Coconut Lethal Yellowing Phytoplasma Disease in Mozambique

Diversity, Host Range, and the Impact of Farming Practices on Disease Incidence

João Bila
Faculty of Forest Sciences
Department of Forest Mycology and Plant Pathology
Uppsala

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2016

Acta Universitatis agriculturae Sueciae
2016: 33

Cover: Final stage of coconut lethal yellowing disease symptoms
(photograph: João Bila)

ISSN 1652-6880
ISBN (print version) 978-91-576-8568-1
ISBN (electronic version) 978-91-576-8569-8

Print: SLU Service/Repro, Uppsala 2016

Coconut Lethal Yellowing Phytoplasma Disease in Mozambique. Diversity, Host Range, and the Impact of Farming Practices on Disease Incidence

Abstract

The coconut palm (*Cocos nucifera*) is a major cash crop that is widely grown in coastal tropical regions of the world, including Mozambique. Outbreaks of coconut lethal yellowing disease (CLYD) are threatening the industry and the livelihood of a large part of the Mozambican population. The aim of this thesis was to study different epidemiological aspects of CLYD in Mozambique. Phylogenetic analyses of the 16S and *secA* genes were performed on plant and insect samples collected from different agro-ecological zones of Mozambique and Tanzania. A socio-economic survey was conducted to assess the effect of farming practices on CLYD incidence in Mozambique. The results revealed a high diversity of phytoplasma species associated with CLYD in Mozambique, namely: '*Candidatus* Phytoplasma palmicola' 16SrXXII-A, Tanzanian lethal disease (LD) phytoplasma 16SrIV-C and a novel strain closely related to '*Ca*. Phytoplasma pini' 16SrXXI-A, which was found in a mixed infection with '*Ca.* Phytoplasma palmicola'. Population genetics analyses confirmed the observed high phytoplasma diversity associated with CLYD in Mozambique. The studies also revealed that '*Ca*. Phytoplasma palmicola' and Tanzanian LD phytoplasma occur in both Mozambique and Tanzania. A search for alternate hosts revealed that the palm species *Elaeis guineensis* and *Borassus aethiopum* are alternate hosts of CLYD phytoplasma in Mozambique. Previously, the potential insect vector of CLYD in Mozambique was thought to be the *Platacantha lutea* bug; however, our findings suggest that the potential alternate vector is the planthopper *Diostrombus mkurangai*. Finally, the results revealed that farm age, the presence of other palm species on the coconut farm, type of coconut variety grown, root cut practices and intercropping all had a significant (P<0.05) effect on CLYD incidence. This research study has increased our knowledge of CLYD epidemiology in Mozambique, which should help with the development of a more sustainable CLYD management strategy.

Keywords: Coconut palm, Mozambique, coconut lethal yellowing phytoplasma, '*Candidatus* Phytoplasma palmicola', phylogeny, alternate host, insect vector, farming practices

Author's address: João Bila, SLU, Department of Forest Mycology and Plant Pathology, P.O. Box 7026, SE-75007 Uppsala, Sweden
E-mail: joao.bila@slu.se

Dedication

To my Mom **Maria Bila** and posthumously Dad **Jacinto Macuanine Bila**

“The mind that opens to a new idea never returns to its original size”
Albert Einstein

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Bila J, Mondjana A, Samils B, and Högberg N. 2015b. High diversity, expanding populations and purifying selection in phytoplasmas causing coconut lethal yellowing in Mozambique. *Plant Pathology* 64:597–604.

II Bila J, Högberg N, Mondjana A, and Samils B. 2015a. African fan palm (*Borassus aethiopum*) and oil palm (*Elaeis guineensis*) are alternate hosts of coconut lethal yellowing phytoplasma in Mozambique. *African Journal of Biotechnology*, 14(52): 3359–3367.

III Bila J, Mondjana A, Samils B, Högberg N, Wilson MR, and Santos L. 2016. First report of '*Candidatus* Phytoplasma palmicola' detection in the planthopper *Diostrombus mkurangai* in Mozambique (*Manuscript submitted*).

IV Bila J, Persson I, Högberg N, Mondjana A, Manuel L, Tostão E, Johansson N, Söderlind J, and Santos L. 2016. Effect of farming practices and farm history on the incidence of coconut lethal yellowing in Mozambique. *African Crop Science Journal*, 24(2) (In press).

The contribution of João Bila to the papers included in this thesis was as follows:

I Contributed to the study design, performed field and lab work, data analysis, paper writing in cooperation with co-authors and submission.

II Contributed to the study design, performed field and lab work, data analysis, paper writing in cooperation with co-authors and submission.

III Contributed to the study design, performed field and lab work, data analysis, paper writing in cooperation with co-authors and submission.

IV Contributed to the study design, involved in the data collection, data analysis, paper writing in cooperation with co-authors and submission.

Abbreviations

CLYD	Coconut lethal yellowing disease
CSPWD	Cape Saint Paul Wilt Disease
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
FISP	Farmer Income Support Project
IPM	Integrated pest management
LD	Lethal disease
LY	Lethal yellowing
LYD	Lethal yellowing-like diseases
ML	Maximum likelihood
MZT	Mozambique tall
NJ	Neighbour joining
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism

1 Introduction

1.1 Phytoplasma disease worldwide

Phytoplasma infection is often fatal for plants, including many economically important crops, resulting in huge losses in agricultural production around the world (Seemuller *et al.*, 2002; Bertaccini, 2007; Oshima *et al*., 2013). Phytoplasma-related diseases are expected to increase because the phytoplasma insect vectors are likely to be favoured by the predicted trend of rising global temperatures (Hodgetts *et al.*, 2008). Plants infected with phytoplasma diseases show a wide range of symptoms, including stunting, yellowing, witches' broom (proliferation of shoots), phyllody (formation of leaf-like tissues or shoots from floral organs), virescence (greening of floral organs), purple top (reddening of leaves and stems), phloem necrosis, sterility, fruit size reduction and malformation, malformation of stem ends and plant decline (Garnier *et al.*, 2001; Hogenhout *et al.*, 2008; Ntushelo, *et al.*, 2013). There are three known mechanisms associated with phytoplasma transmission: the most common route is via insect vectors; however, phytoplasmas can also be transmitted by vegetative propagation or grafting of infected plant material or via vascular connections between infected host plants, parasitic plants and non-infected host plants (Weintraub & Beanland, 2006). Seed propagation is also suspected to be a means of phytoplasma transmission, although as yet there is no scientific evidence to support this idea (Nipah *et al*., 2007).

Phytoplasmas are also associated with palm lethal yellowing (LY) disease in several tropical and sub-tropical areas of the world and infection is usually disastrous. The typical coconut lethal yellowing disease (CLYD) symptoms start with premature nut drop and blackening (necrosis) of new inflorescences, followed by progressive yellow discoloration from the most basal to the youngest leaves, rotting of the stem apical tissues (heart) and wilting and collapse of the palm crown (Figure 1) within three to six months of the initial appearance of symptoms (Hunt *et al*., 1973).

Figure 1. CLYD symptoms: Healthy or symptomless coconut palm (a); progressive yellowing discoloration from the oldest to the youngest leaves followed by skirt-shaped brown discoloration (necrosis) of the older leaves (b); death of the apical meristem (bud) followed by wilting and collapse of the entire crown (c) leaving an empty stem (d). Photographs: João Bila.

Lethal yellowing symptoms can be confused with the symptoms of other palm diseases, mainly those caused by fungal basal stem rot (*Ganoderma zonatum*), or by the abiotic deficiencies of boron (early nut fall) or potassium (discoloration and early death of the older leaves). Basal stem rot (*G. zonatum*) causes early death of the older leaves and the spear leaves, resulting in canopy wilting (Broschat *et al.*, 2010).

1.1.1 Background of phytoplasma

When phytoplasmas were first discovered in 1967 by Doi *et al.*, they were initially named mycoplasma-like organisms (MLOs) because of their similarity to mycoplasmas infecting animals. However, as a result of advances in the development of molecular tools, their phylogeny was redefined in 2004 by the International Research Programme on Comparative Mycoplasmology (IRPCM), which led to the designation of a new genus within the class of Mollicutes named '*Candidatus* Phytoplasmas'. Phytoplasmas are pleomorphic, cell-wall-less bacteria with a bead-like, filamentous or multi-branched appearance under the electron microscope, with small adenine-thymine (AT)-rich genomes (Thomas & Norris, 1980; Lee *et al.*, 2000; Seemuller *et al.*, 2002; Weintraub & Beanland, 2006; Hogenhout *et al.*, 2008; Oshima *et al.*, 2013).

1.1.2 Phytoplasma group classification

The first comprehensive and rapid tool for assigning a phytoplasma to a particular group was based on restriction fragment length polymorphism (RFLP) analyses of PCR-amplified 16S rDNA and ribosomal protein gene sequences (Lee *et al.*, 1998). Since then, several universal and group-specific markers have been designed for routine detection of phytoplasmas on the basis of 16S rRNA and other less conservative genes (Duduk & Bertaccini, 2011).

Lee *et al.* (1998) differentiated phytoplasmas into 14 16Sr groups and 32 sub-groups using RFLP analyses. With the aid of putative restriction sites estimated via the MapDraw program, Lee *et al.* (1998) were able to discriminate phytoplasma into 14 groups and 41 sub-groups. Later, using computer-simulated RFLP analysis of the 16S rRNA gene, Wei *et al.* (2007) expanded the phytoplasma group classification to include 10 new phytoplasma groups, representing three recently described and seven novel putative '*Candidatus* Phytoplasma' taxa. So far, 30 reliable '*Ca.* Phytoplasma' genera and more than 100 subgroups have been published (Zhao *et al.*, 2010; Duduk & Bertaccini, 2011).

Classification based on 16S groups does not always provide the molecular distinction necessary for phytoplasma strain characterization in epidemiological studies and, hence, the subgroup designation is necessary (Duduk & Bertaccini, 2011). For further separation and classification of phytoplasmas into subgroups, additional genetic markers such as ribosomal protein (rp) genes, *sec*Y, tuf, groEL, 16S-23S rRNA intergenic spacer region, *secA*, *nusA*, *hflB*, *gcp* and *imp* gene sequences have been used (Schneider *et al.*, 1997; Hodgetts *et al.*, 2008; Hodgetts & Dickinson, 2010; Ntushelo *et al.*, 2013). To date, the phytoplasma taxa associated with palm species have been

members of the 16S rRNA group I, IV, XI, XIV, XXII or XXXII (Mpunami *et al.*, 1999; Cronjé *et al.*, 2000; Mehdi *et al*., 2012; Nejat *et al.*, 2009, 2013; Harrison *et al.*, 2014,). Phytoplasmas on coconut in Mozambique and Nigeria, belonging to 16S rRNA group XXII-A, have been formally described as '*Ca.* Phytoplasma palmicola' whereas other coconut phytoplasmas in West Africa belonging to group XXII-B, have been described as '*Ca.* Phytoplasma palmicola'-related strains (Harrison *et al.*, 2014). The origin, evolution and diversity of the LYD Mozambican phytoplasmas still need to be clarified. The putative phytoplasma species causing CLYD in Mozambique were characterized and population genetics analyses performed to elucidate aspects of phytoplasma biology and evolution (paper I).

1.1.3 Phytoplasma genome and evolution

Phytoplasma lack important metabolic genes, mainly because of their parasitic host-dependent life-cycles (Moran, 2002; Oshima *et al*., 2013). Studies of phytoplasma genomic features also support their host dependence with respect to many vital functions (Hoshi *et al*., 2007; Oshima *et al*., 2011, 2013). The challenge imposed on phytoplasma, to navigate between hosts in Animalia and Plantae kingdoms, limits the speed at which phytoplasmas evolve (Christensen *et al.*, 2005). Oshima *et al*. (2011) reported that about 33% of the gene expression profile changed when '*Ca*. Phytoplasma asteris' switched from a plant to an insect host. Selection against microbe or pathogen-associated molecular patterns that may trigger host responses (Jones & Dangl, 2006) might be an adaptation by phytoplasma to escape recognition by the host receptors.

Phytoplasmas contain the YidC and Sec secretion systems, which mediate the interaction with the host and are responsible for incorporating phytoplasma membrane proteins into the host cells. The Sec system also secretes proteins into the host cytoplasma cells that are associated with pathogenicity factors (Oshima *et al*., 2013). The mechanisms involved in both the YidC and Sec secretion systems, as well as the secreted protein are important for understanding phytoplasma biology (Oshima *et al*., 2013) and their ability to reconcile two diverse environments: the phloem of plants and the digestive tract of different insects (Oshima *et al*., 2011).

1.2 Molecular tools for detection and identification of phytoplasmas

Detection of phytoplasmas for diagnostic purposes has been complicated by several factors, including failure to cultivate these bacteria in artificial media,

their small size and low concentrations in plant tissues (Thomas & Norris, 1980; Christensen *et al.*, 2005). Although still at the experimental stage, Contaldo *et al.* (2012) have shown that phytoplasma can be grown in specific artificial media under certain conditions. The inability to culture phytoplasmas has obstructed reliable and conclusive detection and proof of pathogenicity, making the progress of phytoplasma biology research slow (Christensen *et al.*, 2005; Hoshi *et al*., 2007; Contaldo *et al.*, 2012; Oshima *et al*., 2013). However, the polymerase chain reaction (PCR) nucleic acid-based techniques are simple, adequate and widely used for phytoplasma detection (Weintraub & Beanland, 2006; Duduk & Bertaccini, 2011). PCR techniques for phytoplasma detection can be used as direct, nested, semi-nested, multiplex nested or real-time PCR, or can involve enzymatic digests of the amplified PCR products.

The main goal of any PCR protocol is to concentrate phytoplasma DNA while reducing enzyme inhibitory plant polyphenolic and polysaccharide molecules. The nested-PCR assay, which is designed to increase phytoplasma concentration, primer sensitivity and specificity, is crucial for the amplification of phytoplasmas from host DNA (Khan *et al.*, 2004; Weintraub & Beanland, 2006). The membrane-feeding assay is a rapid, non-destructive method for indirect putative insect vector detection that involves allowing the insect to feed through a membrane on sucrose media, followed by testing for phytoplasma presence on the media using PCR (Tanne *et al*., 2001).

The detection of phytoplasmas using serological techniques has been unsuccessful. The low phytoplasma titre and the presence of host-derived antigens, results in the production of less-specific polyclonal antibodies that cross-react with host-derived non-target antigens (Weintraub & Beanland, 2006). To overcome the cross-reaction problem, Wei *et al*. (2004) developed a more sensitive anti-SecA polyclonal antiserum that can be used routinely to detect even novel phytoplasma species.

1.3 The coconut palm

The coconuts (*Cocos nucifera*) found in the Indian and Pacific Ocean basins are genetically distinct, suggesting two independent centres of origin. The Indian Ocean basin covers the Southern periphery of India, Sri Lanka, and the Maldives whereas Malaysia represents the Pacific Ocean basin (Gunn *et al*., 2011). The occurrence of coconuts on the Atlantic coasts of West Africa and America appears to be the result of recent introductions by European navigators (Lebrun *et al*., 1998). The coconut palm is a major cash crop that is widely grown in coastal tropical regions of the world, including Mozambique, and contributes to the economy, livelihood, tourism and food security of

millions of rural inhabitants. The coconut is also known as "The Tree of Life", which reflects the many different uses that different parts of the tree can be put to: for example, food, construction, wood, medicinal uses, agricultural inputs, industrial applications and biofuel (Myrie, 2014; Smith, 2015).

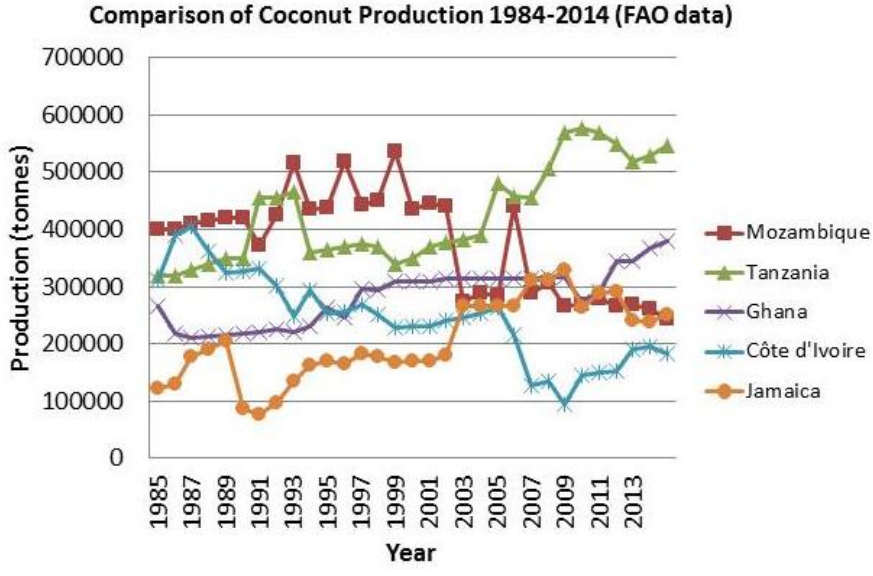

Figure 2. Coconut production (1984–2014) in five countries with CLYD (Source: FAOSTATA, 2016).

Outbreaks of coconut lethal yellowing disease (CLYD) have resulted in waves of epidemics and in the death of more than eight million coconut trees, threatening the industry and the livelihood of a larger part of the Mozambican population. Mozambique used to be the biggest coconut producer in Southeast Africa (Figure 2); however, since 2003 the production levels have dropped below that of Tanzania (FAO, 2016). To address the CLYD epidemic status of Mozambique, in 2007 the Millennium Challenge Corporation (MCC) signed a five-year compact ($20.8 million) with the Government of Mozambique that ended in September 2013 (Abt Associates, 2014). The aim of the compact, named the Farmer Income Support Project (FISP), was to rescue coconut production, while promoting diversification into the production of other cash-crops, within selected zones of the Zambezia and Nampula provinces.

To mitigate the negative impact of CLYD, the FISP approach consisted of cutting and chopping down diseased and dead coconut trees, respectively, and providing seedlings to replace the coconut trees that had been chopped down

(Abt Associates, 2014; Smith, 2015). However, even though small-scale and commercial farms occur side by side, the FISP was only targeted at selected small-scale farms, which was epidemiologically questionable given that the private-sector farms are likely to have been a source of inoculum for the replanted seedlings on the small-scale farms. For several reasons the FISP failed to mitigate the CLYD epidemic.

1.4 Coconut lethal yellowing

The most well-known phytoplasma disease of palms is LY, a devastating disease that affects more than 38 species of palms throughout the Caribbean region (Harrison & Oropeza, 2008). Reports of dying coconut palms exhibiting lethal yellowing-like disease (LYD) symptoms date back to the 19th century in the Caribbean (Eden-Green, 1997). Similar devastating LYD of coconut have previously been observed in Africa (Eden-Green, 1997). The symptoms of LYD are similar, but they differ in terms of epidemiology, the resistance of the coconut varieties and the insect vectors (McCoy, 1976; Mpunami *et al*., 2000). PCR analysis of 16S rDNA revealed differences between African and American LYD phytoplasmas, and between phytoplasmas from the East and West African coasts (Tymon *et al.*, 1998). Interestingly, the 16S rDNA RFLP and sequence analyses revealed that the Mozambican and Ghanaian samples showed more similarity compared with samples from Tanzania and Kenya (Mpunami *et al.*, 1999).

According to De Carvalho and Mendes (1958), cited by Eden-Green (1997), records of a disease resembling CLYD in Mozambique date back to the mid-1950s. The current CLYD outbreak was first detected in the Cabo Delgado province, northeast of Mozambique in 1992. Since then the disease has spread to many coastal areas in central and northern Mozambique. In the main coconut-growing areas of the Zambezia province, CLYD has resulted in the complete loss of the crop at several production sites. In 2010, a new CLYD outbreak was detected in the second biggest coconut-producing province of Inhambane, in the southeast of Mozambique. LYD are considered to be the most significant factors limiting coconut production worldwide (Oropeza *et al.*, 2005).

1.5 Coconut lethal yellowing disease cycle

Phytoplasmas share a two-host life cycle (Figure 3) involving plants and insect vectors (Christensen *et al.*, 2005; Garnier *et al.*, 2001; Oshima *et al.*, 2011). In the Caribbean, lethal yellowing-type phytoplasmas have been detected in grass

species associated with coconut farms, such as *Emilia fosbergii* and *Synedrella nodiflora* (Brown *et al.*, 2008). Moreover, transmission of coconut phytoplasma between different host species has been observed in Malaysia, where the causal agent of coconut LY-type diseases has also been observed in Bermuda grass (*Cynodon dactylon*) and oil palm (*Elaeis guineensis*) (Nejat *et al.*, 2009). These findings highlighted the need to search for potential alternate host plant species for CLYD in Mozambique (paper II).

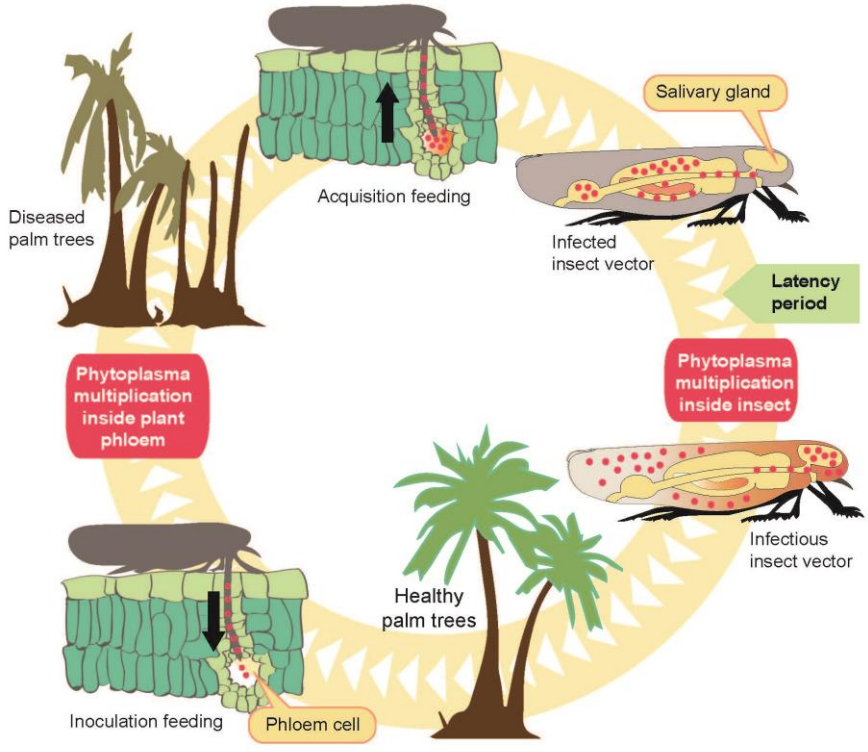

Figure 3. Coconut lethal yellowing disease cycle. Phytoplasma within insect vector tissue or plant phloem are represented by red dots.

The most common phytoplasma transmission mechanism is dependent on phloem-feeding insect vectors of the order Hemiptera, which harbour and spread the bacteria to different plants in a plant–insect–phytoplasma relationship known as the matrimonial triangle (Garnier et al., 2001). Insects belonging to the order Hemiptera share several characteristics that make its members efficient vectors of phytoplasmas (often both immature stages and adults are efficient phytoplasma vectors) (Weintraub & Beanland, 2006): (a) nymphs and adults feed similarly and are usually in the same physical location

(hemimetabolous); (*b*) they feed selectively on certain plant tissues, which makes them efficient vectors of pathogens living in those tissues; (*c*) their feeding is non-destructive, which enables them to avoid triggering plant defensive responses; (*d*) they have a propagative and persistent relationship with phytoplasmas; and (*e*) they can support transovarial transmission of phytoplasmas.

Vectors of phytoplasma diseases are mostly leafhoppers (Membracidae, Cicadellidae), planthoppers (Delphacidae, Derbidae, Cixiidae and Flatidae) and Psyllidae (Philippe *et al.*, 2007; Weintraub & Beanland, 2006; Lee *et al.*, 2000). Phytoplasmas circulate inside their insect vectors, mainly in the digestive tract, multiply in diverse tissues, traverse to the barriers of the salivary glands and are subsequently injected into healthy plants when the insect feeds on phloem (Figure 3) (Seemuller *et al.*, 2002; Zhao *et al.*, 2010; Oshima *et al.*, 2013). In the Caribbean region and in Florida, lethal yellowing disease is vectored by Cixiidae *Haplaxius crudus* (*Myndus crudus*) and potentially by the *Cedusa* species of derbids (Brown *et al.*, 2006). Mpunami *et al.* (2000) associated LD in Tanzania with the planthoppers *Diostrombus mkurangai* and *Meenoplus* sp., both members of the Derbidae family. In the Cabo Delgado province, northern Mozambique, pentatomids of the species *Platacantha lutea* were found carrying the same phytoplasmas as those identified in the diseased coconut on which they were found (Dollet, *et al.*, 2011). However, the presence of phytoplasma DNA in an insect does not prove that the phytoplasma can be transmitted to plants by the insect. Hence, potential CLYD insect vectors in Mozambique (paper III) were further investigated.

1.5.1 Ory*ctes monoceros* beetle accelerates CLYD epidemics and hinders palm restoration

Oryctes monoceros (Coleoptera, Dynastinae) causes damage and crop losses in coconut palms, primarily as result of the adult beetle making feeding galleries in the soft apical section (unfolded fronds) of seedlings and young palms, but also in old palms when populations are large (Allou *et al.*, 2006, 2012).

The final stage of CLYD development is the rotting of the stem apical tissues (heart), followed by wilting and death of the palm crown (Hunt *et al.*, 1973). This leaves an empty stem, which is the preferred breeding sites, and habitat of the larvae of the *O. monoceros* that develop in the decaying dead palm wood (Figure 4). The emerging *O. monoceros* adult beetles prefer to attack the young coconut palms that were planted to replace the coconut palms that had died as a result of CLYD (Figure 4). The combined effect of the beetle and CLYD phytoplasmas is making CLYD management in Mozambique rather

challenging. Eden-Green (2006) observed that 35–50% of the local Mozambique tall (MZT) coconut cultivar can survive continued LYD phytoplasma exposure, although the survivors are usually weakened or killed by the large population of *O. monoceros*. A positive association between CLYD and coconut beetle damage was reported in the current CLYD epidemic in Mozambique (Smith, 2015).

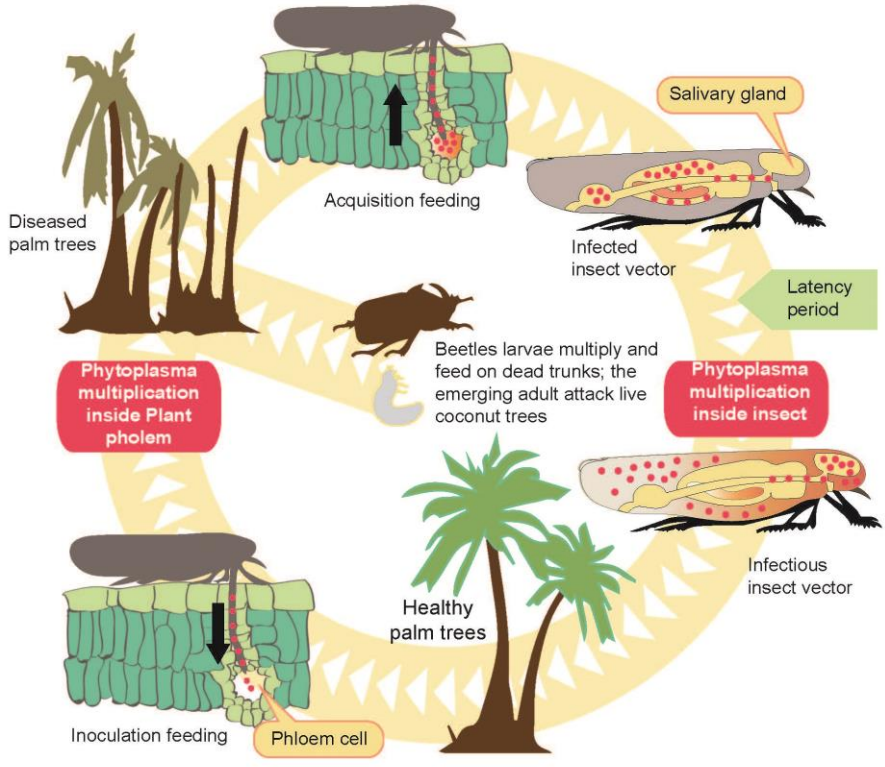

Figure 4. Effect of the *Oryctes* beetle on coconut lethal yellowing disease progress. The coconut beetle is not a CLYD phytoplasma vector, but is a major coconut palm tree pest. Phytoplasmas within insect vector tissues or plant phloem are represented by red dots.

1.6 Management of lethal yellowing palm phytoplasma diseases

The primary approach for controlling phytoplasma diseases is often prevention, which includes the use of resistant varieties, controlling the insect vectors and alternative plant hosts, and clearing out and destroying infected plants (Garnier *et al.*, 2001; Lee *et al.*, 2000). Currently, the most common method for LYD control in Latin America and the Caribbean involves replanting with varieties

that are considered to be resistant to the disease, such as the cultivar Malayan Yellow Dwarf (MYD) and a hybrid Maypan (MYD x Panama Tall). However, LY resistance breakdown in Maypan has been reported in plantations in Jamaica (Lebrun *et al.*, 2008). Finding germplasm that is resistant to the palm LYD in Africa has been a challenge. The varieties known to be tolerant to LY in the Caribbean region have not shown the same degree of resistance in Africa (Eziashi & Omamor, 2010), which may be partly because of the involvement of different phytoplasmas. Swarbrick *et al*. (2013) identified specific markers that can be used for validating the purity of breeding materials that are resistant to Cape Saint Paul Wilt Disease (CSPWD) in Ghana to avoid distributing contaminated breeding material to coconut famers.

In Mozambique, the recommended management strategy for CLYD is to cut and burn any symptomatic coconut trees that are suspected of being infected. The local green MZT variety is still considered to be tolerant because it survives relatively longer when infected with CLYD compared with other varieties. Therefore, this variety is widely used to replace dead coconut palms in Mozambique.

The intensity of CLYD in Mozambique varies significantly among and within the affected areas despite the largely similar agro-ecological conditions in these areas (Bila *et al*., 2015b). Broschat *et al*. (2002) observed that LYD does not kill all susceptible palms in a given year, losses occur over time for as long as the disease remains active at a particular site. The recurrence of CLYD in replanted devastated coconut farms in Mozambique, coupled with the isolation of lethal yellowing-type phytoplasmas in grass species in the Caribbean and Asia (Brown *et al.*, 2008; Nejat *et al*., 2009), supported the hypothesis that other factors such as farming practices could have an impact on CLYD incidence in Mozambique, which was addressed in paper IV.

Knowledge about insect vectors and alternate hosts is crucial to limit the progress of LYD. Some studies have reported that integrated pest management (IPM) can be achieved by manipulating the habitats occupied by the insects vectoring the diseases (Howard & Oropeza, 1998; Caudwell, 2000). Manipulating the composition of the vegetation within and near a phytoplasma-infected farm can affect the presence and dispersal of vectors, their natural enemies and, thus, the insect–pathogen interaction (Weintraub & Beanland, 2006; Bertaccini & Duduk, 2009). These findings also support the hypothesis that farming practices could have an impact on LYD prevalence (paper IV) at a given site.

2 Objectives

Understanding the biological aspects related to CLYD establishment and development up to epidemic status is crucial for efficient disease management. The aim of this thesis was to study different epidemiological aspects of CLYD in Mozambique. Such knowledge could be used to design a sustainable CLYD management strategy, boosting hope for future coconut production in the country.

2.1 The specific objectives

- To characterize the phytoplasma species that are the causal agents of CLYD in Mozambique. In this study, the hypotheses tested were: (i) the phytoplasma species causing CLYD in Mozambique are more similar to the West African type compared with the Tanzanian or Caribbean types and (ii) the observed epidemic can be detected using population genetics analysis.
- To investigate the diversity of the plant secondary hosts of the phytoplasma species causing CLYD in central Mozambique. In this study, the hypothesis that there are other palmae and *Pinus* species that are alternate hosts for CLYD phytoplasma species in Mozambique was tested.
- To search for the potential insect vectors of the CLYD phytoplasmas in Mozambique. In this study, the hypothesis that Derbidae and Pentatomidae insects are potential vectors for phytoplasma species causing CLYD in Mozambique was tested.
- To evaluate the effect of coconut farming practices on CLYD incidence in Mozambique. In this study, the hypothesis that coconut-farming practices and their related history have an impact on the CLYD incidence in Mozambique was tested.

3 Materials and methods

3.1 Fieldwork

3.1.1 Plant and insect sampling

For the phytoplasma characterization study, 136 samples were collected from an equal number of coconut trees in Mozambique. The samples were collected at representative agro-ecological sites in the affected areas, covering both rainy and dry seasons in 2012. An additional 24 samples were collected in the neighbouring country of Tanzania in the coastal regions (Figure 5). Samples were collected by boring into the trunk using a 10-cm-long drill bit that was 10 mm in diameter (Mondjana *et al*., 2010).

For the alternative host study, a sample was collected from 41 palm and 14 pine trees. Plants that showed symptoms or that were propagated in the vicinity of diseased coconut palms were selected for this study. The survey was conducted in the Mozambican Nicoadala and Maganja da Costa districts of the Zambezia province during April 2014 (Figure 5).

Figure 5. Study sites in the different projects covered in this thesis: phytoplasma characterization study (🌴), alternative host search study (🌿) and putative insect vector study (🐛).

For palm tree species (Figure 6a–c), sampling was performed as described for coconut palm samples, whereas bush-like palm species (Figure 6d and e) were sampled by cutting the spear leaves. Finally, pine tree (Figure 6f) samples were collected from needles and from the phloem region of the stem. Both the collected trunk tissues and spear leaves were dried in tubes containing silica gel (Mondjana *et al*., 2010) and then maintained at room temperature until DNA extraction (Bila *et al*., 2015b).

Figure 6. Plant species examined in the alternative host study. (a) African fan palm (*Borassus aethiopum*) showing the symptoms of skirt-shaped brown discoloration (necrosis) of the old leaves; (b) African oil palm (*Elaeis guineensis*) exhibiting skirt-shaped brown discoloration of the older leaves and (c) collapse of the necrotic crown; (d) Senegal date palm (*Phoenix reclinata*); (e) Lala palm (*Hyphaene coriacea*) growing in the vicinity of a diseased coconut trees and (f) pine trees (*Pinus* sp.) growing next to a diseased coconut tree. Photographs: João Bila.

For the putative insect vector study, the survey was conducted in the coastal regions of Inhambane and Zambezia Provinces (Figure 5), covering both the rainy and dry seasons of 2014. Insects on the plant inflorescence area and on the underside surface of leaves (Figure 7) were collected from palms showing

typical CLYD symptoms or growing in the vicinity of diseased coconut palms. The specimens were either collected manually using a mouth aspirator or with the aid of large conical flasks containing lethal insect poison. The insects were preserved in 96% alcohol until DNA extraction. Prior to DNA extraction the insect species were taxonomically grouped based on morphological features.

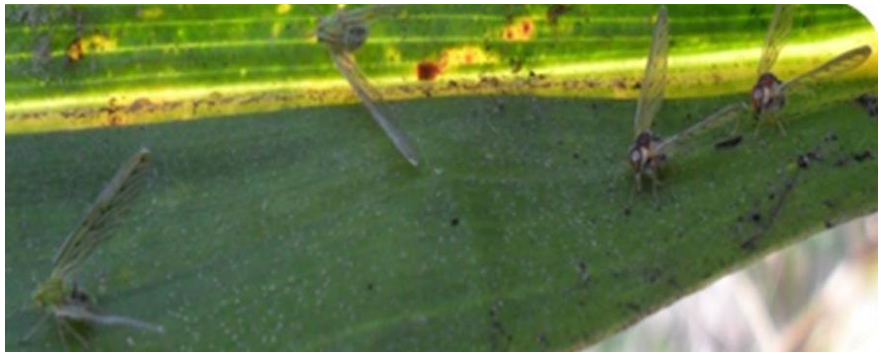

Figure 7. Derbidae insects on the underside surface of coconut leaves. Photograph: Luisa Santos.

3.1.2 Questionnaire and disease survey

Fieldwork to assess whether coconut farming practices effect the incidence of CLYD in Mozambique consisted of two main activities: (a) a socioeconomic survey of households to capture information on farming systems, coconut production, and the household perception of the symptoms and the methods for controlling CLYD; and (b) direct observations on the palm farms to estimate the incidence and severity of CLYD. Both the survey and the direct observation were conducted simultaneously at each household, which had been randomly selected from the target population. A sample of 50 enumeration areas and 10 households from each enumeration area was drawn in two random steps to make a total of 500 observations. The target population consisted of all the households in the coastal districts of Zambezia and Nampula provinces (Figure 5) that were currently involved in coconut production or that had been involved in the recent past. The fieldwork was carried out during October and November 2012.

3.2 DNA extraction and PCR analysis

DNA extraction for both the phytoplasma species characterization study and the alternative host study was performed using the CTAB extraction procedure described by Harrison & Oropeza (2008), with minor modifications (Bila *et al.*,

2015b). Similarly, insect DNA extractions were performed either with CTAB (Harrison & Oropeza, 2008; Bila *et al*., 2015b) or using the DNeasy Blood and Tissue Kit QIAGEN. Insect samples were tested either as individual insects or in sets of five.

There are limitations to phylogenetic analyses that are based solely on a highly conserved gene such as the 16S rRNA (Hodgetts & Dickinson, 2010), therefore, this study combined analyses of the 16S rDNA and the *secA* gene sequences. The phytoplasma DNA was amplified from total DNA extracts using direct and nested PCR (Paper I, II & III). Each final PCR product was analysed on agarose gel containing Nancy-520 (Sigma). The DNA was visualized by Ultraviolet transillumination and photographed.

3.3 Sequencing and sequence analysis

Regardless of sample material (from insect or plants), purification, sequencing and sequence analysis were performed as follows. The PCR products from 16S rRNA and *secA* genes were purified using spin columns (Cycle-Pure Spin PCR purification kit). Sanger sequencing was performed on both strands of the PCR products using the PCR primers.

Sequences retrieved from the PCR products were assembled using the SEQMAN PRO software (DNASTAR Lasergene 10 or 12 core suite) and aligned by CLUSTAL-W (Larkin *et al*., 2007). Sequences were analysed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990) at the National Centre for Biotechnology Information (NCBI). Phylogenetic analyses were performed with MEGA v. 6.06 (Tamura *et al.*, 2013) using the neighbour-joining (NJ) and maximum-likelihood (ML) methods, evaluated using at least 1000 bootstrap replicates.

The phytoplasma groups were assigned using the *i*PhyClassifier (Zhao *et al.*, 2009) online interactive software tool. Furthermore, for the phytoplasma species characterization study, molecular evolutionary analyses of sequences were performed using DNA sequence polymorphism analysis (DnaSP v. 5.10.1) software (Librado & Rozas, 2009), where the numbers of polymorphic (segregating) sites were estimated according to Nei (1987). Tajima's test (Tajima, 1989) was used to identify whether the distribution of polymorphisms was consistent with the theory of neutral evolution at equilibrium between mutation and genetic drift. Tajima's D test was used to distinguish between DNA sequences evolving randomly ("neutrally") and those evolving in a non-random process. Finally, codon-based selection (dN/dS) in the *secA* gene was analysed and evaluated using a Z-test (Kryazhimskiy & Plotkin, 2008).

3.4 Statistical analyses for the study of farming practices

The data analysis was performed using SAS 9.4 software (SAS Institute Inc. ©2013, Cary, NC 27513, USA). Given that the explanatory variables in the models were both metric and non-metric and the dependent variable was ordinal (ordered categorical) (Table 1), the list of statistical techniques was limited to logistic regression and discriminant analysis (Hair *et al.*, 2014). However, the discriminant analysis technique requires that the explanatory variables are metric and that there are no large variations in the group sizes (Table 2). Thus, the choice of statistical method favoured logistic regression.

Table 1. *Dependent and explanatory variables used in the models*

Variable	Code	Definitions/meaning
Disease incidence		An ordered categorical variable with three levels denoting: (1) disease incidence ranging from 0–5% of infected trees, (2) disease incidence of between 5% and 40%, and (3) disease incidence of more than 40%.
Farm age	age	Coconut farm age consisting of three categories: (i) less than 10 years (coded as 1); (ii) between 10 and 40 years (coded as 2), which was used as the reference group; and (iii) more than 40 years (coded as 3).
Other palm species	pspecies	Describes the presence or absence of any other palm species on the farm. The alternatives are Yes (coded as 1) and No (coded as 0).
Planting layout	layout	Describes whether the palm trees are planted in a zigzag pattern (coded as 1) or if the palm trees stand in lines (coded as 2).
Level of weed	weed	Level of weed consisting of three categories: (i) clean, a farm without any weed, which is referred to as the reference group (coded as 1); (ii) the presence of creeping and/or tall grass on the farm (coded as 2); and (iii) the presence of a higher degree of weed, including woody plants (coded as 3).
Coconut variety	variety	Type of coconut variety on the farm, consisting of three categories: (i) dwarf (coded as 1); (ii) tall (coded as 2), which is the reference group; or (iii) a hybrid between dwarf and tall (coded as 3).
Removing mature leaves	prune	Describes whether farmers cut the mature leaves for other purposes such as fencing or house roofs. The alternatives are Yes (coded as 1) and No (coded as 0).

Variable	Code	Definitions/meaning
Holes in stem for climbing	climbing	Describes whether farmers dig holes into the stem for climbing purposes. The alternatives are Yes (coded as 1) and No (coded as 0).
Inflorescence cut	sura	Describes whether farmers cut fresh inflorescences for local wine "sura" production purposes. The alternatives are Yes (coded as 1) and No (coded as 0).
Root cut	root	Describes whether farmers cut roots for other purposes such as medicinal. The alternatives are Yes (coded as 1) and No (coded as 0).
Soil type	soil	Describes the soil on the farm, consisting of three categories: (i) sand (coded as 1); (ii) between sand and soft clay (coded as 2), which is the reference group; or (iii) soft clay (coded as 3).
Intercropping on the farm	intercropping	Describes whether the farmer cultivates crops other than coconut. The alternatives are Yes (coded as 1) and No (coded as 0).

Table 2. *Distribution of significant explanatory variables by CLYD incidence*

Explanatory variables with significant effect	Disease incidence level (%)			Total
	0–5	>5–40	>40	
Farm age (years)	283	183	63	529
Other palm species	280	179	63	522
Coconut variety	285	180	64	529
Intercropping	285	184	64	533
Root cut	277	180	63	520

Logistic regression was used to predict the probability of higher disease incidence from the impact of the explanatory variables in model 1 (equation 1, Table1). The outcome is interpreted in terms of odds, where the odds are defined as the ratio of the probability of two outcomes of events.

Odds of having higher disease incidence = $e\hat{}(\beta_0 + \beta_1 age + \beta_2 pspecies + \beta_3 layout + \beta_4 weed + \beta_5 variety + \beta_6 prune + \beta_7 climbing + \beta_8 sura + \beta_9 root + \beta_{10} soil + \beta_{11} intercroping)$ (equation 1)

Model 1 (equation 1) did not satisfy the group-by-group size assumption for logistic regression analysis (Table 2) (paper IV), therefore, the second and third models (equations 2 and 3, respectively) were estimated using only the significant variables (Table 5) from the first model. A single model including all five significant explanatory variables from model 1 would fail to satisfy the group-by-group assumption (Hair *et al.*, 2014; paper IV), which is why two separate models were developed (equations 2 and 3).

Odds of having higher disease incidence $= e\hat{}(\beta_0 + \beta_1 age + \beta_2 pspecies + \beta_3 variety + \beta_4 root)$ (equation 2)

Odds of having higher disease incidence $= e\hat{}(\beta_0 + \beta_1 age + \beta_2 pspecies + \beta_3 variety + \beta_4 intercroping)$ (equation 3)

4 Results

4.1 PCR and phylogenetic analysis

4.1.1 Phytoplasma characterization study (paper I)

About 50% of the palms sampled yielded PCR products of the expected size for both 16S rRNA and *secA* genes. Most of the PCR negative samples were also cox negative indicating a lack of plant DNA. Furthermore, from the 16S rDNA PCR positive samples, 40 phytoplasma sequences and 11 other bacterial sequences were retrieved. Similarly, from the *secA* gene PCR positive samples, 46 phytoplasma sequences and 17 other bacterial sequences were retrieved. However, not all samples yielded PCR products using both primers sets; 12 samples yielded only the 16S rDNA PCR product, whereas 21 samples yielded only the *secA* PCR product (Table S1 in Paper I). In total, 80 phytoplasma sequences were used in this study because the other sequences were discarded owing to low quality. All sequences were deposited in GenBank under the accession numbers KJ528950–KJ528985 for 16S rDNA and KJ776677–KJ776720 for *secA* genes.

Phylogenetic analyses of the 16S rDNA sequences revealed that two different phytoplasma species were associated with CLYD in Mozambique. '*Ca.* Phytoplasma palmicola' 16SrXXII-A, which clustered together with the Nigerian phytoplasma sequence (Y14175.1), was the most prevalent species, whereas the '*Ca*. Phytoplasma pini'-related strain (MZ-Z7a1) was found in mixed infection with '*Ca*. Phytoplasma palmicola' (MZ-Z7a2) in just one palm (Figure 8).

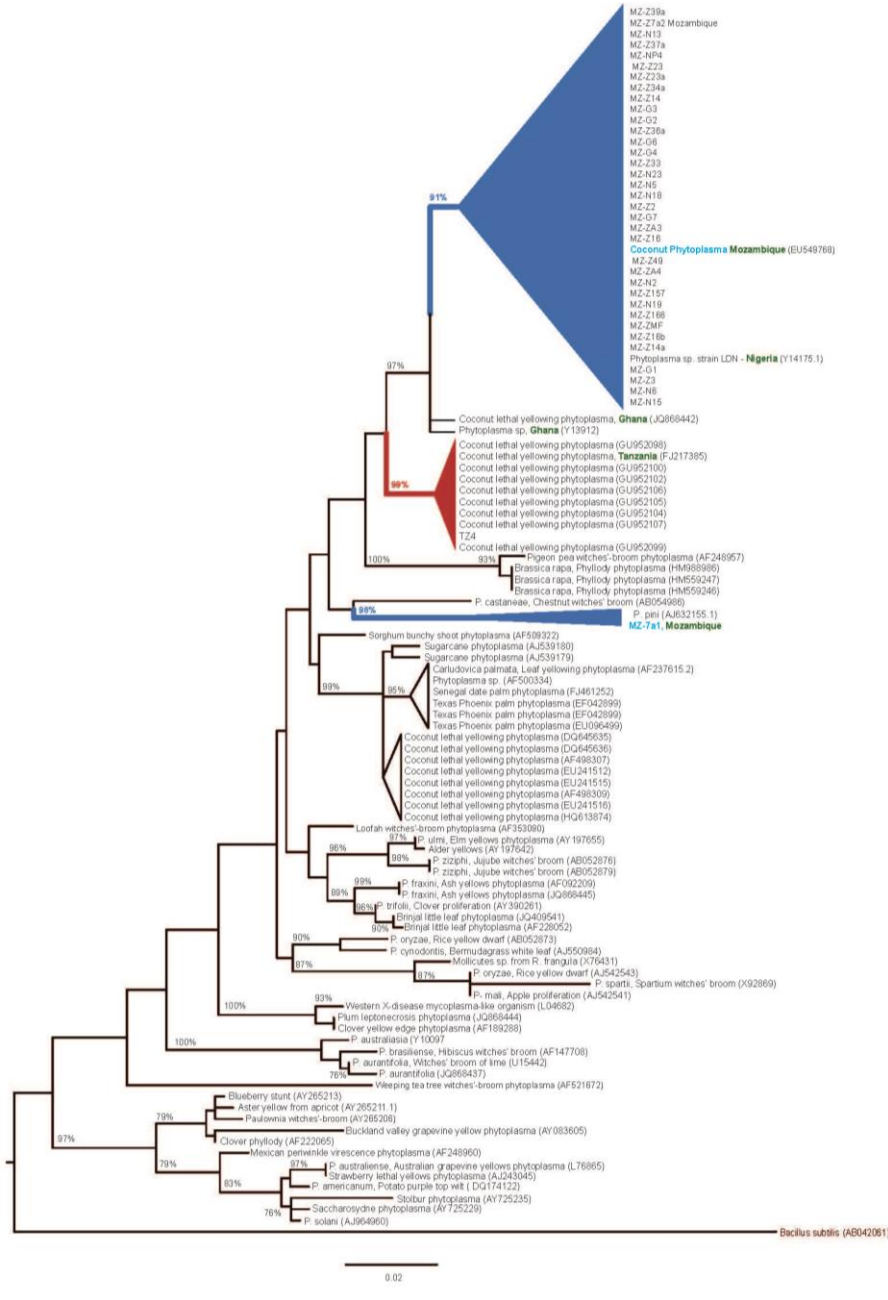

Figure 8. Dendrogram constructed using the maximum-likelihood method showing the phylogenetic relationships among the 16S rDNA sequences for Mozambican coconut phytoplasma compared with representatives from other 16S groups. The Mozambican and Tanzanian samples used to construct the dendrogram are indicated by MZ and TZ, respectively.

The GenBank accession numbers are shown in parentheses. Bootstrap values greater than 70% based upon 1000 replicates are shown.

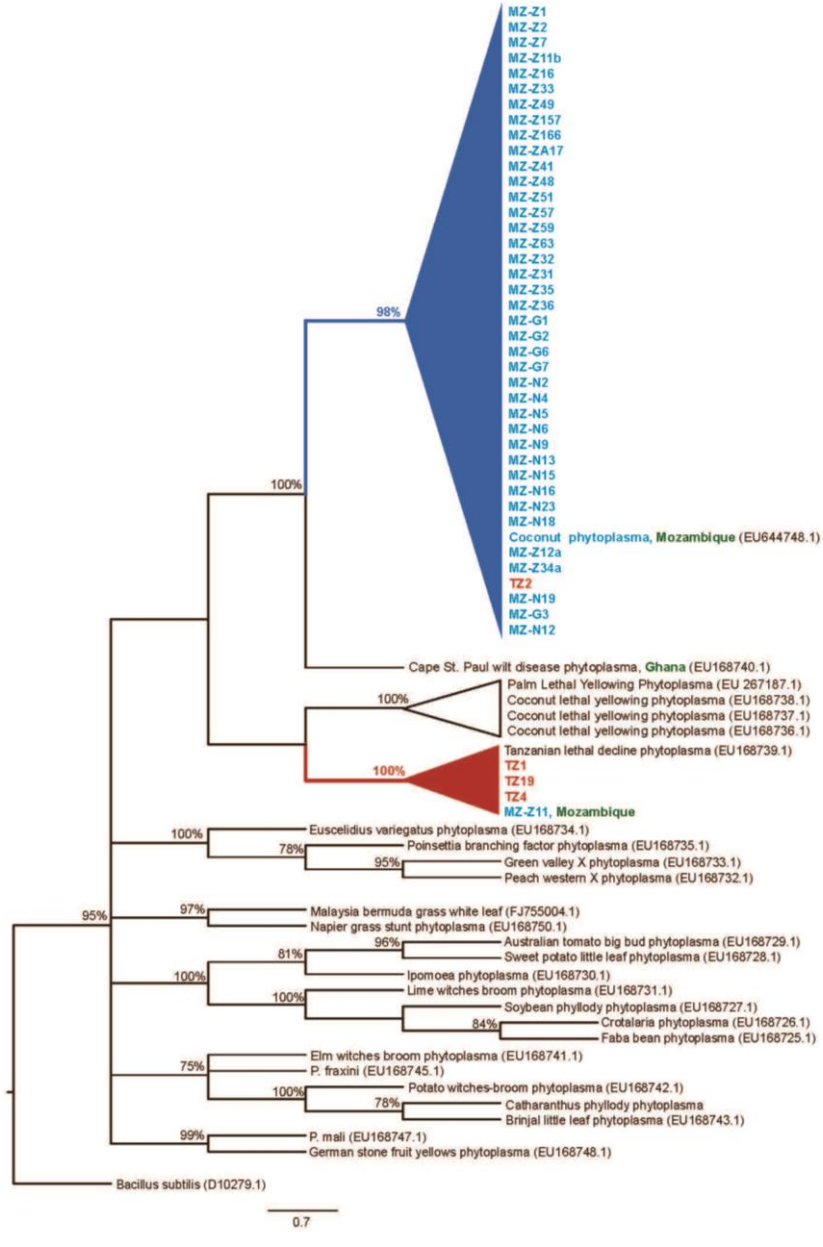

Figure 9. Dendrogram constructed using the maximum-likelihood method showing the phylogenetic relationships among the *secA* gene sequences for Mozambican and Tanzanian coconut phytoplasma compared with representatives from other 16S groups. The Mozambican

and Tanzanian samples used to construct the dendrogram are indicated by MZ and TZ, respectively. The GenBank accession numbers are shown in parentheses. Bootstrap values greater than 70% based upon 1000 replicates are shown.

Phylogenetic analyses of the *secA* sequences also revealed two different phytoplasma species causing CLYD in Mozambique (Figure 9). '*Ca* Phytoplasma palmicola' 16SrXXII-A, which closely resembled the CSPWD Ghanaian phytoplasma group 16SrXXII-B (EU 168740.1) was the most predominant species, and a Tanzanian LD-related phytoplasma strain (MZ11) was found in a single palm sampled in the Mozambican Zambezia province. Furthermore, sample TZ2 collected in Tanzania clustered together with the Mozambican and West African groups with 98% bootstrap support, suggesting that it was carrying a '*Ca.* Phytoplasma palmicola'-related strain.

Together the 16S rDNA and *secA* sequence datasets revealed four main coconut lethal phytoplasma species: three phylogenetically distinct phytoplasmas detected in Mozambique, namely '*Ca.* Phytoplasma palmicola' 16SrXXII-A, Tanzanian LD phytoplasma 16SrIV-C and a novel strain most closely related to '*Ca.* Phytoplasma pini" 16SrXXI-A; and a group containing the North American, Central American and Caribbean strains, which were included in the phylogenetic analysis for comparison purposes.

Table 3. *Molecular evolutionary analysis of the Mozambican 'Ca. Phytoplasma palmicola' and related phytoplasma group*

16S sequence polymorphism data	*secA* sequence polymorphism data
Number of polymorphic (segregating) sites, S: 23	Number of polymorphic (segregating) sites, S: 6
Total number of mutations, Eta: 27	Total number of mutations, Eta: 6
Average number of nucleotide differences, k: 1.69412	Average number of nucleotide differences, k: 0.30000
Nucleotide diversity, Pi: 0.00509	Nucleotide diversity, Pi: 0.00087
Tajima's D: –2.58601 (***, $P < 0.001$)	Tajima's D: –2.10096 (*, $P < 0.05$)

'*Ca.* Phytoplasma palmicola' and related strains, the largest group in both 16S rDNA and *secA* sequences, was used for population genetic analyses (Table 3). For the Mozambican population, the Tajima's D value for the 16S rDNA was negative (–2.6) (Table 3) and highly significant ($P<0.001$). The sequence variation indicated that the population experienced a bottleneck, size expansion and/or purifying selection because the average numbers of polymorphisms observed in pairwise comparisons were lower compared with the total number of polymorphic sites. A codon-based (dN/dS) selection on the *secA* sequences

revealed significant purifying selection on this gene as evaluated by a Z-test (Table 3).

4.1.2 Alternative host study (paper II)

The phytoplasma sequences used in this study were retrieved from samples MZ-Eg19 and MZ-Ba20, detected by G813/Awka SR and LY16Sf/LY16Sr primer pairs. The two phytoplasma sequences were deposited in GenBank under the accession numbers KP938847 and KP938848. The retrieved sequences were used in a phylogenetic analysis together with representatives of other 16S rDNA phytoplasma groups, where the NJ and ML trees had similar topologies (data not shown); the ML tree is shown in Figure 10.

Phylogenetic analysis of the 16S rDNA sequences showed that the phytoplasmas detected in *B. aethiopum* (MZ-Ba20) and *E. guineensis* (MZ-Eg19) belong to the same clade as '*Ca*. P. palmicola', which causes CLYD in Mozambique, the Nigerian phytoplasma sequence (Y14175.1) and closely resembled the CPSWD in Ghana (Y13912) (Figure 10).

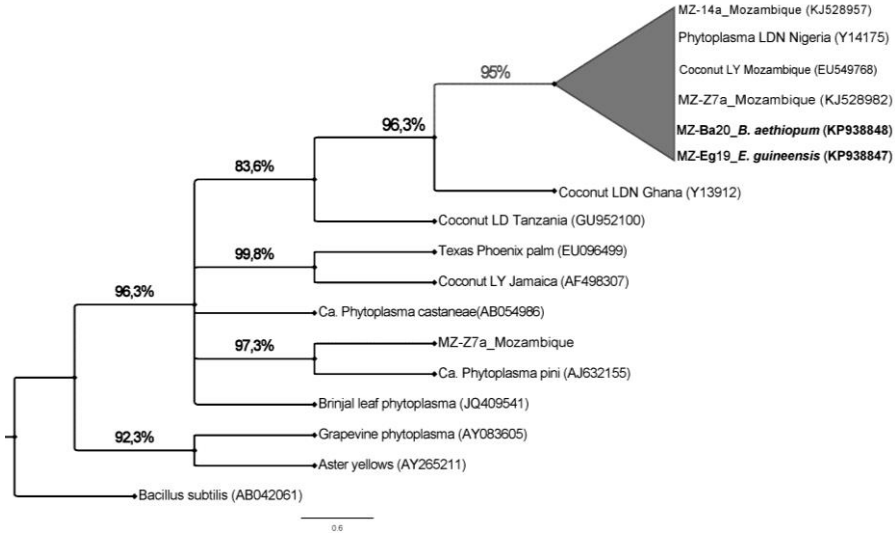

Figure 10. Dendrogram constructed using the maximum-likelihood method showing the phylogenetic relationships among the 16S phytoplasma sequences from wild naturalized palm samples from Mozambique compared with representatives from other 16S groups. The Mozambican samples used to construct the dendrogram are indicated by MZ-Ba20 and MZ-Eg19. The GenBank accession numbers are shown in parentheses. Bootstrap values greater than 80% based upon 1000 replicates are shown.

Based on the *i*PhyClassifier online software tool, the phytoplasma sequence from sample *B. aethiopum* (MZ-Ba20) was classified as '*Ca.* P. cocosnigeriae'

16Sr XXII-A (Y14175). The results indicate that *E. guineensis* and *B. aethiopum* palm species are alternate hosts of CLYD phytoplasma (Figure 10) in Mozambique.

4.1.3 Putative insect vector study (paper III)

The insects collected belonged to the Derbidae (*Diostrombus mkurangai*, *D. abdominalis*, *Lyddastrombus* sp. and *Zoraida* sp.) and Pentatomidae families. *Diostrombus* spp. were by far the most abundant taxa. PCR positive bands were only detected from *D. mkurangai*, *D. abdominalis* and *Lyddastrombus* sp. Phytoplasma sequences were retrieved from two *D. mkurangai* specimens and were deposited in GenBank under the accession numbers KP938847 and KP938848. Other PCR-positive samples were determined to belong to Gram-positive bacteria other than phytoplasmas after sequencing. The retrieved 16S rDNA phytoplasma sequences were used in a phylogenetic analysis together with representatives of other 16S phytoplasma groups. The NJ and ML trees had similar topologies (data not shown); the ML tree is shown in Figure 11.

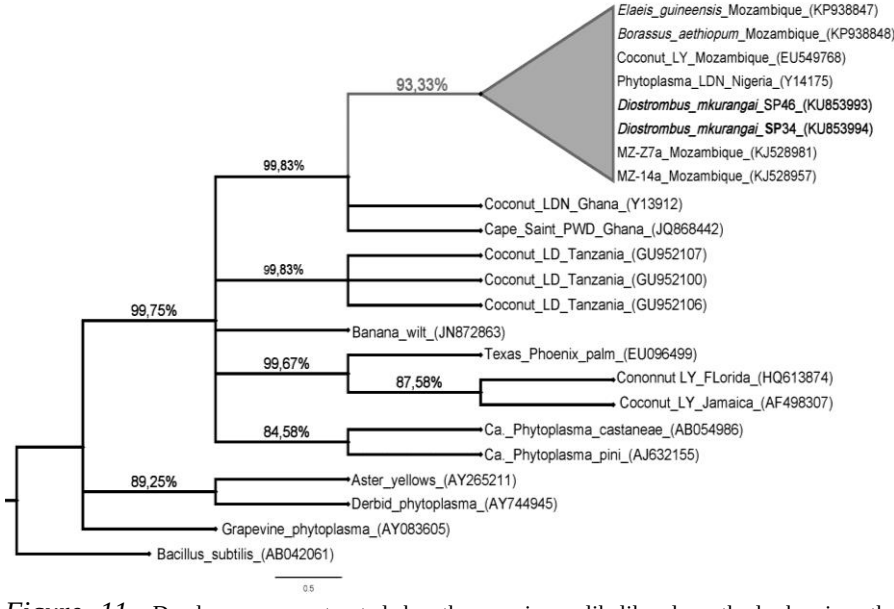

Figure 11. Dendrogram constructed by the maximum-likelihood method showing the phylogenetic relationships of the 16S Mozambican phytoplasma sequences from *Diostrombus mkurangai* compared with Mozambican palm phytoplasma sequences and representatives from other 16S groups. The Mozambican insect samples used to construct the dendrogram are labelled *Diostrombus mkurangai*_SP46 and *Diostrombus mkurangai*_SP34. The GenBank accession numbers are shown in parentheses. Bootstrap values greater than 80% based upon 1200 replicates are shown.

Basic local alignment search tool comparison and phylogenetic analysis of the 16S rDNA gene PCR products revealed that *D. mkurangai* is a potential vector of the coconut phytoplasmas in Mozambique (Figure 11). Based on the iPhyClassifier online software tool, the phytoplasma sequence detected on the *D. mkurangai* SP46 specimen was classified as *'Ca.* Phytoplasma palmicola' reference strain (KF751387) and assigned to the 16Sr group XXII-A (Y14175).

4.2 Effect of farming practices on disease incidence (paper IV)

Five out of the 11 explanatory variables tested had a significant (P<0.05) effect on the odds for CLYD incidence (Table 4), namely: farm age, presence of other palm species on the coconut farm, type of coconut variety grown, root cut practices and intercropping.

Table 4. *Analysis of maximum likelihood and odds ratio estimates based on model 1.*

Explanatory variables		Degree of freedom	Wald Chi-square (*P-value*)	Odds ratio estimates	Confidence intervals
Farm age (years)	0–10 against >10–40	1	6.518 (*0.011*)	2.761	1.189–6.41
	>40 against >10–40	1	6.005 (*0.014*)	0.872	0.599–1.268
Other palm species		1	5.499 (*0.019*)	1.792	1.101–2.919
Planting layout	Zigzag against in line	1	0.658 (*0.417*)	1.285	0.701–2.354
Level of weed	Grasses against clean	1	0.040 (*0.843*)	0.999	0.683–1.462
	Bushes against clean	1	0.064 (*0.800*)	0.916	0.449–1.872
Coconut variety	Dwarf against tall	1	6.175 (*0.013*)	1.414	0.876–2.282
	Hybrid against tall	1	5.162 (*0.023*)	0.421	0.173–1.027
Removing mature leaves (prune)		1	0.211 (*0.646*)	0.917	0.633–1.328
Holes in stem for climbing		1	0.587 (*0.444*)	0.828	0.51–1.343
Inflorescence cut for local wine ("sura") production		1	3.029 (*0.082*)	2.037	0.914–4.537
Root cut		1	6.660 (*0.010*)	2.777	1.278–6.032
Soil type	Sand against between sand & soft clay	1	0.117 (*0.733*)	0.743	0.203–2.713
	Soft clay against between sand & soft clay	1	0.313 (*0.576*)	0.704	0.192–2.588
Intercropping		1	8.779 (*0.003*)	1.842	1.23–2.759

The odds of coconut farms that were up to 10 years old having a higher disease incidence than the reference category (10–40 years old) were about three times greater (odds ratio = 2.727). By contrast, the odds of coconut farms that were more than 40 years old having a higher disease incidence than the reference category were almost 10% lower (odds ratio = 0.926) (Table 5).

Other palm species were found on about 17% of coconut farms. These farms had almost twice the odds (odds ratio = 1.691) of having a higher disease incidence level than that of farms without other palm species (Table 5). The other palm species frequently found on the coconut farms in Mozambique were the African oil palm (*E. guineensis*), African fan palm (*B. aethiopum*) and Senegal date palm (*P. reclinata*) (paper II).

Coconut farms planted with the dwarf variety had around 40% higher odds (odds ratio = 1.433) of having a higher disease incidence than the farms with the tall variety, whereas farms growing the hybrid variety of coconut had around 60% lower odds of having a higher disease incidence compared with the farms with the tall variety (odds ratio = 0.385) (Table 5).

Coconut farms where farmers cut roots for other purposes had three times higher odds (odds ratio = 2.805) of having higher disease incidence levels compared with farms that did not cut the roots (Table 5).

Finally, coconut farms with some kind of intercropping had almost twice the odds (odds ratio = 1.819) of having higher disease incidence levels compared with farms without intercropping (Table 5). The proportion of farmers managing the farms using intercropping was 26%. The crops most commonly intercropped with coconut were grain cereals, grain legumes and root tubers (data not shown).

Table 5. *Analysis of maximum likelihood and odds ratio estimates based on models 2 and 3.*

Explanatory variables		Model 2			Model 3		
		Wald Chi-square (*P-value*)	Odds ratio estimates	Confidence intervals	Wald Chi-square (*P-value*)	Odds ratio estimates	Confidence intervals
Farm age (years)	0–10 versus >10–40	6.406 (*0.011*)	2.727	1.199–6.202	5.371 (*0.021*)	2.416	1.080–5.404
	>40 versus >10–40	5.269 (*0.022*)	0.926	0.643–1.332	4.826 (*0.028*)	0.899	0.625–1.293
Other palm species		4.590 (*0.032*)	1.641	1.043–2.583	5.357 (*0.021*)	1.691	1.084–2.638
Coconut variety	Dwarf versus tall	5.886 (*0.015*)	1.317	0.824–2.107	7.357 (*0.007*)	1.433	0.902–2.279
	Hybrid versus tall	5.772 (*0.016*)	0.383	0.157–0.933	6.215 (0.013)	0.385	0.159–0.935
Root cut		7.401 (*0.007*)	2.805	1.334–5.896			
Intercropping					9.111 (0.003)	1.819	1.234–2.684
Goodness of fit statistics							
Somers' D		0.212			0.216		
Gamma		0.257			0.245		
Tau-a		0.123			0.125		
C		0.606			0.608		
Likelihood ratio (Odds)		0.7561			0.6231		

5 Discussion

This is the first report of the detection of more than one phytoplasma type causing CLYD in Mozambique. Previous studies (Mpunami *et al*., 1999; Marinho *et al.*, 2008; Dollet *et al.*, 2011; Harrison *et al.*, 2014) have only associated the causal agent of CLYD in Mozambique with '*Ca.* Phytoplasma palmicola' and related strains (16Sr XXII-A and 16Sr XXII-B). High phytoplasma diversity has also been reported in Malaysia, where Nejat *et al.* (2009) observed that the coconut yellow decline syndrome was associated with phytoplasmas from the 16SrXIV group and novel taxonomic groups, which were similar to the 16SrV, 16SrVI, 16SrVII and 16SrXI groups.

This is also a pioneer study revealing both the occurrence of the '*Ca.* Phytoplasma palmicola'-related strain in Tanzania and the Tanzanian LD phytoplasma type in Mozambique. Interestingly, in the putative insect vector study (paper III), we found that *D. mkurangai*, the potential vector of LD phytoplasma in Tanzania (Mpunami *et al.*, 2000), was also associated with CLYD in Mozambique. As such, it is plausible that *D. mkurangai* in Mozambique may also carry the Tanzanian LD phytoplasma type; likewise the *D. mkurangai* in Tanzania may possibly harbour '*Ca.* Phytoplasma palmicola' or related strains. The most abundant insect species on Mozambican coconut farms belonged to Derbidae planthoppers. This finding is also in agreement with the study conducted in Tanzania by Mpunami *et al.* (2000), in which the most prevailing taxa were *Diostrombus* spp. and *Lydda woodi*, both derbids. As such, it is likely that a potential CLYD vector in Mozambique and Tanzania might belong to the Derbidae family. Philippe *et al.* (2007) also suspected *Diostrombus* sp. as potential CPSWD in Ghana. Harrison *et al*. (2002) associated the fluctuations in vector abundance with the increased incidence of LY disease in coconut palms in the Caribbean region. Nevertheless, the presence of phytoplasma DNA in insects does not prove its transmission ability. In order to be transmitted to healthy plants, the phytoplasma must circulate and multiply in the insect digestive tract, cross the barriers of the

salivary glands, and subsequently be inoculated into healthy plants when the insect feeds on phloem (Garnier *et al*., 2001; Oshima *et al*., 2011).

At least three phytoplasma groups are present in Mozambique. The dispersal of phytoplasmas among Mozambique and countries in East and West Africa might be mediated by the exchange of plant material or by unknown insect vectors. Dispersal by exchanging plant material is unlikely because these exchanges are mainly of coconut seed, and there is no evidence that the disease can be transmitted from the coconut seed to the seedling (Nipah *et al.*, 2007; Myrie *et al.*, 2011; Nan *et al.*, 2014). Our finding in the putative insect vector study is in line with this assumption given that we revealed that CLYD is also potentially transmitted by *D. mkurangai* in Mozambique (paper III). Dollet *et al.* (2011) have previously suggested that the *Platacantha lutea* Pentatomid bug is a potential CLYD vector in Mozambique; however, our study did not confirm this. Philippe *et* al. (2007) also failed to confirm Pentatomid bugs as potential vector of CSPWD in Ghana. In the Caribbean region and Florida, lethal yellowing (LY) disease is spread by the vector *H. crudus* and potentially by the *Cedusa* species of derbids (Brown *et al*., 2006). Mpunami *et al.* (2000) associated lethal disease in East Africa with *D. mkurangai* and *Meenoplus* spp. Apart from the planthopper *H. crudus* in Florida, which is the only vector confirmed by transmission experiments, other potential vector(s) of different lethal yellowing palm phytoplasmas have not yet been conclusively demonstrated.

The discovery of a novel '*Ca.* Phytoplasma pini'-related type in Mozambican coconut palms suggested that this phytoplasma might be transmitted between other plant hosts to coconut palms or vice versa. The transmission of phytoplasma between different host species was observed in Malaysia, where the causal agent of coconut LYD phytoplasma 16SrXIV group has also been observed in Bermuda grass, oil palm and Madagascan periwinkle plants (Nejat *et al*., 2009). However, some phytoplasmas are transmitted by vectors to dead-end hosts (Alma *et al.*, 2000). Schneider *et al.* (2005) observed that pine phytoplasma strains were closely related to '*Ca.* Phytoplasma castaneae' (AB054986) and to phytoplasma that cause palm lethal yellowing. Yet, in our alternative host search project (paper II), although *Pinus* sp. samples were PCR positive, sequencing did not confirm that *Pinus* sp. was harbouring CLYD phytoplasma. Nevertheless, from the topology of the phylogenetic tree, it is likely that the strain detected in coconut was not '*Ca.* Phytoplasma pini' but a related strain. As such, it was expected that this strain may have a different plant host range than that of '*Ca.* Phytoplasma pini'.

Analysis of sequence variations within '*Ca.* P. palmicola' and related strains group provided insights into the population biology of the Mozambican

coconut lethal yellowing phytoplasmas. The observed nucleotide polymorphism distribution was consistent with the observed population expansion in Mozambique. Furthermore, sequence variation in the *secA* gene was consistent with purifying selection, which suggests that secretion of proteins across the phytoplasma membrane is important for phytoplasma fitness. As observed by Oshima *et al*. (2013), phytoplasma secreted proteins may manipulate the host defence mechanisms and mediate pathogen virulence. Multiple coconut lethal yellowing phytoplasma infections observed in one Mozambican infected sample suggests that pathogen genes might be horizontally transferred between the co-infecting strains, enabling rapid evolution. However, in Ghana, two geographically distinct foci of CSPWD phytoplasma have been identified. Sequence analyses of the CSPWD phytoplasma strains suggested that the two disease foci evolved independently without exchanging phytoplasma genes (Pilet *et al.*, 2011).

Our alternative host study (paper II) revealed that *B. aethiopum* and *E. guineensis* palm species were alternate hosts of CLYD in Mozambique. Therefore, it was not unexpected that the presence of other palm species increased the odds for higher CLYD incidence (paper IV) given that the other palm species could act as reservoirs of inoculum for the healthy coconut palms. Other than *C. nucifera*, this was the first report associating *'Ca*. P. palmicola' with wild naturalized palm species. However, other palm species have been found to harbour lethal yellowing phytoplasma in other parts of the world. More than 38 palm species are susceptible to lethal phytoplasmas (Howard, 1992; Harrison & Oropeza, 2008). *E guineensis* has previously been reported to be susceptible to oil palm stunting disease in India (Mehdi *et al*., 2012) and to Malaysian coconut yellow decline (Nejat *et al.*, 2009). Coconut LY phytoplasmas 16SrIV have also been found causing LY-like symptoms in silver date (*Phoenix sylvestris*), edible date (*Phoenix dactylifera*), queen palms (*Syagrus romanzoffiana*), Mexican fan palms (*Washingtonia robusta*) sabal palms (*Sabal palmetto*), Bismarck palm (*Bismarckia nobilis*), royal palm (*Roystonea regia*) and foxtail palm (*Wodyetia bifurcata*) (Harrison *et al.*, 2008; Myrie *et al.*, 2014).

Phylogenetic analyses based on the 16S rRNA and *secA* gene sequences provided a basis for the exploration of previously unknown phytoplasma diversity related to the coconut lethal yellowing disease in Mozambique. The phylogenetic analyses based on both genes were consistent, providing more support for the different groups and subgroups observed. The lack of consistency between the number of PCR products and phytoplasma sequence recovered could mainly be associated with primer specificity. Thus, the primer pair G813/Awka SR routinely used to detect LYD phytoplasma in

Mozambique was designed to target the Ghanaian '*Ca.* P. palmicola'-related strain 16Sr XXII-B, which is different from the Mozambican 16Sr XXII-A strain (Harrison *et al*., 2014). Developing primers that are more specific for the diverse Mozambican CLYD phytoplasmas could minimize this inconsistency. Furthermore, the use of real-time PCR could help to enhance the PCR detection rate (Myrie *et al.*, 2011; Nair *et al*., 2016). The use of multiplex PCR approaches to study two or more genes simultaneously might also contribute to accurate identification of diseased trees in Mozambique. The observation in the current study that PCR-products contained false-positives highlights the necessity of using sequencing for unequivocal identification of phytoplasmas.

The effect of farm age, presence of other palm species on the coconut farm, type of coconut varieties grown, root harvest practices, and intercropping on CLYD incidence (paper IV) show that scientifically based changes to agricultural practices may lead to a reduced incidence of CLYD. Eziashi *et al.* (2013) observed that the abundance of decaying palm logs, pruned fronds, spathes and inflorescence could predispose coconuts to a series of infections and the possible transmission of LYD to healthy coconut palms. In the Caribbean palm plantations, maintaining grass that inhibits the development of *H. crudus* larvae, which is the vector for the LY phytoplasma, has been a successful practice (Howard, 1990).

Mature coconut farms (≥ 40 years old) were more tolerant of CLYD than recently established coconut farms, which supports the findings reported by Nipah (2000). In addition, younger coconut palms are the preferred hosts for adult *O. monoceros* beetles (Allou, *et al.*, 2006, 2012), a major coconut pest in Mozambique, which may weaken the palm defence response. Although young and immature palms are the preferred targets of the *O. monoceros* beetle, they are also found in mature palms when beetle populations are large (Allou *et al.*, 2006). Smith (2015) inferred that CLYD expression in Mozambique coconut palms was accelerated when stressed by *O. monoceros* beetle attacks.

The observed high tolerance of tall coconut palm varieties to CLYD phytoplasma is in line with the current CLYD management strategy in Mozambique, which involves the removal of symptomatic coconut trees and replacement with the tolerant local green MZT variety. Eden-Green (2006) observed that although the MZT variety can survive persistent exposure to LYD in Mozambique, the survivors are usually killed by *O. monoceros* beetles.

The root-cut practices increased the chances of a higher CLYD incidence, suggesting that the root may play a vital role during CLYD development. Indeed, Oropeza *et al*. (2011) observed that at certain LY development stages, a higher phytoplasma DNA titre was found in the root apex. The injuries caused by the root-cutting practices and by the phytoplasma infection may be

entry points for other pests, which might weaken the plant defence response owing to the multiple infections (Gitau *et al*., 2009). Several researchers (Garnier *et al.*, 2001; Weintraub & Beanland, 2006; Bertaccini, 2007; Nejat & Vadamalai, 2010) have reported the dependence on phloem-sucking insect vectors for phytoplasma transmission. However, examples of pathogen transmission vectored by non-sucking insects feeding at plant wounds or open cuts have also been reported (Gitau *et al*., 2009).

Coconut farms with some kind of intercropping were more vulnerable to CLYD phytoplasma infection. However, this result conflicts with the successful use of intercropping as part of an IPM strategy for some palm pests (Gitau *et al*., 2009). In line with our study, Oleke *et al*. (2012) found that although intercropping coconut with cassava, maize, cashew nut, sorghum and pineapples provided alternative crops to offset the decreasing coconut production trend caused by coconut mite and LD in Tanzania, intercropping was not a promising disease management strategy. Similarly, in Ghana, Andoh-Mensah & Ofosu-Budu (2012) found that intercropping coconut with citrus did not lower CSPWD incidence, but did contribute to a substantial part of the fruit income as insurance against CSPWD. One quarter of the farmers in our study intercropped coconut palm, mostly with the same crops that failed to control LD in Tanzania (Oleke *et al*., 2012). It is also important to note that some of the crops used by the farmers for intercropping could also be hosts for as yet unknown CLYD insect vectors in Mozambique. Maybe, better CLYD management could be achieved by intercropping coconut with similar height plants, which are likely to affect the sensory ability and the movement of the insect from one palm to another.

6 Conclusions

This project has substantially increased our knowledge of LYD epidemiology. Mozambique has the highest observed LYD phytoplasma diversity in Africa. The high phytoplasma diversity, including novel phytoplasma species associated with CLYD in Mozambique support the idea that after Asia the greatest diversity of palm LY phytoplasma is found in Africa. The high level of diversity suggested that different insect vectors and alternate hosts might be associated with their dispersal, providing further challenges for the management approaches being practiced. Alternate hosts for CLYD in Mozambique were revealed, which are other sources of inoculum when the primary host, infected coconuts trees, are absent. The identification of CLYD alternate hosts and a potential insect vector are essential information needed to work towards sustainable CLYD management, which is one of the major gaps on the disease management side. Given that both '*Ca.* Phytoplasma palmicola' and Tanzania LD phytoplasma occur in Mozambique and Tanzania, there is a high probability that *D. mkurangai*, which was found carrying LYD phytoplasma in both countries, is the vector for this disease in this region. Our finding that the potential CLYD insect vector in Mozambique is likely to be *D. mkurangai* rather than *Platacantha lutea* should help to define the insect vector search focus. Our increased awareness of the impact that farm age, presence of other palm species on the coconut farm, type of coconut varieties grown and root harvest practices have on CLYD incidence, combined with the identification of alternate hosts, needs to be capitalized on in future IPM strategies aimed at controlling this disease.

7 Future perspectives

Future challenges in this field include widening the search for insect vectors and alternative hosts, and conducting transmission trials to determine whether *D. mkurangai* does vector CLYD in Mozambique. Understanding the molecular mechanisms behind phytoplasma host switching, a vital stage in the phytoplasma life cycle, could make a significant contribution toward efficient CLYD control. Because the disease in Mozambique is caused by multiple phytoplasma species, further studies are required to analyse multiple infections and their impact on disease epidemiology. The observation of co-infections and CLYD alternate hosts suggests that genetic recombination could be used to promote rapid pathogen evolution, which is an interesting topic for further study. Another important aspect is the study of resistance and/or tolerance against different types of phytoplasmas. The initial screening of coconut lethal yellowing phytoplasma diversity in Mozambique has paved the way for much broader studies.

The primer pair G813/Awka SR (Tymon *et al*., 1997) routinely used to detect CLYD phytoplasma in Mozambique was designed to target the '*Ca.* P. palmicola'-related strain 16Sr XXII-B, which is different from the Mozambican 16Sr XXII-A strain (Harrison *et al*., 2014). Hence, there is a need to develop more specific primers for the different LYD phytoplasmas occurring in Mozambique. Rapid and specific detection protocols should greatly support the current CLYD management strategy by ensuring replanting with disease-free seedlings as well as early detection, even on symptomless infected palm plants.

The effect of intercropping on CLYD management is still unclear and requires additional research to be explained fully.

References

Abt Associates. (2014). Performance evaluations: Mozambique Farmer Income Support Project. Bethesda. [Online]. Available from: http://catalog.ihsn.org/index.php/catalog/6151/study-escription#page=sampling&tab=study-desc, [Accessed on 1st of March 2016].

Alma, A., Marzachì, C., D'Aquilio, M. & Bosco, D. (2000). Cyclamen (*Cyclamen persicum* L.): a dead-end host species for 16Sr-IB and -1C subgroup phytoplasmas. *Annals of Applied Biology*, *136*, pp. 173–178.

Allou, K., Morin, J. P., Kouassi, P., N'Klo, F. H. & Rochat, D. (2006). Oryctes monoceros trapping with synthetic pheromone and palm material in ivory coast. *Journal of Chemical Ecology*, *32*(8): 1743–1754.

Allou, K., Issali, A.E., Lekadou, T., Konan, K.J.L., Zakra, N., Kouassi, K.P., Bourdeix, R., Morin, J.P. & Saraka, Y.D.M. (2012). Comparative synergetic effect of coconut palm (*Cocos nucifera* L.) slices and bunches residue of oil palm (*Elaeis guineensis* JACQ.) associated with two kinds of pheromone traps on *Oryctes monoceros* OLIVIER trapping in Côte d'Ivoire. *International Journal of Emerging Technology and Advanced Engineering*, *2*(6), pp. 2–7.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, pp. 403–410.

Andoh-Mensah, E. & Ofosu-Budu, G.K. (2012). Evaluation of coconut citrus intercropping systems in context of coconut lethal yellowing in Ghana. *African Journal of Food, Nutrition and Development*, *12*(7), pp. 6945─6962.

Bertaccini, A. (2007). Phytoplasmas: diversity, taxonomy, and epidemiology. *Frontiers in Bioscience*, *12*, pp. 673─689.

Bertaccini, A. & Duduk, B. (2009). Phytoplasma and phytoplasma diseases : a review of recent research. *Phytopathologia Mediterranea*, *48*(3), pp. 355–378.

Bila, J., Högberg, N., Mondjana, A. & Samils, B. (2015a). African fan palm (*Borassus aethiopum*) and oil palm (*Elaeis guineensis*) are alternate hosts of coconut lethal yellowing phytoplasma in Mozambique. *African Journal of Biotechnology*, *14*(52), pp. 3359–3367.

Bila, J., Mondjana, A., Samils, B. & Högberg, N. (2015b). High diversity, expanding populations and purifying selection in phytoplasmas causing coconut lethal yellowing in Mozambique. *Plant Pathology*, *64*(3), pp. 597–604.

Broshat, T.K., Harrison, N.A. & Donsalman, H. (2002). Losses to Lethal Yellowing Cast Doubt on Coconut Cultivar. *PALMS*, *46*(4): 185–189.

Broschat, T.K., Elliott, M.L. & Maguire, I. (2010). Symptoms of diseases and disorders. *In*: A Resource for Pests and Diseases of Cultivated Palms. University of Florida, Identification Technology Program, USDA. [Online]. Available from: http://itp.lucidcentral.org/id/palms/symptoms. [Accessed on 18th March 2016].

Brown, S.E., Been, B.O. & McLaughlin, W.A. (2006). Detection and variability of the lethal yellowing group (16Sr IV) phytoplasmas in the *Cedusa* sp. (Hemiptera: Auchenorrhyncha: Derbidae) in Jamaica. *Annals of Applied Biology*, *149*(1), pp. 53–62.

Brown, S.E., Been, B. & McLaughlin, W.A. (2008). First report of the presence of the lethal yellowing group (16SrIV) of phytoplasma in the weeds *Emilia fosbergii* and *Synedrella nodiflora* in Jamica. *Plant Pathology*, *57*, pp. 56–58.

Caudwell, R. (2000). The successful development and implementation of an integrated pest management system for oil palm in Papua New Guinea. *Integrated Pest Management Reviews*, *5*(4), pp. 297–301.

Christensen, N.M., Axelsen, K.B., Nicolaisen, M., & Schulz, A. (2005). Phytoplasmas and their interactions with hosts. *Trends in Plant Science*, *10*(11), pp. 526–535.

Contaldo, N., Bertaccini, A., Paltrinieri, S., Windsor, H.M. & Windsor, G.D. (2012). Axenic culture of a plant pathogenic Phytoplasma. *Phytopathologia Mediterranea*, *244*, pp. 607–117.

Cronjé, P., Dabek, A.J., Jones, P. & Tymon, A. M. (2000). First report of a phytoplasma associated with a disease of date palms in North Africa. *Plant Pathology*, *49*, pp. 801.

Doi, Y., Teranaka, M., Yora, K. & Asuyama, H. (1967). Mycoplasma or PLT-group-like organisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows or paulownia witches' broom. *Annals of the Phytopathological Society of Japan*, *33*(4), pp. 259–266.

Dollet, M., Macome, F., Vaz, A. & Fabre, S. (2011). Phytoplasmas identical to coconut lethal yellowing phytoplasmas from Zambesia (Mozambique) found in a pentatomide bug in Cabo Delgado province. *Bulletin of Insectology*, *64*(1), pp. 139–140.

Duduk, B. & Bertaccini, A. (2011). Phytoplasma classification: taxonomy based on 16S ribosomal gene, is it enough? *Phytopathogenic Mollicutes*, *1*, pp. 3–13.

Eden-Green, S.J. (1997). History, distribution and research on coconut lethal yellowing-like diseases of palms. In: International Workshop on Lethal Yellowing-like Disease, Chatham, UK, pp. 9–25.

Eden-Green, S.J. 2006. An assessment of Coconut Lethal Yellowing-type Disease (LYD) in Mozambique. Consultant's report by EG Consulting, 24pp, UK.

Eziashi, E. & Omamor, I. (2010). Lethal yellowing disease of the coconut palms (*Cocos nucifera* L.): An overview of the crises. *African Journal of Biotechnology*, *9*(54), pp. 9122–9127.

Eziashi, E.I., Omamor, I.B., Aisueni, N.O., Aisagbonhi, C.I., Airede, C.E., Ikuenobe, C.E., Ataga, C.D., Oruade-Dimaro, E.A., Odewale, J.O. & Osagie, I.J. (2013). Potential weed species as alternate hosts of insect vectors of the Lethal Yellowing Disease (LYD) of coconut palms (*Cocos nucifera* L.) in Nigeria. *British Jounal of Applied Science & Technology*, *3*(1), pp. 123–130.

Food and Agriculture Organization of the United Nations. 2016. FAOSTAT. [Online]. Available at: http://faostat3.fao.org/download/Q/QC/E.

Garnier, M., Foissac, X., Gaurivaud, P., Laigret, F., Renaudin, J., Saillard, C. & Bové, J. M. (2001). Mycoplasmas, plants, insect vectors: A matrimonial triangle. *Comptes Rendus de l'Academie Des Sciences - Serie III*, *324*(10), pp. 923–928.

Gitau, C.W., Gurr, G.M., Dewhurst, C.F., Fletcher, M.J. & Mitchell, A. (2009). Insect pests and insect-vectored diseases of palms. *Australian Journal of Entomology*, *48*(4), pp. 328–342.

Gunn, B.F., Baudouin, L. & Olsen, K.M. (2011). Independent origins of cultivated coconut (*Cocos nucifera* L.) in the old world tropics. *PLoS ONE*, *6*(6), e21143. doi:10.1371/journal.pone.0021143

Hair, J.F., Black, W.C., Babin, B.J. & Anderson, R.E. (2014). Multivariate data analysis. 7th ed. Harlow, Pearson Education Limited.

Harrison, N.A. & Oropeza, C. (2008). Coconut lethal yellowing. In: Harrison, N.A., Rao, G.P. and Marcone C., eds. *Characterization, Diagnosis and Management of Phytoplasmas*. Houston, Studium Press, pp. 219–248.

Harrison, N.A., Womack, M., Carpio, M.L. (2002). Detection and characterization of a lethal yellowing (16SrIV) group phytoplasma in Canary Island date palms affected by lethal decline in Texas. *Plant Disease*, *86*, pp. 676–681.

Harrison, N.A., Davis, R.E., Oropeza, C., Helmick, E.E., Narváez, M., Eden-Green, S., Dollet, M. & Dickinson, M. (2014). '*Candidatus* Phytoplasma palmicola', associated with a lethal yellowing-type disease of coconut (*Cocos nucifera* L.) in Mozambique. *International Journal of Systematic and Evolutionary Microbiology*, *64*, pp. 1890–1899.

Harrison, N. A., Helmick, E. E. & Elliott, M. L. (2008). Lethal yellowing-type diseases of palms associated with phytoplasmas newly identified in Florida, USA. *Annals of Applied Biology*, *153*, pp. 85–94.

Hodgetts, J., Boonham, N., Mumford, R., Harrison, N. & Dickinson, M. (2008). Phytoplasma phylogenetics based on analysis of secA and 23S rRNA gene sequences for improved resolution of candidate species of '*Candidatus* Phytoplasma'. *International Journal of Systematic and Evolutionary Microbiology*, *58*, pp. 1826–1837.

Hodgetts, J. & Dickinson, M. (2010). Phytoplasma phylogeny and detection based on genes other than 16S rRNA. In: Weintraub, P.G. & Jones, P., eds. *Phytoplasmas: Genomes, Plant Hosts and Vectors*, London, MPG Books Groups, pp. 93–113.

Hoshi, A., Ishii, Y., Kakizawa, S., Oshima, K. & Namba, S. (2007). Host–parasite interaction of phytoplasmas from a molecular biological perspective. *Bulletin of Insectology*, *60*(2), pp. 105–107.

Howard, F.W. (1990). Evaluation of grasses for cultural control of *Myndus crudus*, a vector of lethal yellowing of palms. *Entomologia Experimentalis et Applicata*, *56*, pp. 131–137.

Howard, F. (1992). Lethal yellowing susceptibility of date palms in Florida. *Principes*, *36*(4), pp. 217–222.

Howard, F.W. & Oropeza, C. (1998). Organic mulch as a factor in the nymphal habitat of *Myndus crudus* (Hemiptera: Auchennorhyncha: Cixiidae). *The Florida Entomologist, 81*, pp. 92–97.

Hunt, P., Dabek, A. J., & Schuiling, M. (1973). Remission of symptoms following tetracycline treatment of Lethal Yellowing-infected coconut palms. *Phytopathology*, 64, pp. 307-312.

International Research Program on Comparative Mycoplasmalogy. (2004). '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonise plant phloem

and insects. *International Journal of Systematic and Evolutionary Microbiology*, *54*, pp. 1243–1255.

Jones, J. D. G. & Dangl, J. L. (2006). The plant immune system. *Nature Reviews*, *444*, pp. 323–329.

Khan, J.A., Srivastava, P. & Singh, S.K. (2004). Efficacy of nested-PCR for the detection of phytoplasma causing spike disease of sandal. *Current Science*, *86*(11), pp. 1530–1533.

Kryazhimskiy, S. & Plotkin, J.B. (2008). The population genetics of dN/dS. *PLoS Genetics*, *4*(12), e1000304. doi:10.1371/journal.pgen.1000304.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M. Wilm, A., Lopez, R, Thompson, J.D., Gibson, T.J. Higgins, D.G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, *23*(21), pp. 2947–2948.

Lebrun, P., N'cho, Y. P., Seguin, M., Grivet, L. & Baudouin, L. (1998). Genetic diversity in coconut (*Cocos nucifera* L.) revealed by restriction fragment length polymorphism (RFLP) markers. *Euphytica*, *101*, pp. 103–108.

Lebrun, P., Baudouin, L., Myrie, W., Berger, A. & Dollet, M. (2008). Recent lethal yellowing outbreak: Why is the Malayan Yellow Dwarf Coconut no longer resistant in Jamaica? *Tree Genetics and Genomes*, *4*(1), pp. 125–131.

Lee, I.-M., Gundersen-Rindal, D.E., Davis, R.E. & Bartoszyk, I.M. (1998). Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology*, *48*, pp. 1153–1169.

Lee, I.-M., Davis, R.E. & Gundersen-Rindal, D.E. (2000). PHYTOPLASMA: Phytopathogenic Mollicutes. *Annual Review of Microbiology*, *54*, pp. 221–255.

Librado, P. & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, pp. 1451–1452.

Marinho, V.L.A., Fabre, S. & Dollet, M. (2008). Genetic variability among isolates of Coconut lethal yellowing phytoplasmas determined by Heteroduplex Mobility Assay (HMA). *Tropical Plant Pathology*, *33*(October), pp. 377–380.

McCoy, R.E. (1976). Comparative epidemiology of the lethal yellowing, Kaincope and cadang-cadang diseases of coconut palm. *Plant Disease Reporter*, *60*, pp. 498–502.

Mehdi, A., Baranwal, V. K., Kochu Babu, M. & Praveena, D. (2012). Sequence analysis of 16S rRNA and secA genes confirms the association of 16SrI-B subgroup phytoplasma with oil palm (*Elaeis guineensis* Jacq.) stunting disease in India. *Journal of Phytopathology*, *160*(1), pp. 6–12.

Moran, N.A. (2002). Microbial minimalism: Genome reduction in bacterial pathogens. *Cell*, *108*(5), pp. 583–586.

Mondjana, M., Santos, L., Vaz, A., Caniço, A., Mangana, S., Nuila, V., Massingue, F. & Massinga, J. (2010). Manual Prático Para a Identificação e Maneio de Amarelecimento Letal de Coqueiro. Minestério de Agricultura, Maputo, Moçambique.

Mpunami, A., Tymon, A., Jones, P. & Dickinson, M.J. (1999). Genetic diversity in the coconut lethal yellowing disease phytoplasmas of East Africa. *Plant Pathology*, *48*, pp. 109–114.

Mpunami, A., Tymon, A., Jones, P. & Dickinson, M.J. (2000). Identification of potential vectors of the coconut lethal disease phytoplasma. *Plant Pathology*, *49*(3), pp. 355–361.

Myrie, W., Oropeza, C., Sáenz, L., Harrison, N., Roca, M. M., Córdova, I., Ku, S. & Douglas, L. (2011). Reliable improved molecular detection of coconut lethal yellowing phytoplasma and reduction of associated disease through field management strategies. *Bulletin of Insectology*, *64*(Supplement), pp. S203–S204.

Myrie, W.A. (2014). Lethal yellowing disease of coconut palm. Jamaica. In: *13th Caribbean Week of Agriculture 2014*. 6–12 October 2014, Paramaribo, Suriname. Available at: http://www.cwa.caricom.org/index.php/media-centre/presentations/meeting-and-workshops/14-pest-lethal-yellowing-jamaica/file, [Accessed on 2nd March 2016].

Myrie, W.A., Douglas, L., Harrison, N.A., Mclaughlin, W. & James, M. (2014). First report of lethal yellowing disease associated with subgroup 16SrIV-A phytoplasma in Antigua, West Indies. *New Disease Reports*, *29*, pp. 12.

Nair, S., Manimekalai, R., Soumya, V.P. & Likhitha, K.C. (2016). Dual labeled probe based real time PCR method for detection of 16SrXI-B sub-group phytoplasma associated with coconut root wilt disease in India. *Australasian Plant Pathology*, *45*(2). doi:10.1007/s13313-016-0406-7.

Nei, M. (1987). *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Nan, O.A., Bi, E.G., Kouassi, A.B., Kouassi, A., Philippe, R., Malaurie, B. & Dollet, M. (2014). Isolation of phytoplasma DNA from the coconut palms (*Cocos nucifera* L.) collected from Ghana. *Journal of Experimental Biology and Agricultural Sciences*, *2*(2320), pp. 495–500.

Nejat, N., Sijam, K., Abdullah, S.N.A, Vadamalai, G. & Dickinson, M. (2009). Phytoplasmas associated with disease of coconut in Malaysia: Phylogenetic groups and host plant species. *Plant Pathology*, *58*, pp. 1152–1160.

Nejat, N. & Vadamalai, G. (2010). Phytoplasmas detection in coconut palms and other tropical crops. *Plant Pathology Journal*, *9*(3), pp. 112–121.

Nejat, N., Vadamalai, G., Davis, R.E., Harrison, N.A., Sijam, K., Dickinson, M., Abdullah, S.N.A. & Zhao, Y. (2013). '*Candidatus* Phytoplasma malaysianum', a novel taxon associated with virescence and phyllody of Madagascar periwinkle (*Catharanthus roseus*). *International Journal of Systematic and Evolutionary Microbiology*, *63*, pp. 540–548.

Nipah, J.O. (2000). Some characteristics of the Sri Lanka Green Dwarf (SGD): A variety resistant to the Cape Saint Paul wilt disease of coconut. *Journal of the Ghana Science Association*, *2*, pp. 88–93.

Nipah, J.O., Jones, P. & Dickinson, M.J. (2007). Detection of lethal yellowing phytoplasma in embryos from coconut palms infected with Cape St Paul wilt disease in Ghana. *Plant Pathology*, *56*, pp. 777–784.

Ntushelo, K., Elliott, M.L. & Harrison, N.A. (2013). Palm yellows phytoplasmas and their genetic classification. *African Journal of Biotechnology*, *12*(22), 3376–3382.

Oleke, J.M., Isinika, A., Manyong, V., Hanna, R. & Sabelis, M. (2012). Farmers' perception of coconut mite damage and crop diversification alternatives in the coastal belt of Tanzania. *International Journal of Acarology*, *38*(6) pp. 471–479.

Oropeza, C., Escamilla, J.A., Mora, G., Zizumbo, D. & Harrison, N.A. (2005). Coconut lethal yellowing. In: Batuga, P., Rao, V.R. & Oliver, J., eds. Coconut Genetic Resources, IPGRI-APO, Malaysia, pp. 359–363.

Oropeza, C., Cordova, I., Chumba, A., Narváez, M., Sáenz, L., Ashburner, R. & Harrison, N. (2011). Phytoplasma distribution in coconut palms affected by lethal yellowing disease. *Annals of Applied Biology*, *159*, pp. 109–117.

Oshima, K., Ishii, Y., Kakizawa, S., Sugawara, K., Neriya, Y., Himeno, M., Minato, N., Miura, C., Shiraishi, T., Yamaji, Y. & Namba, S. (2011). Dramatic transcriptional changes in an intracellular parasite enable host switching between plant and insect. *PLoS ONE*, *6*(8), e23242. doi:10.1371/journal.pone.0023242.

Oshima, K., Maejima, K. & Namba, S. (2013). Genomic and evolutionary aspects of phytoplasmas. *Frontiers in Microbiology*, *4*, pp. 1–8.

Philippe, R., Nkansah, J.P., Fabre, S., Quaicoe, R., Pilet, F. & Dollet, M. (2007). Search for the vector of Cape Saint Paul wilt (coconut lethal yellowing) in Ghana. *Bulletin of Insectology*, *60*(2), pp. 179–180.

Pilet, F., Poulin, L., Nkansah-Poku, J. & Quaicoe, R.N. (2011). Ribosomal protein gene sequences reveal a geographical differentiation between CSPWD phytoplasmas in Ghana. *Bulletin of Insectology*, *64*, pp. S219–S220.

Schneider, B., Gibbl, K.S. & Seemuller, E. (1997). Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. *Microbiology*, *143*, pp. 3381–3389.

Schneider, B., Torres, E., Martín, M.P., Schröder, M., Behnke, H.D. & Seemüller, E. (2005). 'Candidatus Phytoplasma pini', a novel taxon from *Pinus silvestris* and *Pinus halepensis*. *International Journal of Systematic and Evolutionary Microbiology*, *55*, pp. 303–307.

Seemuller, E., Garnier, M. & Schneider, B. (2002). Mycoplasmas of plant and insects. In: S. Razin and Hermann, R., eds. *Molecular Biology and Pathology of Phytoplasmas*, Academic/Plenum Kluwer, London, pp. 91–115.

Smith, J. (2015). Fera report to ABT associate Inc. On Impact Evaluation of Farmer Income Support Project. Food and Environment Research Institute. Sand Hutton. fera.

Swarbrick, J.P., Yankey, N.E., Nipah, O.J., Quaicoe, R. & Dickinson, J.M. (2013). Identification of receptor like kinase genes in coconut and development of a marker for validation of breeding materials resistant to a phytoplasma disease in Ghana. *African Journal of Biotechnology*, *12*(45), pp. 6347–6357.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, *30*, pp. 2725–2729.

Tanne, E., Boudon-Padieu, E., Clair, D., Davidovich, M., Melamed, S. & Klein, M. (2001). Detection of phytoplasma by polymerase chain reaction of insect feeding medium and its use in determining vectoring ability. *Phytopathology*, *91*(8), pp. 741–746.

Tajima, F. (1989). Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. *Genetics*, *123*, pp. 585–595.

Thomas D.L. & Norris R.C. (1980). The use of electron microscopy for lethal yellowing diagnosis. *Proceedings of the Florida State Horticultural Society*, *93*, 196–199.

Tymon, A.M., Jones, P. & Harrison, N.A. (1997). Detection and differentiation of African coconut phytoplasmas: RFLP analysis of PCR-amplified 16S rDNA and DNA hybridisation. *Annals of Applied Biology*, *131*, pp. 91–102.

Tymon, A., Jones, P. & Harrison, N. (1998). Phylogenetic relationships of coconut phytoplasmas and the development of specific oligonucleotide PCR primers. *Annals of Applied Biology*, *132*, pp. 437– 452.

Wei, W., Kakizawa. S., Jung, H.Y., Suzuki, S. & Tanaka, M. (2004). An antibody against the SecA membrane protein of one phytoplasmas reacts with those of phylogenetically different phytoplasmas. *Phytopathology*, *94*, pp. 683–686.

Wei, W., Davis, R.E., Lee, I.-M. & Zhao, Y. (2007). Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. *International Journal of Systematic and Evolutionary Microbiology*, *57*, pp. 1855–1867.

Weintraub, P.G. & Beanland, L. (2006). Insect vectors of phytoplasmas. *Annual Review of Entomology*, *51*(1), pp. 91–111.

Zhao, Y., Wei, W., Lee, I.M., Shao, J., Suo, X. & Davis, R.E. (2009). Construction of an interactive online phytoplasma classification tool, *i*PhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *International Journal of Systematic and Evolutionary Microbiology*, *59*, pp. 2582–2593.

Zhao, Y., Wei, W., Davis, R.E. & Lee, I.M. (2010). Recent advances in 16S rRNA gene-based phytoplasma differentiation, classification and taxonomy. In: Weintraub, P.G. & Jones, P., eds. *Phytoplasmas, Genomes, Plant Hosts and Vectors*, London: MPG Books Group, pp. 64–92.

Acknowledgements

First of all, I would like to thank my supervisor, **Nils Högberg** for accepting me as PhD student and for freedom given to explore the phytoplasma knowledge. I appreciate your support provided to make this journey possible. Many thanks to my supervisor **Berit Samils**, for open and fruitful discussion that made this journey enjoyable. For all of you, I appreciate your personal care and moral support which made me feeling less homesick. Finally to Prof. **Ana Mondjana** my home university supervisor for helping me to put my study aligned with the local context.

I would like to express my gratitude to other researchers that contributed to the success of this project. First, to Prof. **Domenico Bosco**, for the opportunity given to visit your lab at "Universitá degli Studi di Torino" in Italy. The hands on experience I got in your lab, from your colleagues specially your Post Doc **Sabrina Bertin** enhanced my inspiration to work with phytoplasma. To Dr. **William J. Baker** from Royal Botanic Gardens, Kew, UK for the provided palm species identification support. Prof. **Inger Persson** from the Department of Statistics, Uppsala University for valuable support provided for statistical analysis. I appreciate the help provided by Dr **Michael R. Wilson** from the National Museum of Wales, UK, for the insect identification. Finally I wish to thanks Prof. **Luisa Santos** from my home University, for the assistance provided to get insect samples throughout the country. Prof. **Luisa Santos** and other colleagues from my home university are also thanked for the support provided to design and conduct the survey for the Farming Practices project.

I am also grateful to the Madal Company and the small-scale coconut producers from Mozambique for access to samples from their coconut farms. I am grateful to Verde Azul Company staff and invited researchers, especially to the Director **Eng. Kemal Vaz**, for the assistance provided to design and conduct the survey for Farming Practices project. I thank the Mikocheni

Agricultural Research Institute of Tanzania for the support they provided to collect the samples in Tanzania.

This work was funded by the “Swedish International Development Agency” (SIDA). I am also thankful for Millennium Challenge Account (MCC-Mozambique) for funding the fieldwork for farming practices study. “Universidade Eduardo Mondlane”, my home university is thanked for the resources and good environment provided for the success of this journey, particularly on the fieldwork conducted in Mozambique. The environment and very inspiring atmosphere found at Swedish University Agricultural of Sciences (SLU), particularly at Mykopat was of upmost importance to become scientist. My gratitude is extended to all past and present **Mykopat colleagues** for the wonderful working environment provided, including the relaxing Fika.

Last but not least, I am thankful to my entire family, especially my wife **Miriam**, and my children **Chuisa**, **Niels**, **Wilma** and **Vadinho** for continuously sending me love, endless support and encouragement. I hope you will get inspired to pave your way as scientists.