 had a staggering eleven base deletion within the L10/L12 ribosomal protein intergenic region.

The results show mono-nucleotide and di-nucleotide substitutions on the 3' side down-stream of the deletions but within the intergenic regions of all Machakos and Kakamega isolates when aligned against "*Candidatus* L. africanus Nelspruit". In two of these substitutions adenine is replaced with cytosine. Substitutions on the *"Candidatus* L. asiaticus" are concentrated more on the 5' side of the intergenic region and are present on thymine and cytosine residues, while the substitutions on the "*Candidatus* L. africanus subsp. Capensis" are positioned in a central location on the intergenic region (Figure 4.24).

The deletions/insertions and substitutions were mainly found within this intergenic region as compared to the rest of the sequenced genes. The intergenic region was therefore the most polymorphic and the least conserved region. The L10 and L12 ribosomal protein genes had little variations and thus were conserved even among the two different species; "*Candidatus* L. africanus" and "*Candidatus* L. asiaticus".

Figure 4.24: Alignment of sequenced ribosomal protein L10 DNA (1-535), L10/ L12 intergenic DNA (536-584) and L12 DNA (585-720) showing variations between the Kenyan, South African and Asian strains of the bacteria.

```
 UM3                 Sample from Machakos district upper midland 3. Altitude 1461 M ASL
 UM4                 Sample from Machakos district upper midland 4. Altitude 1377.7 M ASL
 LH4                 Sample from Kabete Lower Highland 4. Altitude 1650 -2400 M ASL
 LM5                 Sample from Machakos district lower midland 5. Altitude 1309.8 M ASL
 LM3                 Sample from Machakos district lower midland 3. Altitude 1455.6 M ASL
 LM4                 Sample from Machakos district lower midland 4. Altitude 1317 M ASL
 LAN    gi 507766    "Candidatus Liberobacter africanus Nelspruit" ribosomal protein L10 (rplJ) and ribosomal protein L12 (rplL) genes,
 CLA    gi 144454    "Candidatus Liberobacter asiaticus" ribosomal protein and RNA polymerase (rplKAJL-rpoB) gene cluster. 3197 bps
 LAC    gi 7716604   "Candidatus Liberobacter africanus subsp. Capensis" RplA (rplA) gene, partial cds; RplJ (rplJ) and RplL (rplL)
 KLM                 Sample from Kakamega district lower midlands Altitude 1300-1500 M ASL
 KUM                 Sample from Kakamega district upper midlands Altitude 1500-1900 M ASL
UM3:   1 ********************************************************n*** 60  *****n***********nn************************nn*************** 120
UM4:   1 ******n**************n************************************** 60  ***************n******************************************** 120
LH4:   1 nnnnnnnnnn************************************************** 60  ************************************************************ 120
LM5:   1 ***************c****************************nnn************* 60  ************************************************n*********** 120
LM3:   1 *****n*******************************************n********** 60  **********n***********a************************************* 120
LM4:   1 catcgggagatgaaagttgaataggcaagaaaagagtgtnnaaatttctgaattaagtaa 60  gatttttcttcttctggatcagttgttgttgcgcactataagggaattagtgtagcgca 120
LAN: 171 ***************************************gg******************* 230 ************************************************************ 290
CLA:1589 ****t*******************a****g******c**ag******************* 1648**********************a**********a**t*****************t***** 1708
LAC: 182 *********n*ag***********a********a*****a**g********g**g***** 241 ******g***********g********c**************a***********t**a** 301
KLM:   1 **********************nn************************************ 60  ************************************************************ 120
KUM:   1 ************************************************************ 60  *************************************************nnn******** 120

UM3: 121 ***************************************n******************** 180 ***********************************************nn*********** 240
UM4: 121 ************************************************************ 180 **********n************************************************* 240
LH4: 121 ************************************************************ 180 ************************************************************ 240
LM5: 121 ************************nn********************************** 180 ************************************************************ 240
LM3: 121 *****************************************************n****** 180 **********************************************************n* 240
LM4: 121 gataaaagatcttcgaaagaaagtgcgagaagctggtggaggtgtaaaggttgcgaaaaa 180 tcgtcttgttaagattgccgtcagcgatactagtttgaagggtgtttcagatcttttgt 240
LAN: 291 ************************************************************ 350 ************************************************************ 410
CLA:1709 ***t***********g**a**ga****g********************a*****c***** 1768******c**c********ta****t*********a*t*ga**aa****t********c** 1828
LAC: 302 ***c*--*ga***********g**nnn********************g*****nnnnn** 361 ***c*****************tc*t********cg*t****aa*****t**c******** 421
KLM: 121 ************************************************************ 180 ************************************************************ 240
KUM: 121 ************************************************************ 180 ************************************************************ 240

UM3: 241 *******************************************cct************** 300 ******n***************************************************** 360
UM4: 241 *******************************************cct************** 300 ************************************************************ 360
LH4: 241 ********************************************ct************** 300 ***********************************n************************ 360
LM5: 241 *******************************************c*t************** 300 ************************************************************ 360
LM3: 241 *******************************************cct************** 300 ************************************************************ 360
LM4: 241 tgggcaatcattgattgtttattcggttgaccctattgttgctnnnaagatttctgtgag 300 ctttgcgaatgataataaacagtttgtggttcttggtggtattttggagaaagatattct 360
LAN: 411 *******************************************cct************** 470 ************************************c*********************** 530
CLA:1829 ******g**tc*a*****c*******a*agt***g**a*****cct**a*****g**t** 1888****t*******c*****t**a***aga***********gg**g*a*****g*g*g*c** 1948
LAC: 422 **********c*******************t************cct***********a*a 481 ***c**a********c**g**a****a************ag****a**a**t******** 541
KLM: 241 *******************************************cct************** 300 ************************************************************ 360
KUM: 241 *******************************************cct************** 300 ************************************************************ 360
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UM3: 361 ********************nn*************************g************ 420 ***********************nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn* 480
UM4: 361 **********************************************g************* 420 ************************************************************ 480
LH4: 361 **********************************************gg************ 420 ************************************************************ 480
LM5: 361 **********************************************gg************ 420 ************************************************************ 480
LM3: 361 **********************************************gg************ 420 ************************************************************ 480
LM4: 361 tgaccaagattctatcaaacggattgcttcgttgcctaatattgatnntattcgttctat 420 gatcattagtgctattcaatttaattcgactagattggtaaatcttcttaatgcacctca 480
LAN: 531 **********************************************gg************ 590 ************************************************************ 650
CLA:1949 *a*t**************g*aa********t**a**cg**c****ggg******ag**gg 2008******a********c****c****g*a***********t*ga******gg*a*g**a** 2068
LAC: 542 ***t***tg******a****aa********t*****a*********gg***c***g**** 601 t*****a******************g******a****c*c*gg******g***g***** 661
KLM: 361 **********************************************gg************ 420 ************************************************************ 480
KUM: 361 **********************************************gg************ 420 ************************************************************ 480

UM3: 481 *******n**************************************************** 540 ************************************************************ 595
UM4: 481 ************************************************************ 540 ************************************************************ 595
LH4: 481 **********************************-*g**gg*at************n*** 540 *************------*********************g***************n*** 589
LM5: 481 ************************************************************ 540 ************************************************************ 595
LM3: 481 ************************************************************ 540 ************************************************************ 595
LM4: 481 gactaaaattgttcgtgctatttctgcttttgtagataaaaatcaacaaagttagactcc 540 aagttatt-----aataccaacaagaataaggaaaatgtgtgatatgtccaatattgaat 595
LAN: 651 ************************************************************ 710 ********aaaaa****aa*c*************************************** 770
CLA:2069 ****t**g*************************g***********g***g*****ttctg 2128ttca*cg*taaaa*c**ag*ta**a*ca*********a********************** 2187
LAC: 662 *g*ac**g***********c**********************ca***********ct**t 721 ********aaaaa**ctaag**c*a**c****g*****************g********* 781
KLM: 481 ************************************************************ 540 *************----******************************************* 591
KUM: 481 ************************************************************ 540 *************----************************n****************** 591

UM3: 596 ****************************************nn****************** 655 ************************************************************ 715
UM4: 596 ********n*************************************************** 655 *******************************************************g**** 715
LH4: 590 ************************************************************ 649 ************************************************************ 709
LM5: 596 ****************************************n******************* 655 *************n********************************************** 715
LM3: 596 ********n*************************************************** 655 ************************************************************ 715
LM4: 596 caattgttgaaaaattatcgtctcttacgctccttcaagcggcagaactttctaaaagat 655 tagaagaagaatggggagtttctgctgctgctcctgtagctgttgttgcgtctgctgcag 715
LAN: 771 ************************************************************ 830 *******************************************************g**** 890
CLA:2188 ****************g**c********t**ta**g****t**g********a******* 2247*******g********t*********t*******c**tt*c*********c***t***t* 2407
LAC: 782 ****************g***********t**ta*******t******************* 841 ****g*****g*****t*********t**********t****c***************** 893
KLM: 592 ************************************************************ 651 ************************************************************ 711
KUM: 592 ************************************************************ 651 ************************************************************ 711

UM3: 716 ***** 720
UM4: 716 ***** 720
LH4: 710 ***** 714
LM5: 716 ***** 720
LM3: 716 ***** 720
LM4: 716 gggaa 720
LAN: 891 ***** 895
CLA:2408 ca**g 2413
LAC: 894 ***** 899
KLM: 712 ***** 716
KUM: 712 ***** 716
```

Note the 5 base deletion in L10/ L12 intergenic DNA of UM3, UM4, LM5, LM4 and an eleven base deletion in LH4.

4.4.4 Translation and Comparison of L10 ribosomal protein

DNA sequence of L10 gene was translated into its amino acid protein sequences (Figure 4.25) and the sequences were aligned. The sequences had a molecular weight of 18637.6 and an atomic formula: $C_{829}H_{1388}N_{232}O_{248}S_2$. It had an estimated half-life of 30 hours and an instability index of 32.85, which classifies the protein as stable. It had a start codon of TTG at the seventh base from the 5' while the L12 protein has its start codon at the 564th base (Figure 4.25). Comparison of the DNA sequence of the rpL10 protein gene showed that the Kenyan strain isolates from different agro-ecological zones were 100% homologous to one another. In contrast the L10 DNA sequence from the Kenyan strains was 99%, 83% and 83.18% similar to "*Candidatus* Liberobacter africanus" strain Nelspruit, "*Candidatus* Liberobacter africanus subspecies capensis" and "*Candidatus* Liberobacter asiaticus", respectively. Comparisons of the partial 136 base sequence of the rpL12 gene showed 100% homology among isolates from the Kenya's agro-ecological zones, but 99.26%, 91.9% and 84.56% similarity to the "*Candidatus* Liberobacter africanus" strain Nelspruit, "*Candidatus* Liberobacter asiaticus" and "*Candidatus* Liberobacter africanus subspecies capensis", respectively. These results further indicate that the Kenyan strains of the proteobacterium were more closely related to "*Candidatus* L. africanus" than the "*Candidatus* L. asiaticus" in the amino acid sequences of the L10 and L12 ribosomal proteins.

Figure 4.25: Alignment of the translated ribosomal protein L10 Sequences showing amino acid variation.

```
MAC                Translated Ribosomal protein L10 from Machakos district lower midland 4.
LH4                Ribosomal protein L10 from lower highland 4 Altitude  1650 M ASL
LAN    Gi507767    ribosomal protein L10 ["Candidatus Liberobacter africanus"]
CLA    Gi551768    ribosomal protein L10 ["Candidatus Liberobacter asiaticus"]
LAC   Gi7716606    RplJ ["Candidatus Liberobacter africanus subsp. Capensis"]
KAK                Translated Ribosomal protein L10 from Kakamega district Upper midland 0.
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```
MAC:    17 MNRQEKSVEISELSKIFSSSGSVVVAHYKGISVAQIKDLRKKVREAGGGVKVAKNRLVKI 196
LH4:    1  ************************************************************  60
LAN:    1  ************************************************************  60
LAC:    17 ****************L*******************RTSKEGX***************** 196
CLA:    1  ****G*****************I*******************M*****************  60
KAK:    1  ************************************************************  60

MAC:   197 AVSDTSLKGVSDLFVGQSLIVYSVDPIVAPKISVSFANDNKQFVVLGGILEKDILDQDSI 376
LH4:   61  ************************************************************ 120
LAN:   61  ************************************************************ 120
LAC:   197 **R*****E*************************N*************V****V***C** 376
CLA:    61 *IR***IR*I*************DS*VI********S***NE*R****VV**GV*N**** 120
KAK:   61  ************************************************************ 120

MAC:   377 KRIASLPNIDGIRSMIISAIQFNSTRLVNLLNAPQTKIVRAISAFVDKN 523
LH4:   121 **********************************************R** 169
LAN:   121 ************************************************* 169
LAC:   377 *Q***********AI********A*K*LR**S***AQV***L******* 523
CLA:   121 *Q*****DLE***AG******S*A****R**GT***QV*********** 169
KAK:   121 **********************************************R** 169
```

The composition of amino acids in the L10 protein is as follows

Ala (A) 12 7.0%;	Glu (E) 5 2.9%;	Met (M) 2 1.2%;	Tyr (Y) 2 1.2%;
Arg (R) 8 4.7%;	Gly (G) 10 5.8%;	Phe (F) 6 3.5%;	Val (V)21 12.2%;
Asn (N) 9 5.2%;	His (H) 1 0.6%	Pro (P) 4 2.3%;	Asx (B) 0 0.0%;
Asp (D) 10 5.8%;	Ile (I) 19 11.0%;	Ser (S) 22 12.8%;	Glx (Z) 0 0.0%;
Cys (C) 0 0.0%;	Leu (L) 13 7.6%;	Thr (T) 3 1.7%;	Xaa (X) 0 0.0%
Gln (Q) 9 5.2%;	Lys (K) 16 9.3%;	Trp (W) 0 0.0%;	

CHAPTER 5

5.0 DISCUSSION

5.1 HLB disease infects citrus varieties differently in varying AEZS.

This study found that the distribution of the disease was significantly ($P<0.05$) different among the agro-ecological zones in Machakos district and Kakamega districts. The highest AEZ sampled in Machakos (LH5) had a HLB disease mean visual score of 2.3, while the highest in Kakamega (UM1) had a mean visual score of 2.67. These results are validated by the PCR score results and the results on psyllid distribution, which indicated that the psyllid (*Trioza erytreae*; Del Guercio) responsible for the transmission of the bacteria is found more in the upper midlands than the lower midlands. These results are similar with previous findings, which indicated that the disease was more intense in the highlands (Muriuki, 1989; Seif, 1991).

This study found that the distribution of the disease was significanty ($P<0.05$) different among the important citrus varieties in Machakos and Kakamega districts. In Machakos washington navels had the highest mean score (2.619) while in Kakamega, rough lemon showed higher disease scores (2.40). Though the rough lemon did not show HLB morphological symptoms like the other varieties in Machakos it had the highest infestation by psyllid vectors due to its frequent yellow flushes with a mean psyllid score of 0.762. It also had a high PCR score of 0.667. This shows that rough lemon has the highest tolerance to the "*Candidatus* L. africanus" bacterial strain, which causes HLB in Machakos district of Kenya. The results are similar to previous results (Manicom *et al.,* 1990) in South Africa that show that sweet orange and mandarin were severely affected with HLB, followed by, lemon, sour orange which had moderate tolerance to "*Candidatus* L. africanus" strain. Other reports (Miyakawa, 1980; Gonzales *et al.,* 1972; Fraser, 1978) found that lemon is fairly tolerant to the HLB disease to the

extent that in some cases the rough lemon rootstock has been found to induce some degree of tolerance in the sweet orange scion in greenhouse trials (Kapur *et al.,* 1984). The rough lemon in a HLB infected orchard, serves as a reservoir for the bacterium. Due to its frequent flashing and increased psyllid activity an infected rough lemon could easily increase the rate of HLB infection in an orchard.

5.2 Distribution of psyllids varies across varieties and AEZS

The psyllids (*Trioza erytreae),* which are the main vectors of the HLB disease in Kenya, were frequently encountered in AEZs where citrus plants were heavily infected with the HLB disease. Previous reports (McClean *et al.,* 1965; Schwarz *et al.,* 1970) show that psyllids (*Trioza erytreae)* are major vectors for the "*Candidatus* L. africanus", which causes HLB disease in Africa. Therefore the distribution of these insects influences the distribution of the HLB disease.

There were significant ($P<0.05$) mean psyllid score differences among the AEZs in Machakos, this was contrary to the situation in Kakamega where the differences were not significant ($P<0.05$). In Machakos, the mean psyllid score was highest in high altitude areas (LH4 - 0.833) and least in the low altitude areas (LM5 - 0.167). A similar situation was seen in Kakamega where the mean psyllid score was highest in high altitude areas (UM0 - 0.833) and least in the low altitude areas (LM2 - 0.500). This suggests that the psyllid vectors are more active in the highlands than the low lands. These results are similar to the findings by Schwarz (1975) who found that *T. erytreae* thrived well in highlands where most HLB affected orchard were found (Schwarz, 1975; Anon, 1982). The highlands are cooler than the low lands and since *T. erytreae* is sensitive to heat and high temperatures of 32ºC (Moran *et al.,* 1967; Catling, 1969a; Catling, 1972) the insect will optimally function in cool, moist upland regions. This contrasts with

Diaphorina Citri, which is more resistant to extreme temperatures and is more sensitive to high rainfall and humidity (Catling, 1972; Aubert, B. 1987; Regmi *et al.,* 1988; Xia, 1987).

The distribution of psyllid vectors varied significantly ($P<0.05$) among the 4 varieties (tangerine, valencia, washington navel and rough lemon) in Machakos and the variation was insignificant ($P<0.05$) in Kakamega. The psyllids seemed to prefer the young yellowish, light green flushes of rough lemon (which had a mean psyllid score of 0.867 in Kakamega and 0.762 in Machakos) than any other varieties in both districts. For instance the tangerines in Machakos district had a mean psyllid score of 0.190. This could be due to the dark green colour of tangerine leaves, even on the young flashes. These results are similar to previous results (Samways, 1987) which show that lemons probably serve as important reservoirs of infection because of their frequent flushes of new growth which make them attractive to the Psylla vectors which are strongly attracted by yellow green colour of wavelength 550 nm.

5.3 The 16S rDNA shows limited intra-species variability in Liberobacter species

The PCR reaction gave a band of 1100 bp long in the HLB diseased plants from Machakos and Kakamega district. The 1100bp band represented the 16S rDNA of the "*Candidatus* L. africanus" bacteria that is responsible for causing of the HLB disease in Machakos district of Kenya. This 1100 bp DNA band was similar to reports by Jagoueix *et al.* (1994) who indexed "*Candidatus* L. africanus" strains from Nelspruit South Africa. This is a precise method of indexing for the African HLB disease in citrus plants, however the 16S rDNA does not show much variation to differentiate the different strains of bacteria, infact the only report in which the 16S rDNA was used to differentiate the African and the Asian species was by Jagoueix *et al.* (1994) who restricted the PCR product with X*bal* restriction enzyme. Other researchers found

that while the 16S rDNA genes have been used to identify new species of organisms, they show limited variability between strains of bacterial species (Olsen and Woese, 1993; Woese, C.R. 1987; Weisburg *et al.,* 1991). Therefore the procedure was not beneficial in characterizing the "*Candidatus* L. africanus" strains in Machakos and Kakamega districts.

5.4 16S/23S rDNA has insignificant intra-species polymorphism in Liberobacter species

Amplification of the 16S/23S rDNA of the "*Candidatus* L. africanus" strain of the bacteria that cause HLB disease in Machakos district gave a band of 800 bp from diseased samples from Machakos. This band was not present when disease free samples from the greenhouses were used as the PCR template. The band was similar with the results presented by Jagoueix *et al.* (1997) after amplifying the 16S/23S rDNA of the Liberobacter species causing greening in Nelspruit South Africa. The 16S/23S intergenic region in bacteria is highly variable and has been used to characterize bacteria with variable results (Barry *et al.,* 1991; Leblond-Bourget *et al.,* 1996). Jagoueix *et al.* (1997) found that though there was interspecies variation in the 16S/23S rDNA there was insignificant intraspecies variation in the Liberobacter species (Jagoueix *et al.,* 1997). Due to this reason and secondly due to the failure of this PCR reaction to amplify the Kakamega isolates, the utility of the 16S/23S primers in PCR reactions for characterizing the Liberobacter species in Kenya was diminished.

5.5 Amplification of the L10/L12 Ribosomal protein rDNA

The PCR reaction using the L10/L12 rDNA primers amplified a fragment of 716-720bp from all diseased materials from Machakos and Kakamega districts. This band was not present in the citrus plants developed by tissue culture and maintained in the greenhouses. This band was consistent in all the diseased citrus materials from Machakos district. The PCR results on the

distribution of HLB disease among different agro-ecological zones and different varieties was correlated to the visual scores and it gave a positive correlation with a significant ($P<0.05$) regression line. Thus the visual scores can be used to accurately predict the PCR scores and vice-versa in both districts.

The L10/L12 DNA has been previously amplified for the purpose of detection and characterization of the Liberobacter species from South Africa (Garnier *et al.,* 2000; Villechanoux et *al.,* 1993; Planet *et al.,* 1995). In all the three studies much emphasis was focused on the L10 protein and the L12 protein. Little attention was given to the intergenic regions between the ribosomal protein genes.

5.6 Sequences of the ribosomal protein L10/L12 rDNA intergenic region

The 44 base L10/ L12 ribosomal protein rDNA intergenic regions of "*Candidatus* L. africanus" strain from Kenya isolated from agro-ecological zones LM3, LM4, LM5, UM3 and UM4 from Machakos were 100% homologous to one another, but had 92% similarity to the bacterial isolates from LM2 and UM0 agro-ecological zones from Kakamega. Similarly, the 595 basepair intergenic region of the 16S/23S of the "*Candidatus* L. asiaticus" strains from India and the People's Republic of China were 100% homologous to one another, although they were isolated from distant geographic regions (Jagoueix *et al*., 1997). However, the level of homology of the entire 16S/23S intergenic regions of "*Candidatus* L. africanus" and "*Candidatus* L. asiaticus" was 87.46% and was only 498 base pairs long (Jagoueix *et al*., 1997). Because of the high homology between the "*Candidatus* L. asiaticus" strains from India and the People's Republic of China even though the two strains belonged to two different serotypes Jagoueix *et al*. (1997), concluded that probably in "*Candidatus* Liberobacter" the 16S/23S intergenic region does not

vary much within a species. Hence monoclonal antibodies remain the only reagents that allow identification of Liberobacter strains within a given species. However in the current study, the variability in the sequences of "*Candidatus* L. asiaticus" and "*Candidatus* L africanus subspecies Capensis*"* and "*Candidatus* L. africanus strain Nelspruit" were high, 49%, 65.31%, 83.67% and 92% homology respectively, to "*Candidatus* L. africanus" strain from Kenya isolated from LM4, LM3, LM5, UM3 and UM4. These differences were magnified due to the presence of a five base deletion within the ribosomal protein L10-L12 intergenic rDNA of the Machakos strains of the "*Candidatus* L. africanus" and a nine base deletion within that from Kakamega. However the deletions were not the only source of the variation. The deletions and most of the substitution were found on adenine or/and thymine bases. Genetic information derived from the ribosomal protein DNA and its intergenic spacer region can be used to differentiate members of the Liberobacter species (Garnier *et al.,* 2000; Villechanoux *et al.,* 1993; Planet *et al.,* 1995).

These variations show that the Kenyan strain is genetically closer and thereby a more recent descendant of the South African than the Asian strain. This finding strongly suggests that the Huanglongbing disease spread from South Africa to Kenya. The finding is supported by the fact that substantial amounts of citrus planting material were acquired from South Africa in the early sixties. The South African strains of "*Candidatus* L. africanus Nelspruit" (Planet *et al.,* 1995) *and* "*Candidatus* L. africanus subsp. Capensis" (Garnier *et al.,* 2000) are closely related to the Asian strain "*Candidatus* L. *asiaticus"* (Villechanoux *et al.,* 1993) than the Kenyan strain of "*Candidatus* L. africanus" by comparison of the ribosomal protein DNA L10/L12 intergenic region. Polymorphism within this region thereby suggests that the use of universal ribosomal primers to amplify the gene for the ribosomal proteins for indexing of the greening disease in

citrus, as has been the case may give rise to false negative or false positives in PCR reactions. Therefore there is need to develop strain specific primers for amplifying unique regions within the bacterial genome for indexing of the disease and thus ensuring clean seedling delivery to the farmers.

The results indicate that there is little variation within the bacterial L10/L12 ribosomal protein rDNA within regions of less than 1400m ASL in Kenya as is seen with the LM4, LM3, LM5, UM3 and UM4 100% homology. This uniformity subjects the disease to be amenable to control by use of monogenic resistance through conventional breeding or genetic transformation. This is the case due to the absence of local variability within the bacteria. Genes for resistance or tolerance to the HLB disease can be isolated and incorporated into commercial citrus varieties. Citrus resistance to phytophthora and citrus nematode (Grosser and Gmitter, 1990) and *Citrus* tristeza virus (Gutiérrez-E. *et al.,* 1997; Domínguez *et al.,* 2000; Yang *et al.,* 2000) has been achieved by single gene resistance through protoplast fusion and genetic transformation respectively.

5.7 Sequences of the L10 ribosomal protein rDNA

Comparison of the DNA sequence of the rpL10 protein gene showed that the Kenyan strain isolates from different agro-ecological zones were 100% homologous to one another. In contrast the L10 DNA sequence from the Kenyan strains was 99%, 83% and 83.18% similar to "*Candidatus* Liberobacter africanus strain Nelspruit", "*Candidatus* Liberobacter africanus subspecies capensis" and "*Candidatus* Liberobacter asiaticus" respectively. The Kenya strains of the alpha subgroup of HLB disease causing proteobacteria were different from the strains characterized from South Africa strains and the Asiatic strains, but more closely related to the

former than the later. Genes encoding the L10 protein of alpha subgroup proteobacteria have been sequenced and characterized in "*Candidatus* Liberobacter africanus strain Nelspruit" from South Africa (Planet *et al.,* 1995), "*Candidatus* Liberobacter africanus subspecies capensis" (Garnier *et al.,* 2000) and in "*Candidatus* Liberobacter asiaticus (Asiatic strain)" (Villechanoux *et al.,* 1993). It is generally accepted that ribosomal proteins modulate the structure and function of rRNAs (Noller, 1991). The L10 and L12 protein are transcribed from the rplKAJL-rpoBC ribosomal protein-RNA polymerase gene cluster. The most abundant transcript is the 2600 nucleotide tetracistronic L11-L1-L10-L12 mRNA initiated at the upstream major PL11 promoter and terminated at the transcription attenuator in the L12-beta intergenic space (Petterson, 1979).

5.8 L10 ribosomal protein

There is no variation within the L10 amino acid sequence from the different AEZs in Machakos. This means that it is possible that only one strain of *"Candidatus* L. africanus" bacteria is active in Machakos district of Kenya. Nevertheless, the *"Candidatus* L. asiaticus" and the *"Candidatus* L. africanus subsp. Capensis" differed from the Kenyan strains by 15.4% and 19.5% respectively. Similarly, $tRNAs^{Ala}$ identified in the 16S/23S intergenic regions of "*Candidatus* L. africanus" and "*Candidatus* L. asiaticus" region were 87.8%. This is in agreement with the general consensus that variability within protein and RNA encoding genes tends to be low. The results show that though we have a different strain of HLB bacteria in the lower altitude of Kenya, the strain is closely related to that from Nelspruit South Africa than the one from Asia. This gives strong evidence that the HLB disease spread to Kenya from South Africa. The results also indicate that the same strain of bacteria is responsible for causing HLB disease in all sampled varieties of citrus in Machakos district of Kenya.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

The present study shows that Huanglongbing disease is a serious citrus disease in Machakos and Kakamega districts of Kenya, and the levels of infection the HLB disease varied among different citrus varieties and different AEZs. The visual and the PCR scores showed that the disease was most pronounced on washington navel and least on rough lemon varieties in Machakos district. In Kakamega district the rough lemon had higher PCR and visual scores than the washington navels. This suggests that the tolerance exhibited by the rough lemon is overwhelmed in the presence of high levels of disease innoculum found in the highlands.

The agro ecological zones located in high altitude areas (>1500 m ASL), such as UM0, UM1, UM3, UM4, LH4 and LH5 had higher disease score (both PCR and visual) in the two study districts. However, those AEZs in low altitudes (<1500 m ASL) (LM1, LM2, LM3, LM4 and LM5), exhibited low PCR and visual disease scores. Therefore the HLB disease was more pronounced in the highlands, and it decreased with decrease in altitude in both districts. These findings augured well with the findings on the distribution of the HLB disease vector. The psyllid (*Trioza erytreae*) was most encountered on citrus in the AEZs located in altitudes above 1500, while lower psyllid counts were found in AEZs located in altitudes above 1500 in both districts. The psyllid thrives well in highlands than the lowlands. In addition, high psyllid scores were recorded on rough lemon in both districts while the least psyllid score was recorded on tangerines in Machakos district. Psyllids preferred rough lemon due to its frequent flushing and the light yellowish colour of the young shoots which was rare on tangerines.

PCR amplification of the 16S rDNA of the "*Candidatus* L. africanus" bacteria in Kenya, is a precise method of indexing for the African HLB disease in citrus plants, however the 16S rDNA does not show much variation to differentiate the different strains of bacteria. PCR Amplification of the 16S/23S rDNA of the "*Candidatus* L. africanus" gave polymorphism that was neither repeatable nor scientifically justifiable. However, PCR amplification of the L10/L12 ribosomal protein DNA plus its intergenic regions was repeatable and the polymorphism within the intergenic regions was the basis of characterization of the bacteria.

The isolated HLB causing proteobacteria in Kenya was more closely related to the "*Candidatus* Liberibacter africanus" strains from South Africa than the "*Candidatus* Liberibacter asiaticus" strains isolated from Asia with respect to the L10 and L12 DNA, L10 amino acid sequences and the L10-L12 intergenic DNA sequences. There was high homology in L10 and L12 DNA and amino acid sequences and the L10-L12 intergenic DNA sequences of alpha proteobacteria isolated from different agro-ecological zones in Kenya. The HLB disease of citrus in Kenya may have originated from importation of citrus planting materials from South Africa.

6.2 Recommendations

Production of clean planting material through tissue culture systems should be strengthened, and insect control in orchards should be empasized so as to rejuvenate citrus production in the highlands (Kakamega) and to the whole country. In addition, early detection and disease diagnostics systems can be improved by regular use of the PCR indexing of samples from orchards. The Kenya Plant Health Inspectorate Service (KEPHIS) should adopt these diagnostics tools for citrus HLB disease so as to ensure all citrus planting material coming in or leaving the country are free from the disease. Development and optimisation of new primers for

the different strains obtained will considerably improve the utilization of PCR in indexing of the greening disease by improving PCR reaction accuracy and fidelity.

Research should be done on the development of a protein based one-step field diagnostic kit that can be utilized routinely by the farmer for early detection of the diseased material in the orchard.

Research should focus towards genetically modifying citrus for traits such as disease and insect resistance. Identifying and isolating a gene expressing resistance or tolerance to the HLB bacteria and the psyllid vector in citrus would be a base for genetic transformation of citrus. Research shold focus on developing optimal transformation systems for local cultivars. Success in introducing resistance genes in citrus will remedy the Huanglongbing disease scourge in the country and enhance citrus production.

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APPENDIX 1

ANALYSIS OF VARIANCE TABLES FOR MACHAKOS DATA.

Analysis of variance tables for PCR score

Source of variation	d.f.	s.s.	m.s.	v.r.	
AGRO_E_ZONE	6	2.7381	0.4563	2.56	**
VARIETY	3	1.5714	0.5238	2.93	**
AGRO_E.VARIETY	18	5.9286	0.3294	1.84	**
Residual	56	10.0000	0.1786		
Total	83	20.2381			

S.E.=0.1725

Analysis of variance tables for vector score

Source of variation	d.f.	s.s.	m.s.	v.r.	
AGRO_E_ZONE	6	3.4762	0.5794	3.24	**
VARIETY	3	3.7500	1.2500	7.00	**
AGRO_E.VARIETY	18	3.6667	0.2037	1.14	ns
Residual	56	10.0000	0.1786		
Total	83	20.8929			

S.E.=0.1304

Analysis of variance tables for visual score

Source of variation	d.f.	s.s.	m.s.	v.r.	
AGRO_E_ZONE	6	11.9048	1.9841	2.42	**
VARIETY	3	14.2381	4.7460	5.78	**
AGRO_E.VARIETY	18	27.4286	1.5238	1.86	**
Residual	56	46.0000	0.8214		
Total	83	99.5714			

S.E.=0.2797

** significant at $P< 0.05$ ns= not significant at $P<0.05$

APPENDIX 2

ANALYSIS OF VARIANCE TABLES FOR KAKAMEGA DATA.

Analysis of variance PCR score

Source of variation	d.f.	s.s.	m.s.	v.r.	
AGRO_ECOLOGICAL_ZONE	4	0.8000	0.2000	0.86	ns
VARIETY	1	0.0333	0.0333	0.14	ns
AGRO_E_ZONE.VARIETY	4	1.4667	0.3667	1.57	ns
Residual	20	4.6667	0.2333		
Total	29	6.9667			

S.E.=0.2789

Analysis of variance vector score

Source of variation	d.f.	s.s.	m.s.	v.r.	
AGRO_ECOLOGICAL_ZONE	4	0.4667	0.1167	0.50	ns
VARIETY	1	0.8333	0.8333	3.57	ns
AGRO_E_ZONE.VARIETY	4	0.3333	0.0833	0.36	ns
Residual	20	4.6667	0.2333		
Total	29	6.3000			

S.E.=0.1764

Analysis of variance visual score

Source of variation	d.f.	s.s.	m.s.	v.r.	
AGRO_ECOLOGICAL_ZONE	4	7.1333	1.7833	3.15	**
VARIETY	1	2.1333	2.1333	3.76	**
AGRO_E._ZONE.VARIETY	4	8.8667	2.2167	3.91	**
Residual	20	11.3333	0.5667		
Total	29	29.4667			

S.E.=0.275

** significant at P< 0.05 ns= not significant at P<0.05

APPENDIX 3

Regression analysis for the relationship between PCR and Visual scores in Machakos district.

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	1	10.01	10.0105	80.26	***
Residual	82	10.23	0.1247		
Total	83	20.24	0.2438		

s.e. 0.0354

Regression analysis for the relationship between PCR and Visual scores in Kakamega district.

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	1	3.041	3.0413	21.69	***
Residual	28	3.925	0.1402		
Total	29	6.967	0.2402		

s.e. 0.0690

*** = Very significant at P< 0.05

APPENDIX 4

FIELD VISUAL SCORES FORM						
MACHAKOS	***UM 3***	***FARM 1***	***ALTITUDE***		***NAME***	

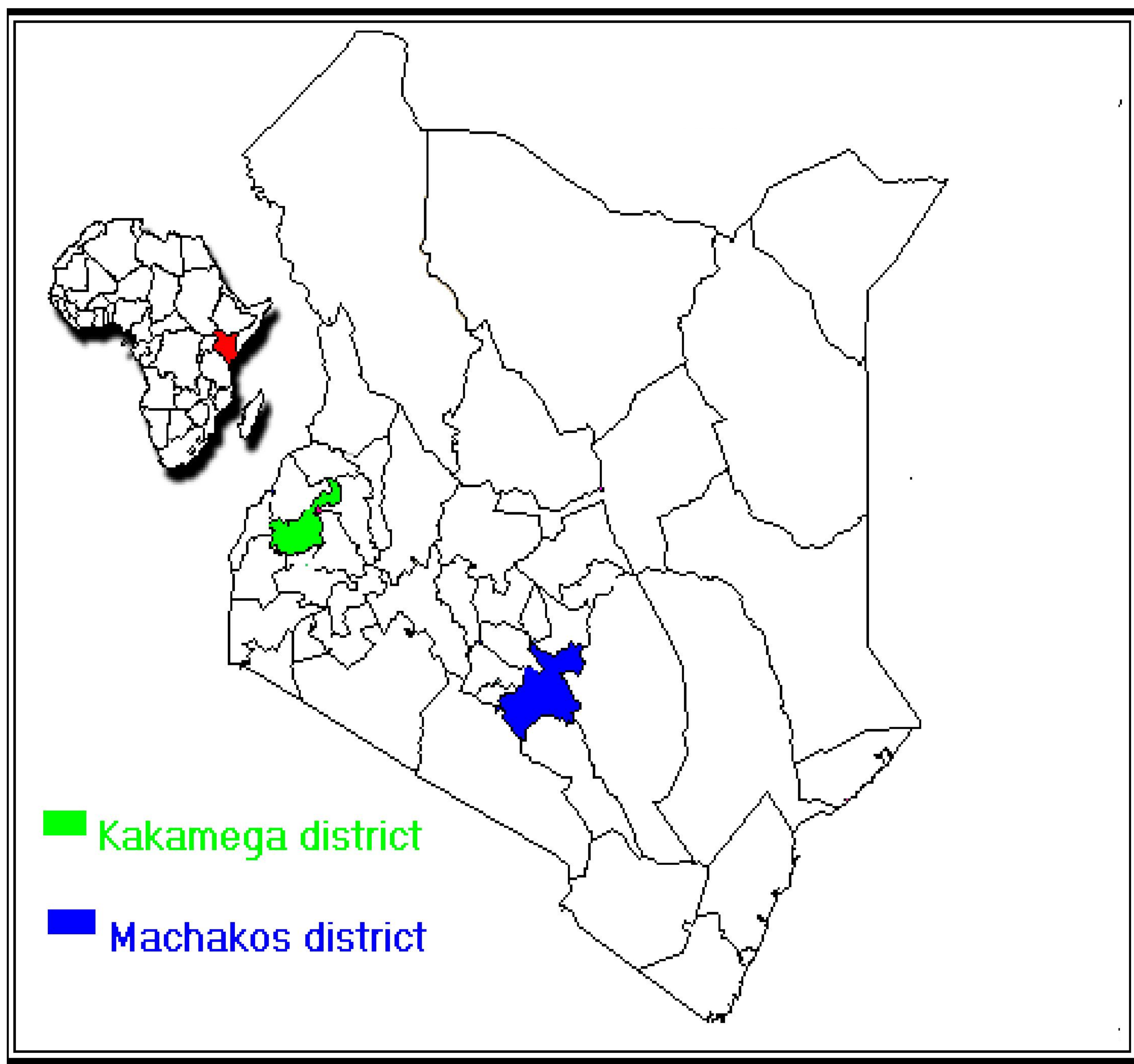
VARIETY / DISEASE	VALENCIA	WASHINGTON NAVEL	ROUGH LEMON	TANGERINE	COMMENTS
CITRUS GREENING					
VECTOR PRESENCE					
TRIZTEZA					
GUMMOSIS					
NUTRITIONAL					
OTHER					

APPENDIX 5 **QUESTIONAIRE**

District:		Name:	
Division:		AEZ :	
FARM INFORMATION			
1.1	How many citrus trees do you have?	1.5	When and how long is the harvesting period?
1.2	Varieties present in your farm?	1.6	What are the average yields per tree?
1.3	What is the average age of your trees?	1.7	How far is the next citrus farm?
1.4	Source of your planting material?	1.8	
FRUIT QUALITY			
2.1	Size of fruit?	2.4	Colour of the outer skin?
2.2	Fruit texture (hard or normal)?	2.5	Colour of the flesh?
2.3	Thickness of the skin?	2.6	Juiciness of the fruit?
MAJOR PROBLEMS IN CITRUS PRODUCTION			
3.1	Diseases?	3.2	Pests?
CULTURAL PRACTICES			
4.1	What do you do with diseased citrus?	4.5	Do you use manure? How much? When?
4.2	Do you mono-crop citrus?	4.6	Do you use fertilizer? How much? When?
4.3	Do you Inter-crop? With what? Is it fertilized?	4.7	Do you use pesticides? How much? When?
4.4	Do you irrigate or is the orchard rainfed?	4.8	Irrigation Source (canal, borehole, other)?
OTHERS			

APPENDIX 6

Kakamega and Machakos district geographical position



APPENDIX 7

Machakos adminisrative boundaries